

THE ROLE OF INDOLEAMINE 2,3-DIOXYGENASE IN MODULATING ALLERGIC RESPONSES

FABIÁN ALBERTO SALAZAR LIZAMA, MSc.

Immunology Department

School of Life Sciences

University of Nottingham

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

JULY 2016

Abstract

Dendritic cells (DCs) are key players in the induction and re-elicitation of T helper (Th) 2 immune responses to allergens. Recent data by our group have shown that different C-type lectin receptors (CLRs) on DCs play a major role in allergen recognition and uptake. Particularly, mannose receptor (MR), through modulation of Toll-like receptor (TLR) 4 signalling pathway, can regulate indoleamine 2,3 dioxygenase (IDO) activity favouring Th2 responses. IDO is the rate limiting enzyme involved in tryptophan (TRP) catabolism and it is well known for its role in modulating immune responses through TRP depletion and generation of immune-regulatory metabolites known as kynurenines (KYN). Interestingly, another CLR named dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) has been suggested to support Th1 responses; however, the mechanisms involved are unknown. Therefore, the main objective of this project was to evaluate the role of CLRs, with focus on MR and DC-SIGN, and TLRs in modulating the IDO pathway in order to understand their involvement in regulating Th2 immune responses. In addition, it was aimed to study how regulation of IDO levels could impact immune responses in order to understand the signalling pathways involved in this process.

The data show that mannosylated allergens can down-regulate TLR4-induced IDO1 and IDO2 expressions as well as IDO activity in human DCs, most likely through binding to the MR. This correlates with data showing increased IDO activity in MR^{low}-DCs (generated by gene silencing) stimulated with mannan compared with control-DCs (CT-DCs). Conversely, DC-SIGN could exert a differential regulation on IDO levels depending on the antigen's sugar moieties. Mannosylated ligands could

up-regulate TLR4-induced IDO activity through DC-SIGN as evidence by a significant reduction in IDO activity in DC-SIGN^{low}-DCs upon stimulation with mannan compared with CT-DCs. In contrast, DC-SIGN-specific fucosylated ligands, such as Lewis-X (Le^X), can down-regulate IDO activity in a TLR4-dependent manner.

It was also found that allergens from diverse source can down-regulate IL-12p70 production by DC, which can further impact T helper cell polarization by reducing IFN-γ production by T cells in autologous co-cultures. Moreover, mannan was also shown to decrease CD86 expression in human DCs, which might impact the IDO pathway, as CD86 is one of the B7 family molecules involved in IDO induction. Furthermore, it was found that the Le^X can potentially down-regulate IL-6 production by DCs. In addition, DCs stimulated with Le^X and LPS induce lower levels of IFN-γ production by autologous T cells than DCs stimulated with LPS only. This effect was reversed in the presence of KYN, showing that the IDO pathway can regulates T helper cell polarization.

TLR4 signalling and LPS exposure have been shown to play a pivotal role in Th2-mediated inflammation and asthma. In addition, it is known that CLRs can modulate TLR-induced responses in an allergy context. Therefore, we further study the role of the TLR signalling pathway in modulating IDO levels in human DCs. TLR4 engagement on human DCs was shown to induce high levels of both IDO isoforms. DCs primed with LPS induce much higher levels of IDO than single LPS controls, inducing a cellular re-programing. This was characterized by an induction of anti-inflammatory mediators such as IL-10 and the transcription factor aryl-hydrocarbon receptor (AhR); the last has been previously linked with the IDO pathway in mouse DCs. Intriguingly, TLR9 engagement with synthetic cytosine-phosphate guanosine

(CpG) A motifs was able to down-regulate IDO and induce a pro-inflammatory phenotype in human DCs. Future studies should aim at evaluating their implications in immune responses under tolerance and during infection.

Finally, the study was aimed at elucidating the intracellular molecules involved in IDO regulation in human DCs. The analysis was first focused on AhR, a ligand-dependent transcription factor that have been suggested to modulate IDO levels in mouse DCs. It was shown for the first time that TLR4 induction of IDO was dependent on AhR in human DCs. In addition, AhR gene expression as well as AhR activity were reduced in DCs stimulated with mannan and LPS compared with LPS only controls. This data suggest that MR can regulate IDO levels by interfering with AhR expression. Furthermore, it was shown that the NF-κB pathway is key in regulating IDO levels through CLRs and TLRs in human DCs. Particularly, RelB, a member of the non-canonical pathway, expression correlates with the levels of AhR, suggesting that a functional and/or physical association between them might be involved in regulating IDO levels in human DCs. Future studies should aim at elucidating such interactions.

To sum up, this project has shown the pivotal role of CLRs and TLRs in modulating the IDO pathway in human DCs, which can affect DC phenotype and function and impact immune responses. In addition, we have gained some understanding into the molecular mechanisms of IDO modulation which might involve cooperation between different transcription factors such as AhR and RelB. These data give new insights into how glycosylated allergens can induce Th2 immune responses, which can pave the way to develop better therapeutic strategies to fight back allergy related diseases.

Publications and Conferences

Publications arising from this thesis:

1. Salazar, F., Hall, L., Negm, O.H., Awuah, D., Tighe, P.J., Shakib, F. & Ghaemmaghami, A.M. (2015). The mannose receptor negatively modulates the Toll-like receptor 4-aryl hydrocarbon receptor-indoleamine 2,3-dioxygenase axis in dendritic cells affecting T helper cell polarization. The Journal of Allergy and Clinical Immunology. doi: 10.1016/j.jaci.2015.10.033.
2. Salazar, F. & Ghaemmaghami, A.M. (2013). Allergen recognition by innate immune cells: Critical role of dendritic and epithelial cells. Frontiers in Immunology, Vol.4, pp.356.
3. Salazar, F., Sewell, H.F., Shakib, F. & Ghaemmaghami, A.M. (2013). The role of lectins in allergic sensitization and allergic disease. Journal of Allergy and Clinical Immunology, Vol.132, No.1: pp.27-36.
4. Salazar, F., Negm, O.H., Tighe, P.J., Shakib, F. & Ghaemmaghami, A.M. (2016). LPS conditioned human dendritic cells adopt an anti-inflammatory phenotype with an induction of the non-canonical NF-κB pathway. Manuscript submitted for publication.
5. Salazar, F., Negm, O.H., Tighe, P.J., Shakib, F. & Ghaemmaghami, A.M. (2016). DC-SIGN regulates indoleamine 2,3-dioxygenase activity in human dendritic cells in a carbohydrate specific manner affecting T helper cell polarization. Manuscript submitted for publication.

Publications and Conferences

National and international conferences attended and awards:

1. This thesis has been awarded the Early Researcher of Excellence Award 2015, School of Life Sciences, University of Nottingham.
2. Salazar, F., Hall, L., Negm, O.H., Tighe, P.J., Shakib, F. & Ghaemmaghami, A.M. (2015). Mannose receptor negatively regulates the TLR4-AhR-IDO axis in DCs with implications in allergic responses. 4th European Congress of Immunology. 6-9 September 2015, Vienna, Austria.
3. Salazar, F., Hall, L., Negm, O.H., Tighe, P.J., Shakib, F. & Ghaemmaghami, A.M. (2015). The role of mannose receptor in modulating the TLR4-IDO axis in allergy. MACS Allergy Day. 19th May 2015, London, UK. (Junior Investigator Award for the best oral presentation).
4. Salazar, F., Hall, L., Shakib, F. & Ghaemmaghami, A.M. (2014). Differential regulation of indoleamine 2,3-dioxygenase by TLRs and CLRs in human DCs, the role of aryl hydrocarbon receptor. 13th International Symposium on Dendritic Cells. 14-18 September 2014, Tours, France.
5. Salazar, F., Shakib, F. & Ghaemmaghami, A.M. (2013). Differential regulation of IDO by TLRs and CLRs in human DCs. 27th Annual Conference of the European Macrophage and Dendritic Cell Society. 10-12 October 2013, Erlangen, Germany.
6. Salazar, F., Shakib, F. & Ghaemmaghami, A.M. (2013). Differential regulation of indoleamine 2,3-dioxygenase in human airway epithelial cells and dendritic cells. Joint Respiratory Research Day. 4th September 2013, Loughborough, UK.

Acknowledgements

I would like to acknowledge all the people who have contributed in one way or another to the completion of my PhD. Firstly; I would like to express my deepest appreciation to my supervisor Professor Amir Ghaemmaghami for his invaluable guidance and continuous support throughout the thesis and for his trust by giving me the opportunity to carry out this project.

I wish to extent my sincere gratitude to the rest of my thesis committee, Professor Farouk Shakib, Dr Bruno Gran and Professor Howard Clark for their insightful comments and encouragement that help me to widen my research from various perspectives.

I would also like to thank my fellow labmates as well as the technicians and post-docs from the Allergy and Tissue Engineering Research Group for listening during the seminars, for their comments and suggestions, for the fresh blood, for the day-outs and the Christmas meals, and for their help and friendship during my studies in the University of Nottingham, it was a real pleasure to work with them. In particular, a sincere thanks to Laurence Hall whose expertise helped me a great deal with some experiments.

I also recognize all the member of the Immunology Division in the School of Life Sciences. Particularly, Dra Luisa Martinez-Pomares for her support and encouragement during my studies, Dra Ola Negm and Dr Paddy Tighe for their help with the microarray and Dr David Onion and Miss Nicola Croxall for their assistance with the Flow Cytometer.

Acknowledgements

I would also like to express my gratitude to the source of funding that allows me undertake this project. In particular, many thanks to the Becas Chile Grant from the National Commission for Scientific and Technological Research (CONICYT, Chile) for funding my studies in the UK. Financial contributions from the British Society for Immunology and the European Macrophage and Dendritic Cell Society are also greatly appreciated, along with the School of Life Sciences at the University of Nottingham for supporting my travel to National and International Conferences.

On a personal note, I am very grateful for all the support and encouragement coming from my family and friends from Chile, specially my parents Teresa Lizama and Luis Salazar, my sisters Perla and Macarena, and of course my nephew Bastian. Last but not least, special thanks to my amazing wife Natalia Mansilla, this project would not have been accomplished without her infinite love and patience, and her tremendous encouragement and support. Certainly, this period of time in the UK has been a wonderful and unforgettable enriching experience for both of us and I feel so fortunate and happy that we have completed it together.

Table of Contents

Abstract....ii
Publications and Conferences....v
Acknowledgements....vii
Table of Contents....ix
Table of Figures....xii
Abbreviations....xiv
1. Chapter I: Introduction....1
1.1 The immune system....1
1.2 Asthma as a syndrome....2
1.3 Type-I hypersensitivity and allergy....2
1.4 Emerging role of innate immune cells in allergy....5
1.5 Role of epithelial cells in allergen recognition....6
1.5.1 Protease activated receptors....6
1.5.2 Toll-like receptor-4....8
1.5.3 C-type lectin receptors....9
1.6 Role of dendritic cells in allergy responses....10
1.7 Pattern recognition receptors....12
1.7.1 Toll-like receptors in allergen recognition....12
1.7.2 C-type lectin receptors in allergen recognition....13
1.7.3 Crosstalk between CLRs and TLRs....22
1.8 Indoleamine 2,3 dioxygenase....23
1.8.1 Regulation of indoleamine 2,3 dioxygenase in dendritic cells....26
1.8.2 The role of indoleamine 2,3 dioxygenase in allergic responses....28
1.8.3 The role of aryl-hydrocarbon receptor in modulating indoleamine 2,3-dioxygenase and implications in allergic responses....30
1.9 Hypothesis and aims....31
2 Chapter II: General Material and Methods....33
2.1 Generation of human DCs from blood samples....33
2.2 Quantification of IDO activity....33
2.3 Viability assay....34
2.4 Flow cytometry analysis....34

2.5 mRNA isolation, cDNA synthesis and PCR....35
2.6 Cytokine measurements....36
2.7 RNA interference....37
2.8 Reverse phase protein microarray....37
2.9 DC-T cell co-culture....38
2.10 Statistical analysis....39
3 Chapter III: The role of MR in modulating IDO and subsequent immune responses....40
3.1 Introduction....40
3.2 Results....42
3.2.1 TLR4 agonist induce IDO1 and IDO2 in human dendritic cells....42
3.2.2 TLR4-induction of IDO1 and IDO2 is down-regulated by allergens from diverse sources....44
3.2.3 Mannose-based ligands down-regulate TLR4-induced IL-12p70 production in human dendritic cells and affect T helper cell polarization....46
3.2.4 Mannose receptor mediates the IDO down-regulation by mannose-based ligands…....48
3.2.5 IDO regulation in human dendritic cells is partially dependent on AhR50
3.2.6 The NF-κB pathway is negatively regulated by MR in human dendritic cells……....53
3.3 Discussion....55
4 Chapter IV: The role of DC-SIGN in modulating IDO and subsequent immune responses....62
4.1 Introduction....62
4.2 Results....64
4.2.1 Fucose-mediated down-regulation of IDO impairs Th1 cell polarization….....64
4.2.2 Fucose-specific DC-SIGN signalling affects the non-canonical NF-κB pathway......66
4.2.3 DC-SIGN engagement with mannose and fucose-based ligands differentially modulates IDO activity in human DCs....68
4.3 Discussion....72
5 Chapter V: The role of TLR4 engagement in modulating IDO....78
5.1 Introduction....78
5.2 Results....80

5.2.1 LPS conditioning induces high levels of both IDO isoforms and AhR in human DCs....80
5.2.2 LPS conditioning induce an anti-inflammatory profile in human DCs..82
5.2.3 LPS conditioning favours the non-canonical NF-κB pathway in human DCs.....83
5.3 Discussion....86
6 Chapter VI: The role of TLR9 engagement in modulating IDO....90
6.1 Introduction....90
6.2 Results....91
6.2.1 CpG-A down-regulates IDO activity in human myeloid DCs without affecting cell viability....91
6.2.2 CpG-A interfere in LPS-induced cytokine production in DCs....93
6.3 Discussion....94
7 Chapter VII: General Discussion....98
8 Chapter VIII: References....109

Table of Figures

Chapter I

Figure 1.1: The allergic cascade..... 3
Figure 1.2: Role of airway epithelial cells (AECs) in allergen sensitization.. 7
Figure 1.3: Schematic representation of the polypeptide and domain structures of membrane-associated CLRs. 14
Figure 1.4: Squematic representation of the polypeptide and domain structures of galectin receptors.. 18
Figure 1.5: Squematic representation of the polypeptide and domain structures of soluble collectins. 21
Figure 1.6: Interactions between CLRs and TLRs in an allergy context.. 23
Figure 1.7: The kynurenine pathway.. 24
Figure 1.8: Jak-STAT signalling pathway of IDO induction by IFN-γ in DCs.. 26
Figure 1.9: Reverse signalling and non-canonical NF-κB signalling pathway of IDO induction in pDCs.. 28

Chapter III

Figure 3.1: TLR4 agonist induces IDO activity in human DCs.. 42
Figure 3.2: TLR4 agonist induces IDO1 and IDO2 in human DCs. 43
Figure 3.3: TLR4-induction of IDO activity is down-regulated by allergens from diverse sources..... 44
Figure 3.4: TLR4-induction of IDO1 and IDO2 is down-regulated by allergens from diverse sources..... 45
Figure 3.5: Mannose-based ligands down-regulate TLR4-induced IL-12p70 production by human DCs.. 46
Figure 3.6: Stimulation of human DCs with mannose-based ligands affect T helper cell polarization. 47
Figure 3.7: MR down-regulation by gene silencing. 48
Figure 3.8: MR down-regulation does not affect the expression of additional relevant molecules. 49
Figure 3.9: Mannose receptor mediates the IDO down-regulation by mannose-based ligands..... 50
Figure 3.10: AhR down-regulation by gene silencing.. 50
Figure 3.11: AhR down-regulation does not affect the expression of additional relevant molecules... 51
Figure 3.12: IDO regulation in human DCs is partially dependent on AhR. 52
Figure 3.13: Mannose-based ligands down-regulate TLR4-induced AhR expression and AhR activity by human DCs.. 52
Figure 3.14: The NF-κB pathway is negatively regulated by MR in human DCs.. 53
Figure 3.15: The NF-κB pathway is negatively regulated by MR in human DCs.. 54

Figure 3.16: The NF-κB pathway is negatively regulated by MR in human DCs. 55
Figure 3.17: MR-mediated IDO down-regulation in human DCs involved the transcription factors AhR and RelB, having an impact on T helper cell polarization..58

Chapter IV

Figure 4.1: Fucose-specific DC-SIGN engagement down-regulates IDO activity in human DCs. 64
Figure 4.2: Fucose-specific DC-SIGN engagement modulates IDO activity in human DCs affecting T helper polarization.. 65
Figure 4.3: Fucose-specific DC-SIGN engagement modulates cytokine production in human DCs.. 66
Figure 4.4: Fucose-specific DC-SIGN signalling involves the non-canonical NF-κB pathway in human DCs.. 67
Figure 4.5: Fucose-specific DC-SIGN signalling involves the non-canonical NF-κB pathway in human DCs. 68
Figure 4.6: Fucose-specific DC-SIGN agonist (Lewis-X) and mannan down-regulate IDO activity in human DCs.. 69
Figure 4.7: DC-SIGN and MR specific knock-down using gene silencing. 70
Figure 4.8: DC-SIGN and MR specific knock-down using gene silencing. 70
Figure 4.9: DC-SIGN and MR differentially regulate IDO activity in response to mannan in human DCs... 71
Figure 4.10: DC-SIGN and MR can modulate TLR4-mediated induction of IDO in human DCs. 76

Chapter V

Figure 5.1: IDO1 and IDO2 are induced in LPS-conditioned human DCs but not monocytes.. 81
Figure 5.2: AhR is induced in LPS-conditioned human DCs. 82
Figure 5.3: LPS-conditioning modulates cytokine production in human DCs.. 83
Figure 5.4: LPS-conditioning favours the non-canonical NF-κB pathway in human DCs. 84
Figure 5.5: LPS-conditioning favours the non-canonical NF-κB pathway in human DCs.. 85

Chapter VI

Figure 6.1: CpG suppresses IDO activity in human myeloid DCs.. 92
Figure 6.2: TLR9 stimulation of human DCs does not affect cell viability. 93
Figure 6.3: TLR9 stimulation potentiates a pro-inflammatory phenotype in response to TLR4 stimulation in human DCs.. 94

Abbreviations

1MT	1-methyl-tryptophan
Ab	Antibody
AFU	Arbitrary fluorescence units
AhR	Aryl hydrocarbon receptor
APC	Antigen presenting cell
ARNT	Ah receptor nuclear translocator protein
BGP	Bermuda grass pollen
CLR	C-type lectin receptor
CpG	Cytosine-phosphate guanosine
CRD	Carbohydrate recognition domain
CT-DC	Control dendritic cell
CTLD	C-type lectin-like domain
CYP1A1	Cytochrome P450 1A1
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin
DRE	Dioxin responsive elements
EC	Epithelial cell
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	German cockroach
GCN2	General control of non-repressed 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDM	House dust mite
IDO	Indoleamine 2,3-dioxygenase
IgE	Immunoglobulin-E
iITAM	Incomplete immunoreceptor tyrosine-based activation motif
IκB	Inhibitor of kappaB
IKK	IκB kinase

Abbreviations

ILC	Innate lymphoid cell
IRAK	Interleukin-1 receptor-associated kinase
KYN	Kynurenine
Le^X	Lewis-X
LPS	Lipopolysaccharide
Man-LAM	Mannose-capped lipoarabinomannans
MBL	Mannose-binding lectin
MD-2	Myeloid differentiation protein-2
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
Mo-DC	Monocyte-derived DC
MR	Mannose receptor
MyD88	Myeloid differentiation primary response protein 88
NF-κB	Nuclear factor kappaB
NIK	NF-κB inducing kinase
NO	Nitric oxide
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PAR	Protease activated receptor
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid DC
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
siRNA	Small interfering RNA
SP	Surfactant protein
TCDD	2,3,7,8-terachlorobenzo-p-dioxin
TDO	Tryptophan 2,3-dioxygenase
TGF-β	Transforming growth factor-β

Th2	T helper type 2
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
Treg	Regulatory T cell
TRP	Tryptophan
TSLP	Thymic stromal lymphopoietin

1. Chapter I: Introduction

1.1 The immune system

We are continuously exposed to an array of microorganisms and foreign substances that are inhaled, ingested or inhabit our skin and mucous membranes. Depending on their pathogenicity and host defence mechanisms, they can infect our organs and cause disease. The immune system is an interactive network of lymphoid organs, organised tissues, cells and soluble and humoral factors that are involved in host defence by counteracting infection as well as tumours. However, under certain conditions it can generate exacerbated responses against innocuous substances or normal tissues resulting in chronic conditions such as allergy and autoimmunity respectively. In addition, when a component of the immune system is absent or not working properly, it can result in an immune deficiency disease (1).

In order to eliminate pathogens and toxins, the immune system relies on detecting structural features on them known as pathogen associated molecular patterns (PAMPs), which help it to discriminate between self and non-self, avoiding damage to own tissues. The mechanisms permitting recognition of microbial, toxic or allergenic structures can be divided into two general categories. The first set of responses constitutes the innate immune system, whose components are encoded by genes in the germline and therefore acts rapidly against invading substances. The following set of responses constitutes the adaptive immune system encoded by gene elements that somatically rearrange to assemble antigen-specific molecules. Thus, the adaptive response is temporal and produces long-lived cells, which confer immunological memory and rapid response in the event of re-exposure (2, 3).

1.2 Asthma as a syndrome

Asthma is a very complex and multifactorial disease of the airways that affects more than 300 million people worldwide, with increased incidence in affluent societies. There are several phenotypes of asthma with distinct pathophysiology defined as endotype clusters of asthma. They have differences in terms of genetic susceptibility, environmental risk factors, age of onset, clinical presentation, prognosis and response to standard and new therapies (4). Genome-wide association and epigenetic studies have identified variations in several genes as well as specific signatures in non-coding elements that can increase the risk of the disease. Environmental factors such as smoke and pollutants can trigger or exacerbate asthma as well as modify the response to therapy (5, 6). People with asthma who smoke exhibit a clinical overlap with chronic obstructive pulmonary disease (7). The hygiene hypothesis proposes that the lack of early childhood exposure to infectious agents together with an augmented use of antibiotics increase susceptibility to allergic diseases in later life (8). In addition, asthma is associated with increased susceptibility to infection with respiratory viruses and with changes in the composition of microbial population colonizing the airways (9). Imbalance in diet that leads to obesity or micronutrient deficiency can promote inflammation, and have also been suggested as factors that influence susceptibility to allergic diseases and asthma (10). Unravelling all these mechanisms could help to better understand the pathogenesis of the disease and develop more effective therapeutic strategies to combat asthma-related pathologies.

1.3 Type-I hypersensitivity and allergy

Hypersensitivity is an undesirable reaction of the immune system. The original Gell and Coomb's classification in the 1960s categorizes hypersensitivity reactions into

four subtypes according to the type of immune response and the effector mechanism responsible for cell and tissue injury. These categories include type-I or immunoglobulin-E (IgE)-mediated hypersensitivity, type-II or antibody (Ab)-dependent hypersensitivity often referred to as cytotoxic, type-III or immune complex-mediated hypersensitivity and type-IV delayed hypersensitivity mediated by memory T cell as well as effector cells such as macrophages, eosinophils and neutrophils. Based on our current understanding of the immune response, this classification has been recently reviewed and new subcategories have been proposed (11-13).

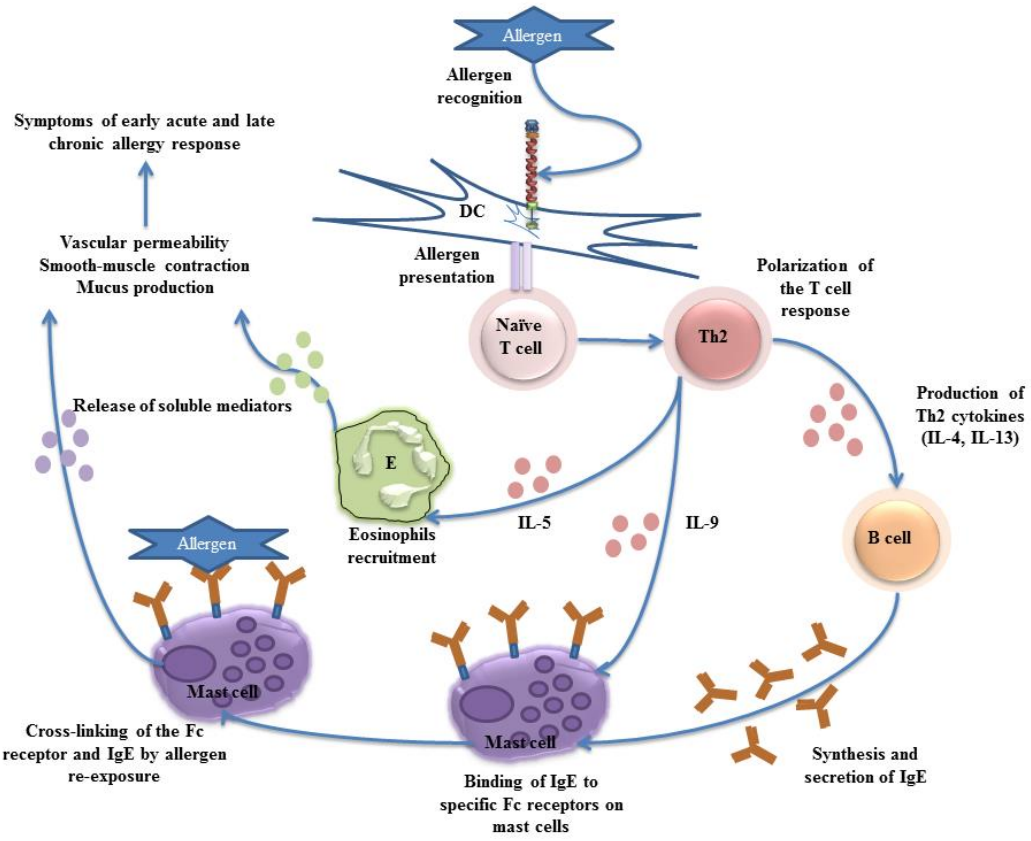

Figure 1.1: The allergic cascade. The allergic response starts with the recognition, processing and presentation of allergens by dendritic cells (DCs), followed by Th2 polarization and IgE production. IgE binds to specific Fc receptors on mast cells and in an event of re-exposure the cross-linking of the receptor with IgE facilitated by the allergen induces the degranulation of mast cells and the release of the soluble mediators of allergy. Adapted from (14).

Type-I hypersensitivity or allergy is an immediate exacerbated immune response against otherwise innocuous antigens in sensitized individuals after re-exposure. By means of direct contact, inhalation, ingestion or injection, allergens can cause an allergic reaction mediated by the synthesis of IgE known as atopy **(Figure 1.1)** (14). The disease starts with allergic sensitization, often accompanied by eczema and followed by allergic rhinitis. It is also associated with asthma, a complex and heterogeneous disorder characterized by a chronic inflammation of the lungs as well as airway hyper-reactivity and remodelling and by symptoms of recurrent wheezing, coughing and dyspnoea. In allergic asthma, the most common form of asthma, allergen specific T helper type 2 (Th2) cells are the central controllers of the disease through the production of diverse cytokines, such as IL-4, IL-5, IL-13 and IL-9. Particularly, IL-4 mediates class switching to allergen-specific IgE production by B cells, IL-13 induces goblet cell metaplasia and mucus secretion leading to airway obstruction and hyper-responsiveness, IL-5 induces eosinophilia contributing to chronic conditions and airway remodelling and IL-9 mediates the recruitment and growth of mast cells **(Figure 1.1)**. However, more recently other innate and adaptive immune cells as well as structural cells have been shown to be pivotal in initiating, maintaining and exacerbating allergic responses (15-19). It is worth mentioning that additional cytokines milieu and cellular features have been observed in diverse endotype of asthma, these includes non-atopic asthma with a Th2 high endotype cluster and eosinophilia as well as non-allergic intrinsic asthma with a mixed Th1 and Th17 cytokine milieu and neutrophilia. In addition, there are some patients that exhibit overlapping cytokine profiles, which are often associated with disease severity (4, 19).

1.4 Emerging role of innate immune cells in allergy

Initially, studies with mice deficient in the recombination-activating gene (RAG) recombinase had suggested that there were additional mechanisms of inducing type-2 cytokines as well as eosinophilia independent of the adaptive immune system (19, 20). Studies over the past five years have identified innate lymphoid cells (ILCs) as part of the innate immune system with the ability to communicate with immune and non-immune cells orchestrating immunity, inflammation and homeostasis in diverse tissues. Non-cytotoxic ILCs consist of three groups (ILC1s, ILC2s and ILC3s) defined on the basis of their differential requirements for transcription factors during development, their pattern of expression of effector cytokines and the acquisition of other effector functions (21). ILC2 cells do not express antigen specific receptors, but like Th2 cells they react to epithelium derived cytokines including IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). They become active at early stages after allergen exposure and their production of Th2 cytokines, such as IL-13, IL-5 and IL-9, can induce allergic asthma in a T cell-independent manner (22-24). In addition, ILC2 cells contribute to the response to respiratory virus, which could explain how viral exposure can induce or exacerbate asthma (25, 26). Despite the rapid increase in knowledge about ILC2 cells in experimental models of type-2 immunity, still very little is understood about their clinical relevance in human asthma. In this context, it has been suggested that ILC2 cells might be involved in a subset of patients with severe non-allergic eosinophilic airway inflammation that exhibit a characteristic Th2 cytokine signature (19).

1.5 Role of epithelial cells in allergen recognition

Airway epithelial cells (ECs) constitute the first line of defence against pathogens and allergens by not only forming a physical barrier, but also through expressing a wide range of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and protease activated receptors (PARs). These receptors enable ECs to recognize microbial motifs and environmental allergens which lead to cascade of events culminating in the release of cytokines including TSLP, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-25, IL-33, IL-1β, chemokine ligands such as CCL2 and CCL20 and dangers signals including adenosine triphosphate (ATP) and uric acid which recruit and activate other immune cells (27-29) (**Figure 1.2**).

1.5.1 Protease activated receptors

PARs are G-protein coupled receptors characterized by a self-activation mechanism following proteolytic cleavage of their extracellular N-terminal domain. There are four PAR members identified to date. PAR-1,-3,-4 respond to the protease thrombin, expressed primarily by cells in the vasculature; and they are mainly involved in thrombosis. Conversely, PAR-2 is activated by trypsin-like serine proteases but not by trypsin and can be found in airways, vascular, skin, and intestinal cells, and mediates proliferative and inflammatory responses linked to tissue damage (30, 31). Due to its ability to respond to serine proteases, proteolytic allergens from diverse sources such as house dust mite (HDM), cockroach, pollen or mold can act as exogenous activators of PAR-2 with implications in allergy and asthma.

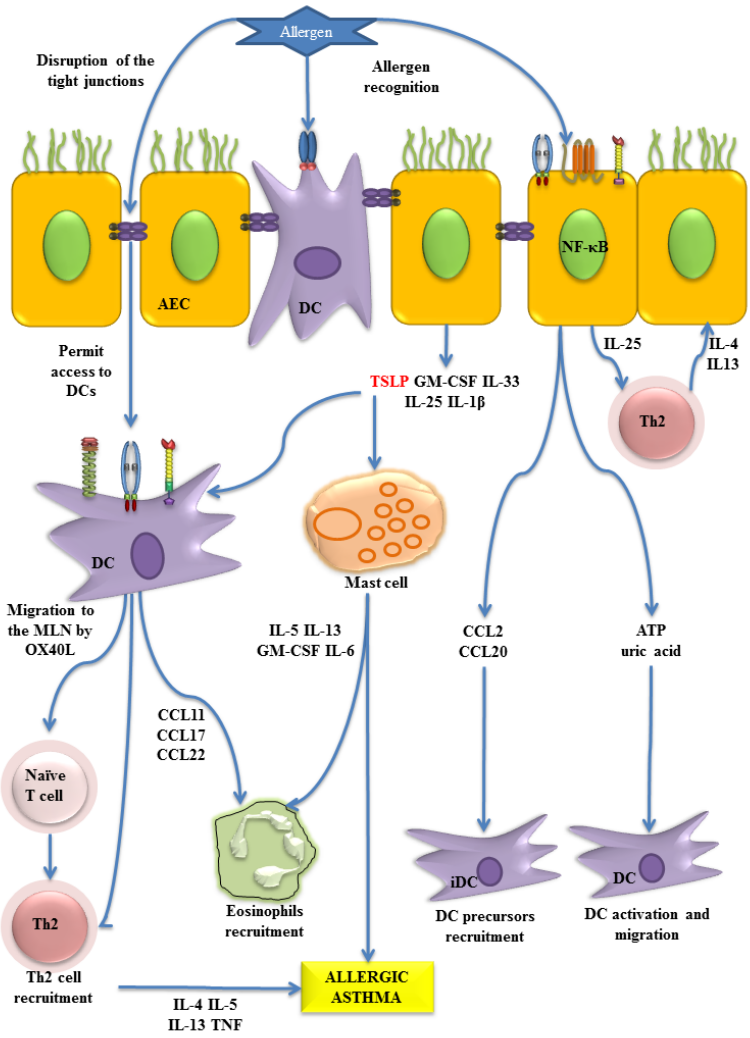

Figure 1.2: Role of airway epithelial cells (AECs) in allergen sensitization. AECs can directly recognize allergens by diverse PRRs inducing the production of cytokines, chemokine ligands and danger signals. TSLP has diverse effects on DCs and mast cells, which lead, in absence of IL-12, to cell recruitment, Th2 polarization and the onset of allergic asthma (27).

Different cysteine and serine proteases from HDM, pollen and the fungus *Penicillium* have been shown to increase epithelial permeability and disrupt the tight junctions by mainly targeting the transmembrane adhesion proteins occludin and zonula occludens-1 (32-38). In addition, it has been demonstrated that allergen-induced cytokine production, cell detachment and morphological changes in ECs is largely dependent

on allergens' protease activity (36, 39-42). For instance, it was demonstrated that IL-33 contributes to protease-dependent allergic airway inflammation *in vivo* (43). Main allergens from HDM, cockroach and the fungus *Penicillium* can activate PAR-2 and induce the release of pro-inflammatory cytokines (44-47). In an *in vivo* model of allergy, only when the allergens are administered through the mucosa, cockroach proteases regulate chemokine production and DC recruitment in a PAR-2-dependent manner (48, 49). More recently, PAR-2-mediated allergic sensitization was shown to be associated with the tumour necrosis factor (TNF) signalling pathway (50, 51).

In addition to the proteolytic activity of some allergens, it was demonstrated that HDM-induced redistribution of E-cadherin, a key molecule involved in EC adhesion, was mediated via epidermal growth factor receptor-dependent activation of PAR-2 and transforming growth factor-β (TGF-β) enhanced this signalling (52). Nevertheless, not only PAR-2 is directly involved in allergen-mediated cytokine production, as PAR-1 and PAR-3 has also been shown to be activated by different fungal allergens (47, 53).

1.5.2 Toll-like receptor-4

LPS is recognised by TLR4 with the participation of accessory proteins including CD14, LPS binding protein and myeloid differentiation protein-2 (MD-2). This leads to the recruitment of the signalling adapter protein myeloid differentiation primary response protein 88 (MyD88), the activation of the transcription nuclear factor kappaB (NF-κB) among others, and the expression of pro-inflammatory cytokines (54). TLR4 (55-58), MyD88 (57, 59, 60) and NF-κB (61-64) have been shown to be crucial in the elicitation of allergic Th2 immune responses. Different studies using knock-out mice have demonstrated the importance of TLR4 expression in both hematopoietic

radiosensitive and structural radioresistant cells in the induction of TLR4-dependent Th2 responses to intranasal allergens in the presence of endotoxin (55-58). In addition, it has been shown that the LPS dosage is crucial in driving either Th1 or Th2 responses, with lower levels of LPS inducing Th2 responses to inhaled allergens in a mouse model of allergic sensitization (65). Furthermore, it was shown that short ragweed pollen acts as a TLR4 agonist, initiating TLR4-dependet TSLP/OX40L signalling pathway, triggering Th2 allergic inflammation (66).

1.5.3 C-type lectin receptors

CLRs are receptors that recognize oligosaccharide moieties among other molecular patterns on antigens including allergens (67). CLR's role in allergen recognition and uptake by DCs is well established (68), which will be further discussed in the upcoming sections. CLRs have also been shown to participate in allergen recognition by ECs. It was demonstrated that HDM induction of CCL20 by ECs was not protease or TLR4/2 dependent; however, it was mediated by β-glucan moieties within HDM extract. This effect was specific for HDM because other allergens, such as cockroach and ragweed, failed to induce this response (69). The authors suggest the involvement of the dectin-1/Syk signalling pathway, since Syk inhibition abrogated the HDM-induced CCL20 production (70).

More recently, our group demonstrated that TSLP secretion by ECs upon stimulation with Der p 1 was at least partly carbohydrate dependent. In addition, DC uptake of deglycosylated Der p 1 was considerably decreased compared with its natural (glycosylated) counterpart, indicating that glycosylation of allergens plays a crucial role in their recognition by immune and non-immune cells (71). Interestingly, a study in patients suffering from cow milk allergy has shown that their duodenal

intraepithelial lymphocytes preferentially bind Galectin-3 expressed by intestinal epithelial cells and macrophages compared to healthy controls (72). These data clearly suggest a potential role for Galectin-3-glycan interactions in modulating the epithelial-immune cell cross-talk during allergic inflammation (72).

1.6 Role of dendritic cells in allergy responses

It is clear that T cell and ILCs play a key role in controlling airway inflammation. However, these cells need to be activated by other cellular components in order to drive inflammation. Particularly, DCs have been shown to be pivotal in the initial steps of allergic recognition and sensitization (27, 68). Antigen recognition and uptake by DCs is an important first step in the sequence of events that leads to the induction of the adaptive immune response **(Figure 1.1)**. Immature DCs reside in the periphery and can effectively sample the microenvironment for antigens. Once taken up by DCs, such antigens are processed into peptides and appear on the surface of DCs in the context of major histocompatibility complex (MHC) molecules. Antigen bearing DCs migrate to the local lymph nodes where through expression of MHC-II-peptide complex, cytokines, and co-stimulatory molecules they can stimulate naïve T cells toward distinct effector T cell subsets (e.g., Th1, Th2, Th17) or induce tolerance through induction of regulatory T cells (Tregs), depending on the nature of the antigen and other microenvironmental factors (73). In an allergy context, the sequence of events starts with the recognition of an allergen by DCs, followed by Th2 cell differentiation, IgE production and mast cell sensitization and triggering. During re-exposure, the cross-linking of Fcε receptor-bound IgE on the surface of mast cells and basophils by allergens promotes the release of soluble mediators, such as histamine,

leukotriene and prostaglandins, and onset of the allergic reaction (16, 74) **(Figure 1.1)**.

Several reports have demonstrated that DCs are necessary and sufficient for induction of Th2 immunity against allergens (75, 76). Depletion of lung $CD11c^+$ DCs during allergen challenge suppresses the features of asthma (77) and persistent activation of DCs can break tolerance to inhaled allergens *in vivo* (78). Besides the key role of DCs in the induction and re-elicitation of Th2-mediated allergic disease, their interaction with other non-immune cells such as ECs has been shown to be pivotal in modulating DC behaviour and subsequent immune responses. Intraepithelial DCs are able to form tight junctions with ECs through expression of proteins like occluding and claudin, which can further facilitates antigen/allergen recognition and uptake (79, 80). Some allergens with proteolytic activity can gain access to DCs by disrupting the tight junctions (32-38). More recently, it has been shown that LPS, through the TLR4 signalling pathway, can also regulate tight junction permeability (81). In addition, ECs have been demonstrated to be able to recognize diverse allergens leading to the release of chemokines, cytokines and danger signals that activate and recruit other immune cells to the site of inflammation (16, 19, 82) (**Figure 1.2**). Epithelium-derived cytokines favours Th2 immunity by inducing OX40L-dependent DCs migration and their subsequent activation and by activating ILC2 cells and basophils to produce Th2 polarizing cytokines such as IL-4, IL-5 and IL-13 (23, 83-85). Despite all the advances in understanding how Th2 immunity to inhaled antigens develops in murine studies, we still do not know if this model is true for all allergens in humans and how genetics and environment can influence diverse cellular cross-talk that leads to allergy.

1.7 Pattern recognition receptors

DCs serve as sentinels of the mucosa surfaces, where they constantly sample their milieu for conserved motifs on antigens by using PRRs, such as TLRs, CLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), RIG-I-like receptors (RLRs) and AIM2-like-receptors (ALRs). These receptors effectively increase their internalization efficiency and deliver information regarding the presence of danger signals (86).

1.7.1 Toll-like receptors in allergen recognition

TLRs are type-I integral membrane glycoproteins, each with an extracellular N-terminal ligand recognition domain constructed of tandem copies of a leucine-rich repeat (LRR) motif that provides ligand specificity, a single transmembrane helix, which participates in nucleic acid PAMPs recognition, and a C-terminal cytoplasmic signalling domain known as Toll IL-1 receptor domain (TIR). The receptors homo or heterodimerize upon ligand binding followed by recruitment and signalling through diverse sets of adaptor proteins leading to different transcriptional outcomes (86).

There are more than ten different TLRs identified in humans so far, with some of them being involved in allergen recognition or pathways that induce allergic responses (27, 68). Within this context, mainly three different mechanisms have been proposed. Der p 2, a major allergen from HDM with lipid binding activity, has been shown to induce signalling through TLR2 (87) and TLR4 (88) depending on the cell type involved. Due to its high homology with MD-2, which is the lipopolysaccharide (LPS)-binding component of the TLR4 signalling pathway, Der p 2 forms a complex with TLR4 that signals similarly to MD-2/TLR4 complex inducing innate immune activation (88). In this regard, it is worth highlighting that TLR4 signalling and LPS exposure have been

shown to play a key role in Th2-mediated inflammation and asthma (65, 89-91). In addition, HDM extracts contaminated with flagellin can induce TLR5-dependent allergic responses in mice, however, the mechanism is still unclear (92). More recently, it has been shown that cleavage of fibrinogen by fungal proteinases elicits allergic responses through macrophage activation in a TLR4-dependent manner (93). The second mechanism involves sensitization by nickel, which may not be relevant in the context of airway sensitization but highlights the importance of TLR4 on DCs in allergic reactions. Nickel and other bivalent metals such as cobalt induce a lipid-independent activation of TLR4, which is dependent on the presence of two histidine residues, promoting TLR4 dimerization and subsequent receptor activation independently of MD-2 (94, 95). Finally, the last mechanism involves allergens such as Fel d 1 and Can f 6 that belong to the lipocalin family and cause enhanced LPS-induced TLR4 activation in an MD-2 dependent and independent manner respectively (96).

1.7.2 C-type lectin receptors in allergen recognition

CLRs constitute a superfamily of more than 1,000 proteins classified into 17 subgroups based on domain organization and phylogeny. They are all characterized by the presence of a C-type lectin-like domain (CTLD) also known as carbohydrate recognition domain (CRD) that enables their recognition and uptake of glycoantigens in a Ca^{2+}-dependent manner (67). Diverse membrane-associated CLRs, including mannose receptor (MR), dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and Dectins, as well as soluble lectins i.e. collectins and galectins, have been shown to act as receptors for allergens in human DCs (27, 68) (**Figure 1.3**).

1.7.2.1 Mannose receptor

MR is a 175 kDa type-I integral transmembrane protein primarily expressed by myeloid cells such as macrophages and DCs. The extracellular portion of MR consists of three regions: a cysteine-rich domain, a fibronectin type II-like domain that mediates collagen binding and eight CRDs. These are followed by a transmembrane region and a short COOH terminal hydrophilic cytoplasmic domain, which participates in receptor internalization and recycling.

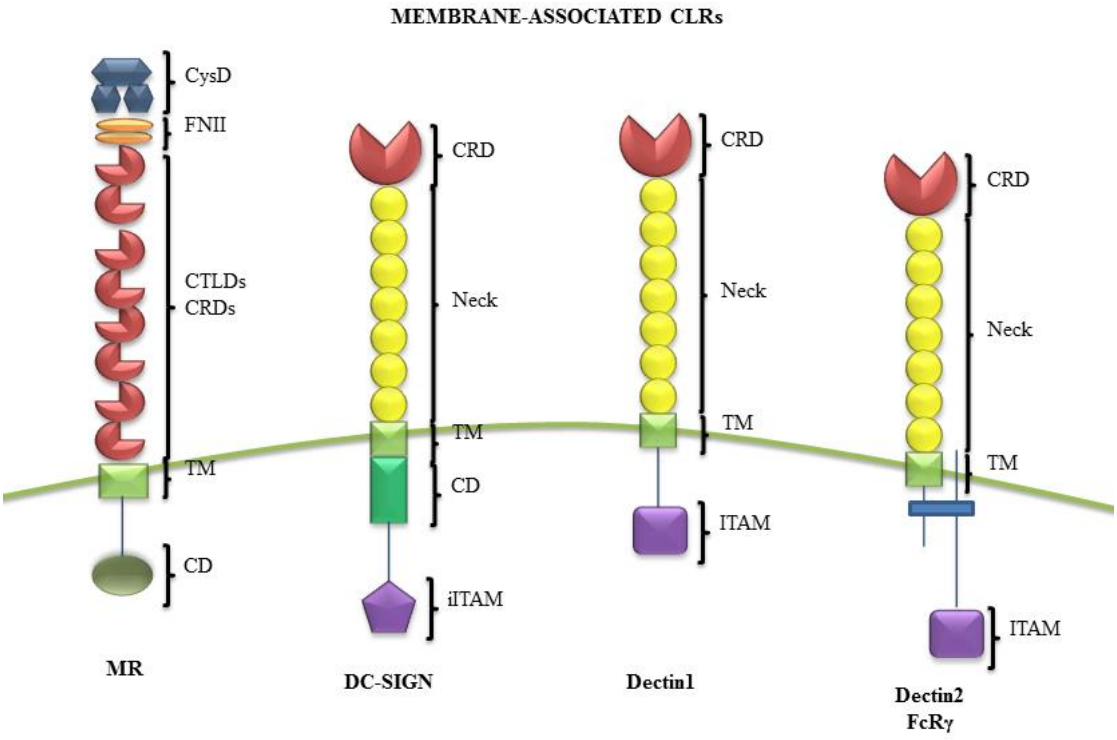

Figure 1.3: Schematic representation of the polypeptide and domain structures of membrane-associated CLRs. CysD: cysteine rich domain, FNII: fibronectin type II-like domain, CTLD: C-type lectin-like domains, TM: transmembrane region, CD: cytoplasmic domain, CRD: carbohydrate recognition domain, iITAM: incomplete immunoreceptor tyrosine-based activation motif (68).

The MR is a multifunctional receptor with two lectin activities involving Ca^{2+}-dependent recognition of carbohydrates terminated in L-fucose, D-mannose or N-acetyl glucosamine through CTLD, as well as Ca^{2+}-independent binding of sulphated acidic glycans through the cysteine-rich domain (27, 68, 97) (**Figure 1.3**).

Interestingly, DCs from patients with HDM allergy have been shown to express higher levels of MR and to be more efficient in allergen uptake than DCs from non-atopic donors (98). Additionally, it was reported that bronchoalveolar lavage fluid from patients with asthma and/or allergic rhinitis contains higher numbers of MR expressing myeloid-DCs compared to healthy controls (99). Gene-mapping linkage analysis in both human subjects (100) and mice (101) have identified *Mrc1* as a positional candidate gene for allergen induced airway hyperresponsiveness, indicating a clear association between MR and asthma. In terms of allergen uptake, *in vitro* studies have found that MR expressed on human monocyte-derived DCs (Mo-DCs) is the main receptor for major allergens from HDM (*Der p* 1), dog (*Can f* 1), cockroach (*Bla g* 2), peanut (*Ara h* 1) and cat (*Fel d* 1) (102, 103). Also, it was shown that MR plays a crucial role in allergen induced Th2 cell differentiation, as demonstrated by a bias towards Th1 response when MR-deficient DCs - where MR expression was knocked down using a synthetic small interfering RNA (siRNA) – were stimulated with Der p 1 and co-cultured with naïve T cells from HDM atopic individuals. Interestingly, the reversal towards Th1 cell polarization by MR-deficient DCs was shown to be partially mediated through the up-regulation of indoleamine 2,3-dioxygenase (IDO) activity in DCs (102). IDO is an enzyme that participates in tryptophan (TRP) metabolism and is involved in many immune-regulatory processes in health and disease, whose role in allergy will be discussed in more detail in the upcoming sections (104, 105). Further studies showed that MR recognition of the

major cat allergen *Fel d* 1 mediated the production of specific IgE and IgG1 Abs in a mouse model of allergy (103). Other studies have shown that omega-1, a glycosylated T2 ribonuclease secreted by *Schistosoma mansoni* eggs, is recognized and internalized by DCs through MR and subsequently interferes with proteins synthesis and conditions DCs for Th2 priming (106). Collectively these data highlight MR's role in allergen recognition and promotion of Th2 mediated immune responses.

1.7.2.2 DC-SIGN

DC-SIGN is a 44 kDa type-II integral transmembrane protein that is mainly expressed by antigen presenting cells (APCs) and consists of four regions: a CRD, a hinge domain and a transmembrane region connected to a cytoplasmic signalling domain. The CRD forms part of two Ca^{2+} binding sites that can recognise mannose and fucose-containing ligands. The cytoplasmic domain contains internalization motifs as well as an incomplete immunoreceptor tyrosine-based activation motif (iITAM) (68, 107) (**Figure 1.3**).

DC-SIGN is mainly expressed by DCs and has been demonstrated to participate in the recognition of allergens from different sources, such as peanut (Ara h 1), HDM (Der p 1 and Der p 2), pollen (BG-60) and dog (Can f 1) (108-110). *In vitro* studies have been shown that DC-SIGN recognition and uptake of Der p 1 induces Th1 cell differentiation. On the contrary, DC-SIGN-deficient DCs bias the response towards a Th2 profile (110). This is in contrast to previous observations in MR-deficient DCs which seem to support Th1 differentiation (102). Interestingly, it has also been shown that Der p 1, using its enzymatic activity, can cleave (111) and induce the down-regulation (112) of cell surface DC-SIGN but not MR (111). In this context, it has been previously proposed that the Th1/Th2 balance in response to allergen exposure

can be determined by the cross-talk between these receptors and the level of their expression on DCs (68, 110). Accordingly, it is important to note that DCs from asthmatic patients show lower expression of DC-SIGN (112), which is in contrast to the high levels of MR expression reported in atopic individuals (98, 99). More recently, it has been shown that fucose-expressing extracellular pathogens by DC-SIGN engagement favour Th2 responses via activation of the atypical NF-κB family member Bcl3, which could be exploited in developing new strategies to prevent inflammation and allergy (113).

1.7.2.3 Dectin receptors

Dectins are type-II transmembrane proteins receptors with an extracellular domain containing a highly conserved CRD (**Figure 1.3**). Dectin-1 and Dectin-2 are both expressed by DCs and have been implicated in infectious and allergic diseases. Dectin-1 is a receptor for yeast β-glucan (70), whereas Dectin-2 is a PRR for fungi. Unlike Dectin-1, Dectin-2 does not have any signalling motif and employs an Fcγ receptor chain signal for internalization and activation of NF-κB signalling which leads to the up-regulation of TNF-α and IL-1ra (114). Furthermore, Dectin-2 has been shown to act as a receptor for HDM (*Dermatophagoides farinae* and *pteronyssinus*) and mould (*Aspergillus fumigatus*) extracts. Interestingly, the same study showed that recognition of allergen extracts by Dectin-2 could trigger cysteinyl leukotriene generation by murine DCs (115). Later, it was shown that Dectin-2, through triggering the generation of cysteinyl leukotrienes, could mediate type-2 immune responses, including pulmonary inflammation, against HDM allergens (116).

1.7.2.4 Galectin receptors

Galectins are a family of proteins that bind β-galactosides. These proteins are highly conserved and are expressed by diverse cell types including monocytes, DCs, macrophages, mast cells, B cells and T cells.

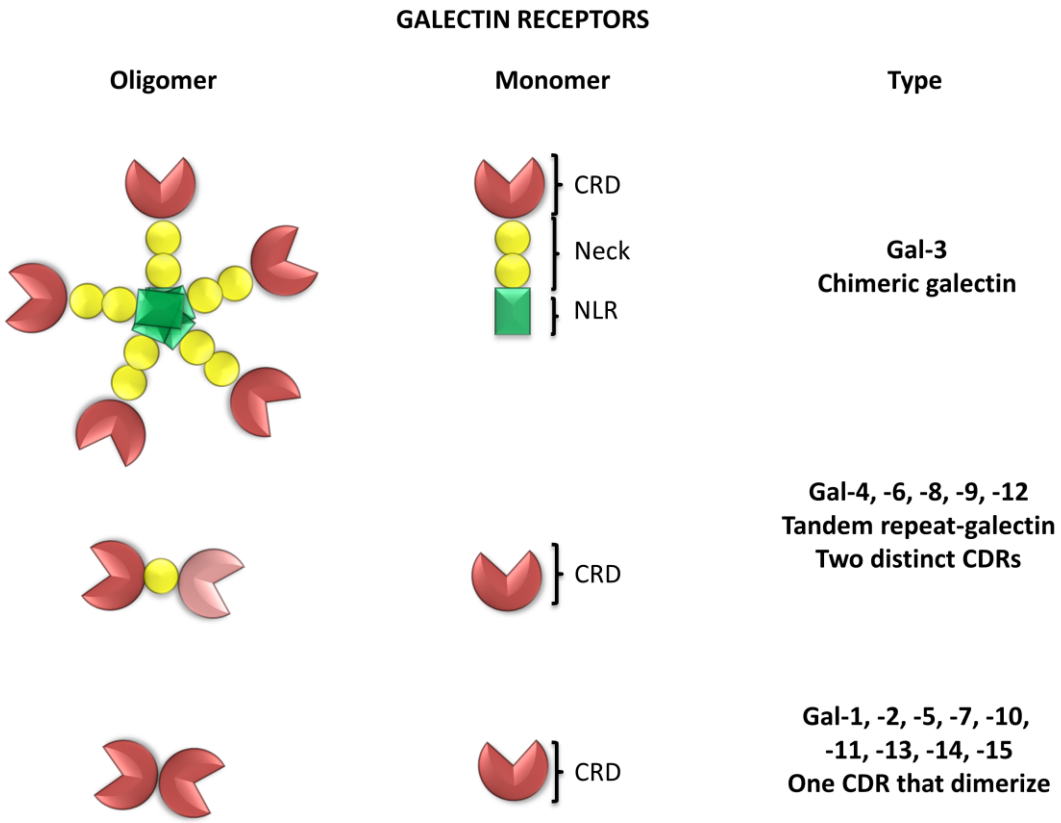

Figure 1.4: Squematic representation of the polypeptide and domain structures of galectin receptors. Gal: Galectin, CRD: carbohydrate recognized domain, NLR: non-lectin region (68).

There are three types of Galectins **(Figure 1.4)**. Galectins that consist entirely of one CRD, such as Galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15, and tandem repeat Galectins, such as Galectin-4, -6, -8, -9 and -12, which contain two homologous CRDs separated by a linker, as well as chimeric Galectins, such as Galectin-3, which

contains a CRD preceded by a non-lectin region consisting of short Pro/Gly-rich tandem repeats (117).

A number of Galectins, particularly Galectin-3 and Galectin-9, have been shown to play an important role in Th2 mediated immune responses. For example, it was shown that bone marrow derived mast cells (118), DCs (119) and leukocytes (120) from Galectin-$3^{-/-}$ mice exhibited a Th1-polarized response, compared with cells from Galectin-$3^{+/+}$ mice. The contribution of Galectin-3 in Th2 responses was further corroborated in murine models of atopic dermatitis, asthma and chronic allergic inflammation (121, 122). Galectin-9 has been shown to be able to efficiently bind IgE and block the formation of IgE-allergen complex alleviating asthmatic reactions in an experimental model of asthma by reducing Th2-associated airway inflammation (123, 124). By contrast, other *in vivo* studies have shown that Galectin-9 is partially involved in prolonged eosinophil accumulation in the lung, whereby Galectin-9 may serve as a recruiter of eosinophil granulocytes hence promoting dominant Th2 responses (125, 126).

1.7.2.5 Collectins

The collectins are a family of mammalian lectins where a CRD is attached to a collagen region via an alpha-coil domain (127) (**Figure 1.5**). Lung surfactant proteins (SP) A and D (SP-A and SP-D) synthesized by alveolar type II cells are amongst the six human collectins that have been described. Apart from their role in surfactant homeostasis, they have been demonstrated to take part in protection against allergens and respiratory pathogens (128). Both SP-A and SP-D have been shown to mediate the binding of pollen grains and pollen-allergen starch granules to alveolar type II cells and macrophages enhancing their phagocytosis (129, 130). They can also bind

directly to HDM extract, purified Der p 1, as well as allergens from *Aspergillus fumigatus* in a carbohydrate-specific and Ca^{2+}-dependent manner, inhibiting specific IgE binding to these allergens, blocking allergen-induced histamine release from basophils and reducing the proliferation of peripheral blood mononuclear cells (PBMCs) isolated from Der p 1-sensitive asthmatic children (131-133). Interestingly, two major mite allergens, Der p 1 and Der f 1, were shown to interact with SP-A and SP-D whereby these allergens, using their cysteine protease activity, could cleave and inactivate both surfactant proteins. This was associated with diminished binding to carbohydrates and reduced capacity to agglutinate bacteria, compromising a potential innate immune defence mechanism against allergens (134).

An *in vivo* study found that SP-$D^{-/-}$ mice were more vulnerable than wild-type mice to *Aspergillus fumigatus* sensitization, whereas SP-$A^{-/-}$ mice were virtually resistant (135). A previous study with a murine model of pulmonary inflammation showed that SP-D only participates in an initial resistance to ovalbumin (OVA), a protein structurally related to various allergens (136). Accordingly, it could be speculated that SP-A and SP-D mediate allergic responses through different mechanisms. Interestingly, it has been shown that SP-A inhibits LPS-mediated maturation of DCs and further allostimulation on T cells (137), while SP-D mediates the binding and uptake of *E.coli* by bone marrow derived mouse DCs, which leads to increase antigen presentation (138). The ability of SP-A and SP-D to decrease specific IgG and IgE levels could be explained by their potential to reduce the proliferation of specific B cells (139), and their ability to reduce IL-2 levels (127, 140, 141). However, shifting cellular responses to a Th1 profile seems to be central in SP-A and SP-D protective mechanisms, because IFN-γ promotes cellular immunity and blocks IL-4-mediated Th2 differentiation, which together with IL-13, is very important for isotype switching

of B cells (127). Accordingly, it has been shown that IL-13 is a potent stimulator of SP-D in the lung, increasing up to 70-fold in a murine model of IL-13 over-expression (142).

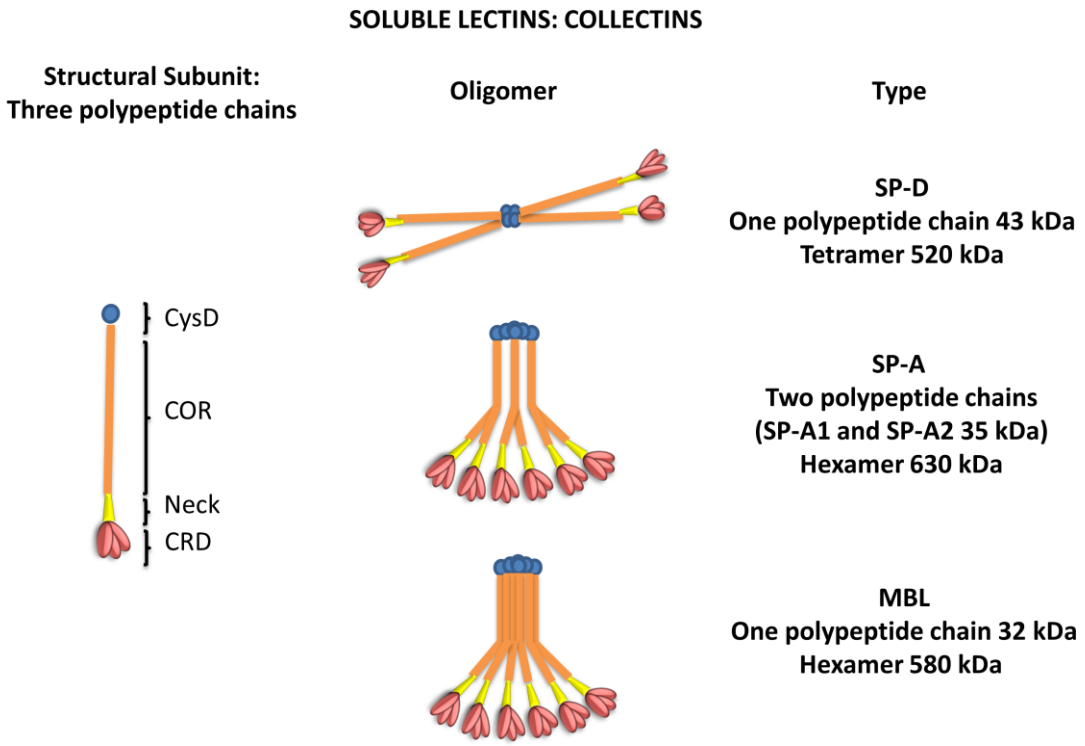

Figure 1.5: Squematic representation of the polypeptide and domain structures of soluble collectins. SP: surfactant protein, MBL: mannose-binding lectin, CysD: cysteine rich domain, COR: collagen region, CRD: carbohydrate recognition domain (68).

Finally, a link has been established between the plasma levels of the collectin mannose-binding lectin (MBL) and susceptibility to allergic responses (143). Moreover, one study identified a new polymorphism in an intronic zone of the MBL gene, which was associated with an increase in the plasma MBL levels and eosinophil counts in patients with bronchial asthma (144). Based on that, the authors suggested

that the high levels of MBL may contribute to the increase in complement activation and eosinophilia (145).

1.7.3 Crosstalk between CLRs and TLRs

It is clear that CLRs can facilitate access to and/or modulate TLR-induced responses, particularly TLR4. For instance, different CLRs, such as the MR (88, 102, 146), DC-SIGN (110, 113), and Galectin-9 (147), have all been linked to TLR signalling in Th2 allergic responses. Der p 2 has been shown to have structural and functional homology with MD-2. This gives Der p 2 an intrinsic adjuvant property and the ability to directly interact with the TLR4 complex and facilitate LPS signalling, which is thought to underpin Der p 2's allergenicity (88). It is interesting to note that Der p 2 has also been shown to be glycosylated, and its uptake by DCs is mediated - at least in part - through MR (102). Therefore, a synergy between binding of Der p 2 to the MR and its functional mimicry to MD-2 is conceivable. In addition, OVA up-regulates MR and TLR2 expression, and MR down-regulation affects allergen induced-DC maturation as well as TLR2 and notch 1 expression (146).

Interestingly, Der p 1 binding to MR in the presence of low levels of LPS can down-regulate IDO activity, which further bias the immune response towards a Th2 profile (102), highlighting the important role of the TLR4 signalling pathway in inducing Th2 allergic immune responses. Conversely, binding of Der p 1 to DC-SIGN in the presence of low levels of LPS skews the response to a protective Th1 profile (110), underlying the antagonistic role of MR and DC-SIGN in allergen recognition and the subsequent events leading to allergic sensitization. More recently, it has been found that fucosylated molecules can induce Th2 immune responses through DC-SIGN in a TLR4-dependent manner involving the activation of the inhibitor of kappaB (IκB)

family member Bcl-3 (113). Collectively, these data provide evidence for synergistic/antagonistic interactions between different PRRs, which most likely work in concert to bias immune responses toward a Th2 phenotype after allergen exposure (**Figure 1.6**).

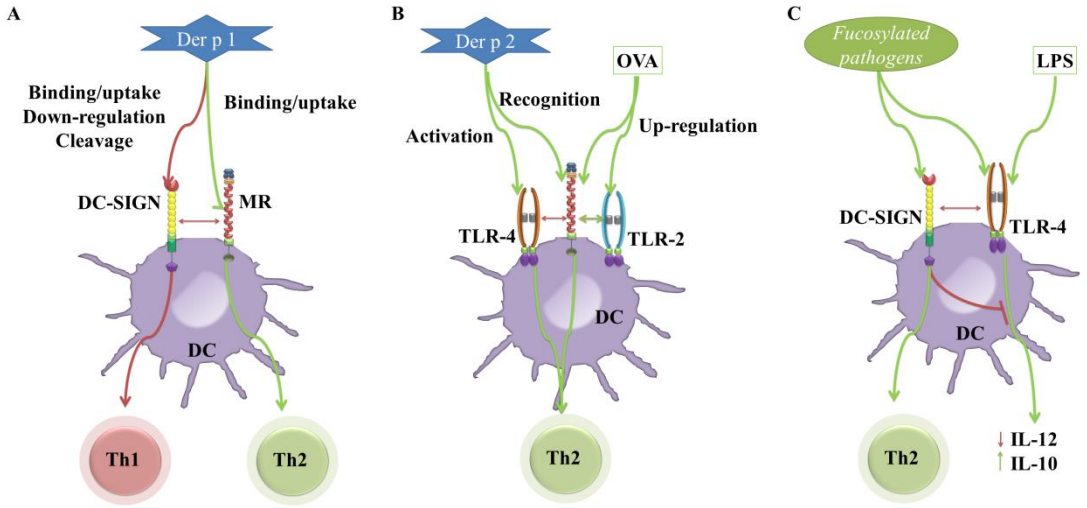

Figure 1.6: Interactions between CLRs and TLRs in an allergy context. A. Antagonistic effect between MR and DC-SIGN. B. Interactions between MR and TLRs. C. Interactions between DC-SIGN and TLR4. Activating signals are shown in green and inhibitory signals are shown in red. Adapted from (68).

1.8 Indoleamine 2,3 dioxygenase

DCs are key regulators of adaptive immunity by promoting or suppressing T-cell responses. One of the mechanisms that account for their induction of immune-suppression is their expression of the enzyme IDO (104, 105). IDO is an enzyme that breakdown the essential amino acid TRP, and plays a critical role in immune suppression and immune tolerance. Initially implicated as a counter-regulatory mechanism in fetal-maternal tolerance (148). IDO has also been found to be crucial in balancing inflammation and tolerance, including transplantation, tumour resistance, chronic infections, autoimmune diseases and allergic asthma (149, 150).

TRP is an amino acid that is indispensable for cell growth and functioning. The primary metabolic route of TRP *in vivo* is the kynurenine (KYN) pathway (**Figure 1.7**), which is essentially controlled by two rate-limiting enzymes: IDO (MW: 42 kDa) and its isoenzyme tryptophan 2,3-dioxygenase (TDO) (MW: 320 kDa) (151). TDO is preferentially synthesized by hepatocytes as a housekeeping gene. On the other hand, IDO is expressed by different cell types and can be up-regulated during the immune response, with IFN-γ being the main inducer (149, 152). Experimental data in the last decade have shown that IDO, but not TDO, has important immunosuppressive properties involved in immune tolerance and Th1/Th2 regulation.

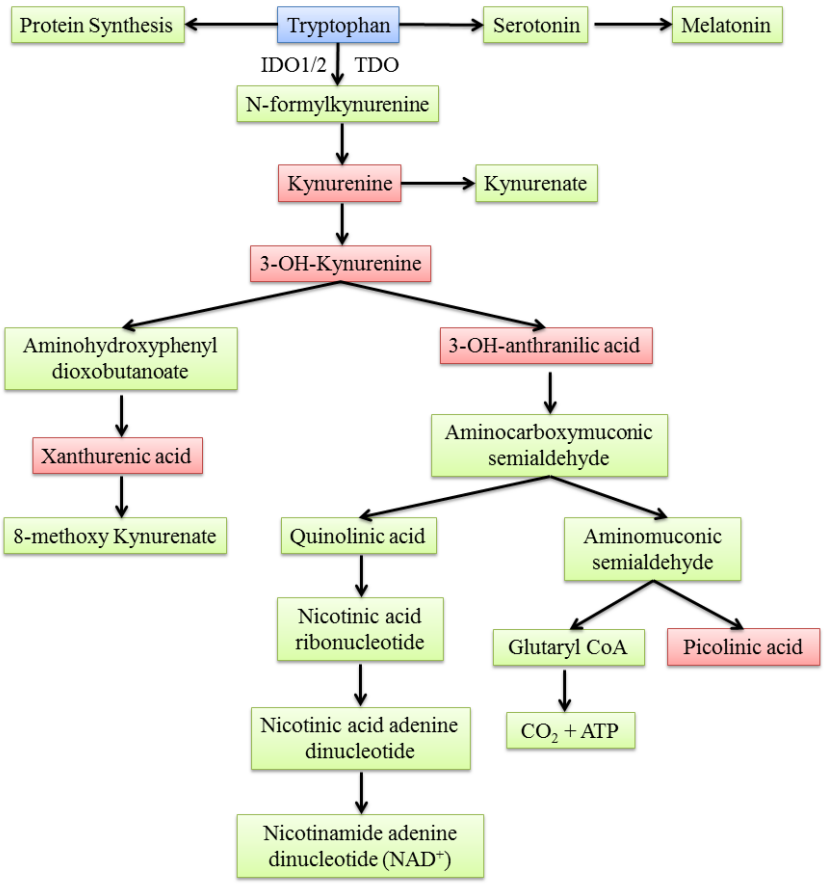

Figure 1.7: The kynurenine pathway. The essential amino acid tryptophan - which comes from the diet - can follow different pathways, such as protein synthesis, serotonin and melatonin synthesis as well as the KYN pathway essentially controlled by the limiting enzyme IDO. Immune-suppressive metabolites are shown in red. Adapted from (152).

Two IDO isozymes have been identified namely IDO1 and IDO2 (153), with IDO1 being the most extensively studied. Both genes have similar structures and are situated adjacent to each other on human and mouse chromosome 8. However, although both proteins have similar enzymatic activity, there are different expression patterns in some pathological condition, which suggest different roles in health and disease. For instance, IDO1 is up-regulated in malaria infection, because of the high levels of IFN-γ (154), however, IDO2 is poorly induced. Moreover, in IFN-$\gamma^{-/-}$ mice the levels of IDO2 are maintained, which suggest that IFN-γ does not exert any regulation over IDO2 (153). As far as substrate specificity is concerned, it has been shown that the Km of IDO2 for TRP is 500-1000 fold higher than that of IDO1, and under physiological conditions the activity of IDO2 is 20% less than that of IDO1 (155). It is interesting to note that 1-methyl-tryptophan (1MT), an IDO inhibitor which has been extensively used, has differential effect on the different IDO isozymes. The stereoisomer L-1MT inhibits IDO1, however, the stereoisomer D-1MT inhibits IDO2 better than IDO1 (156).

Two mechanisms are thought to underlie IDO-mediated regulation of immune responses. IDO mediated TRP depletion that directly impacts survival and function of immune cells by inducing cell stress and activating the general control of non-repressed 2 (GCN2) kinase pathway, which is a sensor of amino-acid deficiency (157). The second mechanism involves the cytotoxic and immune regulatory effects of TRP catabolites derived from the KYN metabolism pathway (158). Particularly in mice, KYN has shown to act through the aryl hydrocarbon receptor (AhR) in T cells leading to AhR-mediated Tregs generation with the participation of TGF-β (159). It is important to mention that both IDO1 and IDO2 trigger GCN2-dependent phosphorylation of the regulatory subunit of the translation initiation factor eIF-2α.

This leads to up-regulation of LIP, a liver inhibitory protein of the nuclear factor for IL-6 expression/CCAAT enhancer-binding protein-β (NF-IL6/CEPBβ) that is up-regulated by amino acid deprivation. However, the up-regulation of LIP by IDO1, but not IDO2, is TRP dependent (156).

1.8.1 Regulation of indoleamine 2,3 dioxygenase in dendritic cells

DCs are the main source of IDO; they express the highest level of IDO in response to different stimuli. Most of the human based studies on DCs have shown that the maturation status of DCs plays a role in IDO expression (160, 161), however the level of expression and the enzymatic activity of IDO depend on the nature stimuli involved.

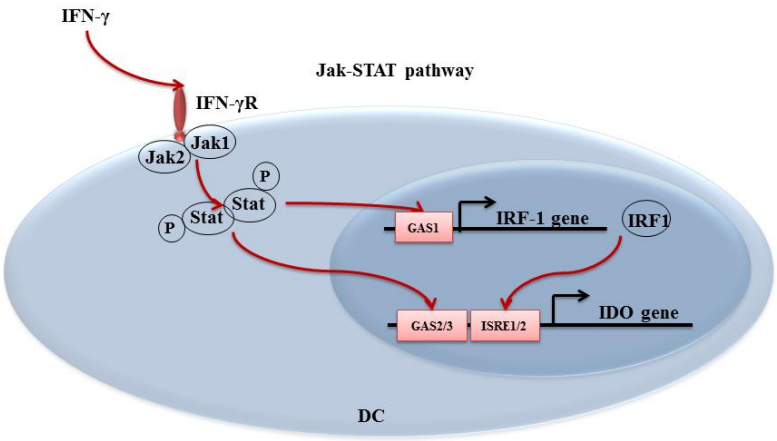

Figure 1.8: Jak-STAT signalling pathway of IDO induction by IFN-γ in DCs. Adapted from (164).

The human and mouse IDO promoters have numerous sequence elements, such as two interferon-stimulated response elements (ISRE1-2) and three gamma activated sequences (GAS1-3), that confer responsiveness to type-I IFN (α/β) and, more potently, to type-II IFNs (IFN-γ) (162-164) (**Figure 1.8**). This is often associated with

the expression of the anti-inflammatory cytokine IL-10 (165, 166). It has been demonstrated that type-I IFNs participate in IDO expression through non-canonical NF-κB in plasmacytoid DCs (pDCs), which synergizes with reverse signalling induced by interaction with Tregs through diverse pair of co-receptors, such as glucocorticoid-induced tumor necrosis factor receptor ligand (GITRL)-GITR, CD40-CD40L and CD80/86-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (150, 167-171). Furthermore, CD200 (172) and 4-1BB ligand (173) both have been shown to contribute to IDO-mediated Tregs generation through pathways that converge on non-canonical NF-κB signalling. By contrast, type-II IFNs have been shown to modulate acute responses of IDO mainly through Jak-STAT1 signalling pathways with the participation of interferon regulatory factor (IRF)-1 (174) (**Figure 1.9**).

There is also an IFN-γ-independent pathway for induction of IDO which is mainly mediated by TLRs such as TLR3 and TLR4 in Mo-DCs (161, 175) and TLR9 in pDCs (176, 177). It has been demonstrated that TLR9 ligands induce expression of IDO in human pDCs, which further induce Treg generation with suppressive functions. This up-regulation of IDO by TLR ligands involves pathways that converge in non-canonical NF-κB (176, 177) (**Figure 1.9**). Furthermore, it was shown that the production of an active form of IDO after stimulation with prostaglandin-E2 (PGE2) requires either TLR or TNF signalling (178). Recently, the role of TGF-β has arisen as a key cytokine in the mediation of IDO regulatory effects, establishing a regulatory phenotype in pDCs with the participation of Tregs (179). This cytokine, which so far appears to be mostly confined to T cells, has been shown to be secreted by mouse splenic $CD8^+$ DCs exerting autocrine and paracrine effects to initiate and maintain the function of Tregs mediated by the combined effect of TRP starvation and KYN metabolites acting through the AhR on T cells (180). Furthermore, it was

demonstrated that treatment of mouse pDCs with TGF-β induced regulatory effects on IDO independently from its enzymatic activity (181).

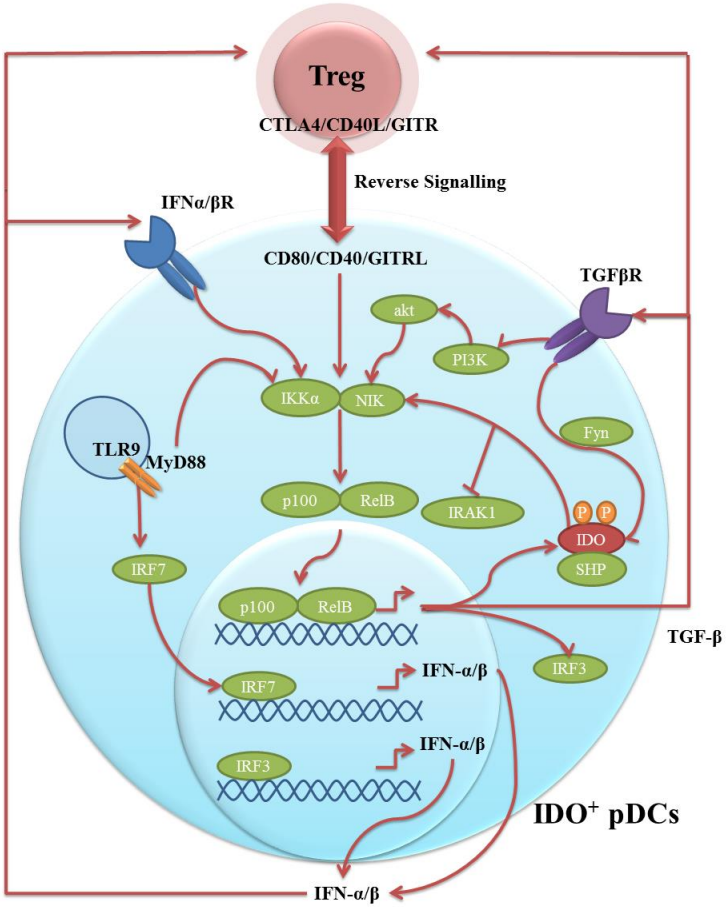

Figure 1.9: Reverse signalling and non-canonical NF-κB signalling pathway of IDO induction in pDCs. Reverse signalling via co-receptor system can maintain IKKα-dependent induction of IDO expression. Signalling through TLR9 can also sustain these events resulting in the production of IFN-α/β, TGF-β and IDO. This can further increase IDO expression forming a positive feedback leading to a sustained generation of Tregs. Adapted from (150).

1.8.2 The role of indoleamine 2,3 dioxygenase in allergic responses

Particularly in allergy and asthma, IDO has been shown to have a protective effect in different models of experimental asthma (167, 182-184). Reverse signalling (167,

184), bidirectional communication between two adjacent cells through co-receptors, and IDO (167) have been demonstrated to have a protective effect in experimental asthma. Moreover, it has been demonstrated that Treg cells (182) and pDCs (185), in an IDO mediated manner, contribute to protection against allergic asthma to the fungus *Aspergillus*. Furthermore, it was demonstrated in a murine model of experimental asthma, induced by OVA, that immuno-stimulatory sequence oligodeoxynucleotide (ODN) which is a TLR9 analogue, induced higher levels of IDO in resident lung cells rather than pulmonary DCs and inhibited Th2-driven experimental asthma (183). Accordingly, several clinical trials have already been initiated, which applied DNA-based immunotherapy to the treatment of allergic disease with promising results (186, 187). Later, different studies demonstrated that metabolites generated in the KYN pathway can either suppress experimental asthma or induce tolerance in an OVA-sensitized mice model (188-190). However, there is also some contradictory data which indicate that IDO is not required to induce immune tolerance to inhaled antigen, but promotes Th2 allergic inflammation in a mouse model (191). Based on that, it could be speculated that a differential role of IDO exists depending on the model being employed.

Altogether these data have demonstrated that IDO is able to protect against allergic reactions in different models of experimental asthma. However, there is not much information regarding the role of IDO in human allergy. In two studies with patients, it was found that during allergen exposure clinically unresponsive but allergen-sensitized atopic individuals had higher systemic IDO activity as well as higher IL-10 levels compared to symptomatic atopic individuals (192, 193). Moreover, the effect of specific allergens on IDO in human DCs has also been studied. Der p 1 was shown to be able to supress IDO in DCs from atopic HDM-sensitized patients with asthma

(194). Recently, data published by our group further demonstrated the participation of IDO in the response to Der p 1. For instance, there was a significant difference in IDO activity after Der p 1 and LPS treatment between control and MR-deficient DCs. These results suggest that MR engagement may contributes to the maintenance of a lower IDO activity. Further work is clearly required to determine how Der p 1 or LPS interfere, through MR engagement, with TLR4 signalling and whether they are able to modulate IDO activity through binding to another receptor (102). In this context, inhibition of experimental asthma has been documented for high doses of LPS but not for low levels in a mice model of allergic sensitization (65).

1.8.3 The role of aryl-hydrocarbon receptor in modulating indoleamine 2,3-dioxygenase and implications in allergic responses

Recently, the AhR has been identified as a new molecule involved in diverse immunological process, such as T cell differentiation and immunosuppression. AhR is a ligand-dependent transcription factor and a member of the Per-Arnt-Sim superfamily of proteins, which are involved in the detection of intracellular or environmental changes (195). 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). Such DREs are present in a large number of genes suggesting widespread influences of AhR activation. A number of endogenous and exogenous ligands for AhR have been identified. AhR is best known as the receptor for the xenobiotic 2,3,7,8-terachlorobenzo-p-dioxin (TCDD), a mechanism that accounts for its toxicity and has also been shown to induce Tregs. Alternatively, engagement with a different endogenous ligand, the UV tryptophan photoproduct 6-formylindolo-[3,1-b] carbazole (FICZ) leads to Th17 cell formation

(196). This demonstrates that the participation of AhR in immunity is complex and depends on the ligands involved. Further endogenous ligands for AhR can be generated through TRP metabolism, such as KYN. The recognition of KYN by AhR has been shown to modulate the immunogenicity of mouse DCs (197) and the differentiation of mouse T cells to Tregs (159) and Th17 (198). In addition, AhR has been shown to mediate IDO activation in response to TCDD (199), LPS and CpG (197) in mouse DCs.

AhR has been shown to be highly expressed in the lung (200). *In vivo* studies have suggested that AhR (201-205) as well as IDO (167, 182, 183, 188-190) could act as protectors against allergic responses. Particularly, stimulation of DCs with AhR ligands or IDO-derived metabolites could impair their phenotype and function, which further affects allergic sensitization (204-209). In addition, metabolites produced in the IDO-dependent TRP degradation pathway, such as KYN and kynurenic acid, can activate AhR leading to diverse effects depending on the immunological context (159, 210, 211). Accordingly, it can be speculated that those metabolites might have disparate effects on allergy responses. Interestingly, an endogenous ligand of AhR has been recently isolated from lung (212).

1.9 Hypothesis and aims

IDO plays a central role in regulating immune responses to antigens including allergens. IDO activity in different cell types seems to be controlled via different receptors and signalling pathways including those linked to PRRs. Here it was hypothesised that, “IDO production by DCs is controlled through engagement of different TLRs and CLRs and once produced it can modulate the events leading to Th2 cell polarisation”. Therefore, the aims of the project were:

1. To evaluate the relationship between diverse PRRs, such as CLRs and TLRs, and IDO in human DCs in order to define the role of these molecules in Th2 immune response.
2. To study how differential regulation of IDO activity/expression by various PRRs in human DCs can influence T cell activation and differentiation.
3. To analyse the intracellular pathways behind the regulation of IDO activity/expression by PRRs in human DCs.

2 Chapter II: General Material and Methods

2.1 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 (102, 213). Briefly, PBMCs were separated by density gradient centrifugation on Histopaque (Sigma-Aldrich, St Louis, Mo). $CD14^+$ monocytes were isolated from PBMCs by positive selection using a magnetic cell separation system (Miltenyi Biotech, Woking, 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 $CD1c^+$ DC isolation kit (Miltenyi Biotech) according to manufacturer's instructions.

2.2 Quantification of IDO activity

DCs ($2.5x10^5$ cells/ml) were seeded in a 24-well plate with complete media supplemented with 100 µM 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 has described previously (102). Allergen extracts were purchased from Greer Laboratories (Lenoir, NC). Typical endotoxin content of HDM preparations was 26.1 EU/mg protein. Mannan from *Saccharomyces cerevisiae* (Sigma-Aldrich) and the trisaccharide Lewis-X (Le^X) (Carbosynth Limited, Compton,

UK) come as low endotoxin/endotoxin free preparations. ODN 2216 was purchased from InvivoGen as an endotoxin free preparation.

2.3 Viability assay

Cell samples were washed with ice-cold PBS and staining was carried out using the Annexin-V-FITC apoptosis detection kit (Beckman coulter) according manufacturer's instructions. Preparations were analyzed within 30 min in a FC 500 Flow Cytometer (Beckman Coulter). Formaldehyde 4% was used as a positive control.

2.4 Flow cytometry analysis

Abs against IFN-γ (clone B27), IL-4 (clone MP4-25D2) and MR (clone 19.2) were purchased from Biolegend (London, 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 (High Wycombe, UK). Abs against PDL2 (clone MIH18), PDL1 (clone MIH1) and ICOSL (clone 2D3/B7-H2) were purchased from BD Biosciences (San Jose, Calif). Ab against AhR (clone FF3399) was purchased from eBioscience (Hatfield, UK). Ab against IDO1 (clone 700838) was purchased from R&D Systems (Abingdon, UK). Ab against TLR4 (clone HTA125) was purchased from AbD serotec (Kidlington, 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) (214). Intracellular staining for cytokines was analysed in a MoFlo XDP Flow Cytometer (Beckman Coulter). All data analysis was done using Weasel Software (Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia).

2.5 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, Manchester, UK) according to manufacturer's instructions. Samples were then DNase treated using the TURBO DNA-free kit (Thermo Fisher Scientific, Waltham, Mass) 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, Stone, 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, Santa Clara, Calif). 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

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (215, 216). Ct values from GAPDH did not change by more than 0.5 to 1 cycle from sample to sample.

Primers were obtained from Eurofin (Luxembourg): 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'), DC-SIGN Forward (5`-CCAAAGGAGGAGACAAGCAG-3`), DC-SIGN Reverse (5`-GGACGACAGCTTCAGTGTGA-3`), MR Forward (5'-CGTTTACCAAATGGCTTCGT-3') and MR Reverse (5'-CCTTGGCTTCGTGATTTCAT-3').

2.6 Cytokine measurements

Cell-free supernatants were collected and stored at -20°C before analysis. Cytokine (IL-6, IL-10, IL-12p70, TGF-β, IFN-α, IL-8, TNF-α and IL-1β) concentration was analysed using the ProcartaPlex Multiplex Immunoassay system (eBioscience)

according to manufacturer's instructions. IFN-γ levels were measured using the DuoSet ELISA System (R&D Systems) according to manufacturer's instructions.

2.7 RNA interference

Gene silencing was carried out as previously described with slight modification (102, 110). MR and AhR siRNA were the SMARTpool: ON-TARGETplus siRNA from GE Healthcare (Little Chalfont, UK). DC-SIGN siRNA was purchased from Eurofin. The DNA-targeted sequence was 5'-GGCAATGGCTGGAACGACGACAAAT-3' from 665 to 689bp. 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.

2.8 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 (217). After denaturation, samples were spotted onto nitrocellulose-coated glass slides (Grace Bio-labs, Bend, Ore) with a microarray robot (MicroGrid 610, Digilab, Marlborough, Mass). 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 (Danvers, Mass). 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, Lincoln,

Neb). The resultant TIFF images were processed with Genepix Pro-6 Microarray Image Analysis software (Molecular Devices, Sunnyvale, Calif). 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. All fluorescent signals are reported as AFU with β-actin normalisation. Heat maps were generated using TMEV software.

2.9 DC-T cell co-culture

Human DCs were treated or not with either mannan (10 µg/ml) or Le^X (50 µg/ml) and co-cultured in the presence of LPS (0.01 µg/ml, Sigma-Aldrich) in 96-well U-bottom plate (Corning Life Sciences, Tewksbury, Mass) 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). In some experiments the IDO metabolite KYN (50 µM) was added at the beginning of the co-culture (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.

2.10 Statistical analysis

Values of the mean ± SEM are shown unless otherwise stated. ANOVA or Student t Test was applied: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001. For all statistical analysis GraphPad Prism 5 software (GraphPad Software, La Jolla, Calif) was used.

3 Chapter III: The role of MR in modulating IDO and subsequent immune responses

3.1 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. DCs are specialized APCs that play an important role in the induction and re-elicitation of Th2-allergic immune responses (19, 27, 68, 218). Recent work by us and others have shown that CLRs such as MR and DC-SIGN play a major role in the recognition and uptake of allergens, the initial steps leading to allergic sensitization (68, 71, 102, 103, 108-110). Interestingly, under certain conditions, DC-SIGN and MR ligation can have opposing effects on T cell polarization (102, 110). MR is a multifunctional endocytic receptor with two independent carbohydrate-binding domains that recognize sulfated and mannosylated structures, respectively (219). Several studies have demonstrated that CLR activation can modulate PRR-induced activation, particularly TLR4 signaling, and the downstream events leading to Th2 cell polarization (67, 68, 88, 102, 113, 146, 147). Our group have previously shown that Der p 1, the main allergen from HDM, in the presence of low levels of LPS down-regulates IDO through engagement of MR on human DCs leading to a Th2 immune response (102). 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 TRP into N-formyl-kynurenine, the first and rate-limiting step of TRP catabolism in the KYN pathway (104, 105). IDO has been demonstrated to be involved in diverse immune-regulatory processes in health and disease (104, 105). In particular, it has been shown that IDO has a protective role in several models of experimental asthma (167, 182, 183, 188-190). 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 (192, 193). However, the mechanism of IDO regulation in these models particularly in a human context has remained elusive.

AhR is a ligand-dependent transcription factor involved in sensing intracellular or environmental changes (200). KYN, one of the main metabolites produced in the IDO-dependent TRP degradation pathway, can activate AhR leading to the generation of Treg (159). In addition, previous observations in mouse DCs have suggested that AhR mediates IDO induction in response to TLR agonists (204, 220). Here it was investigated 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. It was demonstrated that different airborne allergens, most likely through binding 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 priming Th1 responses. Furthermore, it was shown for the first time the co-regulation of AhR and components of the non-canonical 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 highlight 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.

3.2 Results

3.2.1 TLR4 agonist induce IDO1 and IDO2 in human dendritic cells

The 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 Mo-DCs as well as in peripheral blood myeloid DCs (**Figure 3.1** and **Figure 3.2**). This is in contrast with previous reports where co-stimulation with either IFN-γ or prostaglandin E2 was deemed to be essential (169, 178, 221, 222). Additionally, this effect was observed with LPS from different bacterial sources, such as *E.coli* and *S.minnesota* (**Figure 3.1** and data not shown).

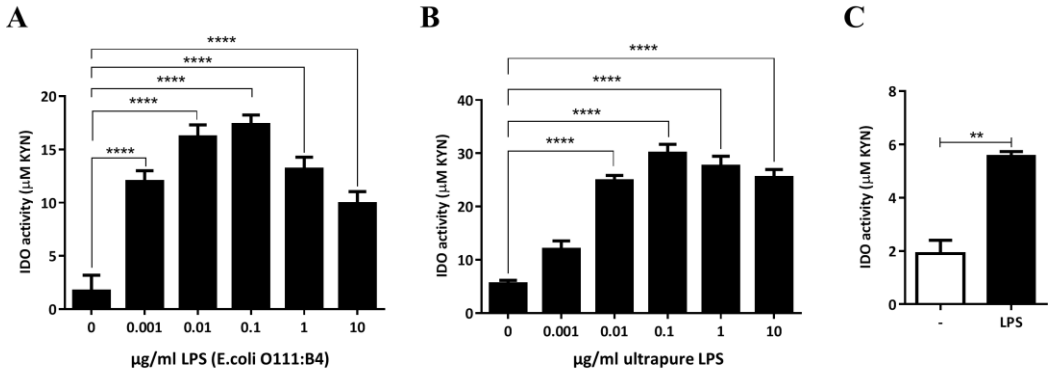

Figure 3.1: TLR4 agonist induces IDO activity in human DCs. A. IDO activity in human DCs stimulated 24 hrs with increasing concentrations of LPS from *E.coli* O111:B4 (n=5). B. IDO activity in human DCs stimulated 24 hrs with increasing concentrations of ultrapure LPS (n=2). C. IDO activity in human peripheral blood myeloid DCs stimulated 24 hrs with LPS (0.01 µg/ml) (n=2).

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, the effects of ultrapure LPS as well as the synthetic diacylated lipoprotein named fibroblast-stimulating lipopetide-1 (FSL-1) as a specific TLR2 agonist were tested.

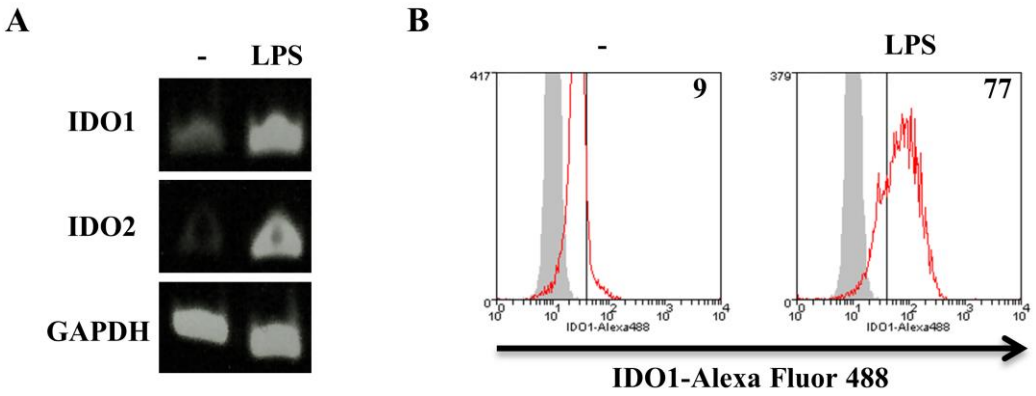

Figure 3.2: TLR4 agonist induces IDO1 and IDO2 in human DCs. A. Conventional PCR analysis of IDO1 and IDO2 gene expression in human DCs stimulated 24 hrs with LPS 0.01 µg/ml. GAPDH was used as housekeeping gene. IDO1 (n=5), IDO2 (n=3). B. Flow cytometry analysis of IDO1 protein expression in human DCs 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).

The data showed similar dose-dependent effect induced by increasing concentrations of ultrapure LPS (**Figure 3.1**), while FSL-1 did not affect IDO activity in human DCs (data not shown). Interestingly, the 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 it was 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).

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

After establishing the role of LPS in IDO regulation, it was investigated how different allergen extracts i.e. 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.

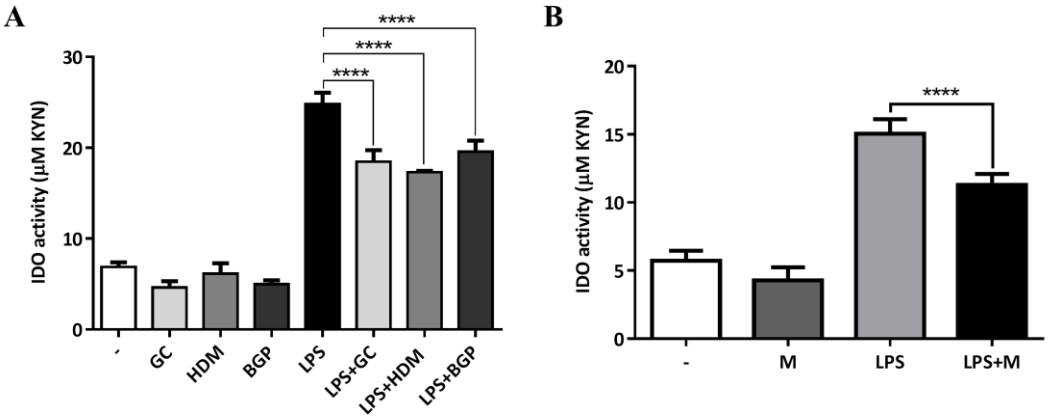

Figure 3.3: TLR4-induction of IDO activity is down-regulated by allergens from diverse sources. A. IDO activity in human DCs stimulated 24 hrs with diverse allergen extracts (10 µg/ml) in the presence and absence of LPS (0.01 µg/ml). German Cockroach (GC) extract from *Blattella germanica*, House Dust 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).

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 (**Figure 3.3**).

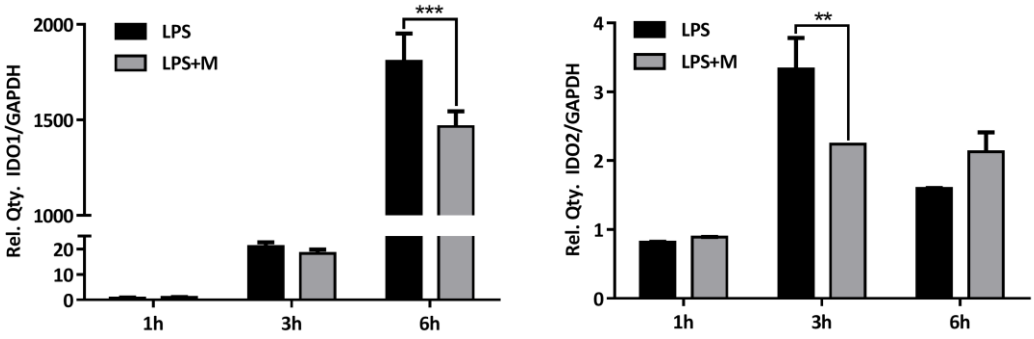

Figure 3.4: TLR4-induction of IDO1 and IDO2 is down-regulated by allergens from diverse sources. qRT-PCR analysis of IDO1 and IDO2 gene expression in human DCs stimulated 24 hrs with mannan (10 µg/ml) in the presence of LPS (0.01 µg/ml). Relative expression of IDO1 and IDO2 were compared with that of GAPDH (n=3). In co-stimulation experiments, cells were stimulated with LPS followed immediately by allergen extracts.

Our group have previously shown that many allergens from diverse sources (including HDM and cockroach) are heavily mannosylated (71) and such sugar moieties play a key role in CLR mediated allergen recognition and uptake by DCs (68, 71) . Accordingly, the impact of highly mannosylated sugars in IDO regulation was studied using mannan as a prototypic high mannose carbohydrate that can be recognized by MR (223, 224). Here it was shown that mannan is able to down-regulate both IDO1 and IDO2 gene expression and activity in human DCs (**Figure 3.3** and **Figure 3.4**). This data suggests a key role for carbohydrates in allergen preparations in modulating IDO in human DCs.

3.2.3 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 (105, 150, 192), while IL-6 has been shown to be involved in IDO degradation (105). Additionally, it has been suggested that specific MR agonists, such as the mannose-capped lipoarabinomannans (Man-LAM), can negatively regulate TLR4-dependent IL-12 production in mouse macrophages (225). Accordingly, the regulation of these cytokines by mannan in human DCs was studied.

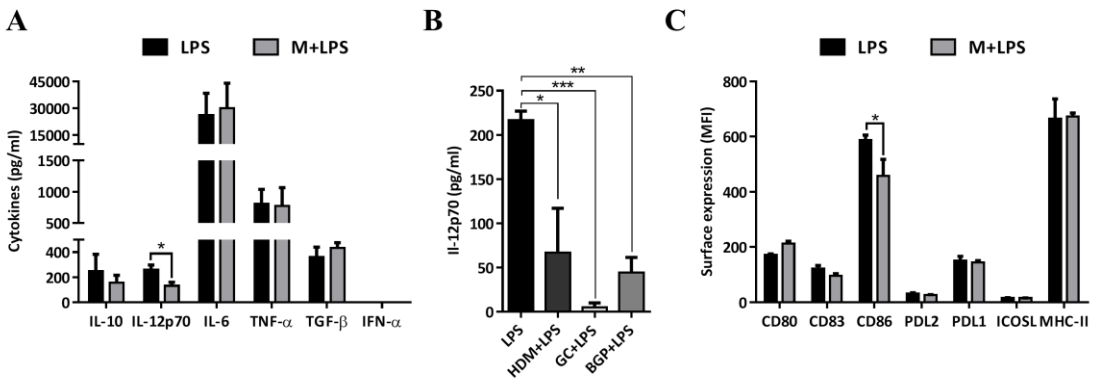

Figure 3.5: Mannose-based ligands down-regulate TLR4-induced IL-12p70 production by human DCs. A. Cytokine production by human DCs stimulated or not with mannan (M) followed by LPS for 24 hrs (n=3). B. IL-12 production by human DCs stimulated with several allergen extracts and LPS (n=3). C. Flow cytometry analysis of costimulatory molecules expression in human DCs stimulated 24 hrs with mannan and LPS. Median fluorescence intensity (MFI) is shown (n=3).

No significant differences were found when DCs were stimulated with mannan alone compared to un-stimulated DCs (data not shown). However, the presence of mannan significantly reduced IL-12p70 production after LPS stimulation compared with LPS

alone (**Figure 3.5**). Similar results were found after stimulating DCs with LPS in the presence of HDM, cockroach and pollen extracts (**Figure 3.5**). Subsequently, it was 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 (**Figure 3.5**).

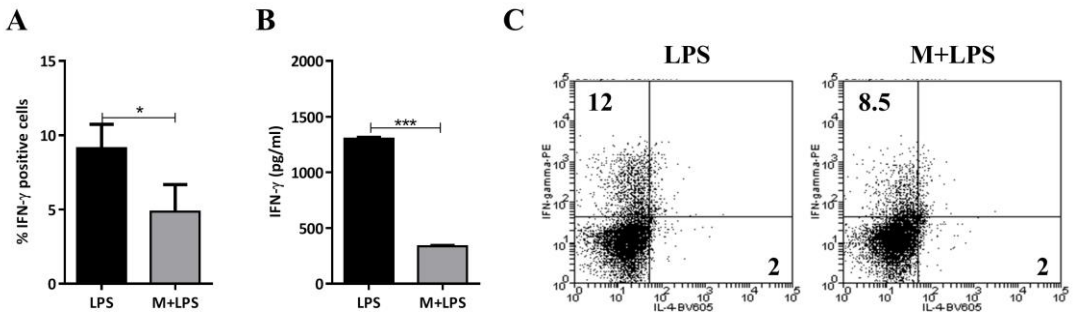

Figure 3.6: Stimulation of human DCs with mannose-based ligands affect T helper cell polarization. A. Percentage of IFN-γ positive cells (n=3). B. IFN-γ production by human T cells co-cultured with DCs stimulated or not with mannan followed by LPS (one experiment representative of three). C. DCs were stimulated or not with mannan 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. Numbers in dot plots represent cell percentage (one experiment representative of three).

Then, co-culture experiments were performed using DCs pre-stimulated with either mannan and/or LPS prior to co-culture with autologous naïve T cells. The 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 (**Figure 3.6**). 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 (**Figure 3.6**). Given the non-atopic status of donors, high levels of IL-4

producing T cells were not detected (**Figure 3.6**), however in one donor 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.

3.2.4 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, it was then determined the role of MR in mannan-mediated modulation of IDO activity in human DCs. Using siRNA MR expression on DCs was knockdown by up to 80% similar to previous work (102) (**Figure 3.7** and **Figure 3.8**).

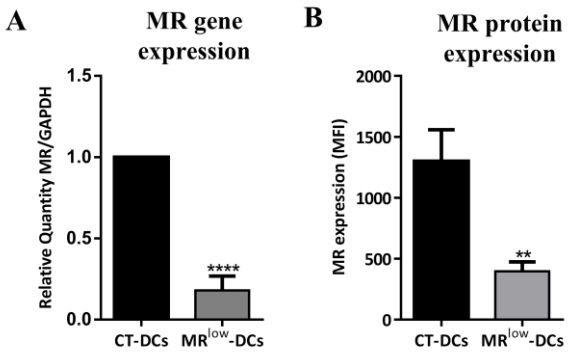

Figure 3.7: MR down-regulation by gene silencing. A. qRT-PCR analysis of MR gene expression in CT and MR^{low}-DCs. Relative expression of MR was compared with that of GAPDH (n=2). B. Flow cytometry analysis of MR protein expression. MFI is shown (n=5).

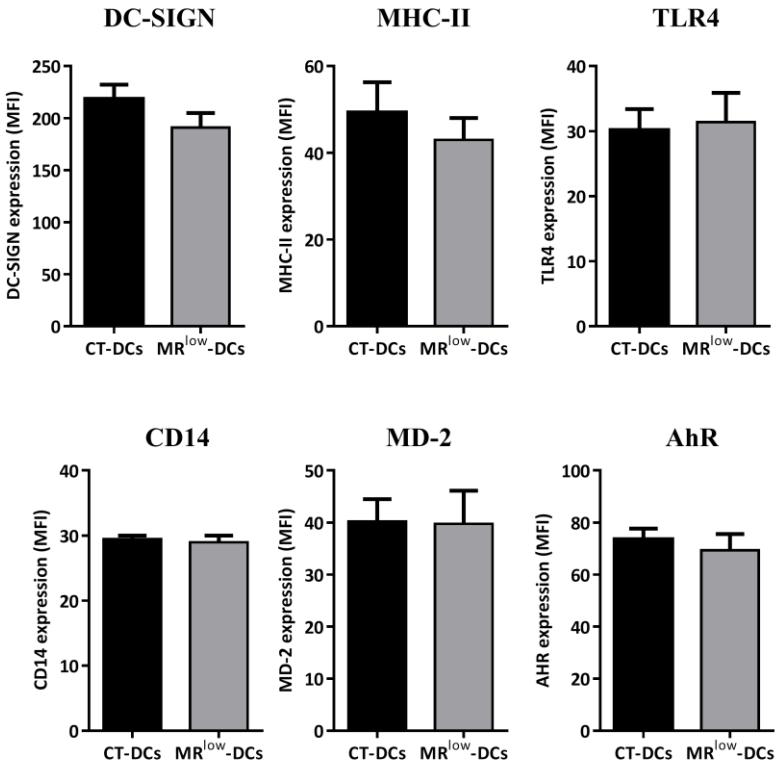

Figure 3.8: MR down-regulation does not affect the expression of additional relevant molecules. Flow cytometry analysis of DC-SIGN, HLA-DR, TLR4, CD14, myeloid differentiation protein-2 (MD-2) and AhR protein expression. MFI is shown

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 control-DC (CT-DC) indicating that MR plays a key role in the IDO down-regulation induced by mannan in human DCs (**Figure 3.9**). Additionally, an inverse correlation between the expression of MR and IDO1 was found in DCs stimulated with mannan and LPS (**Figure 3.9**). 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.

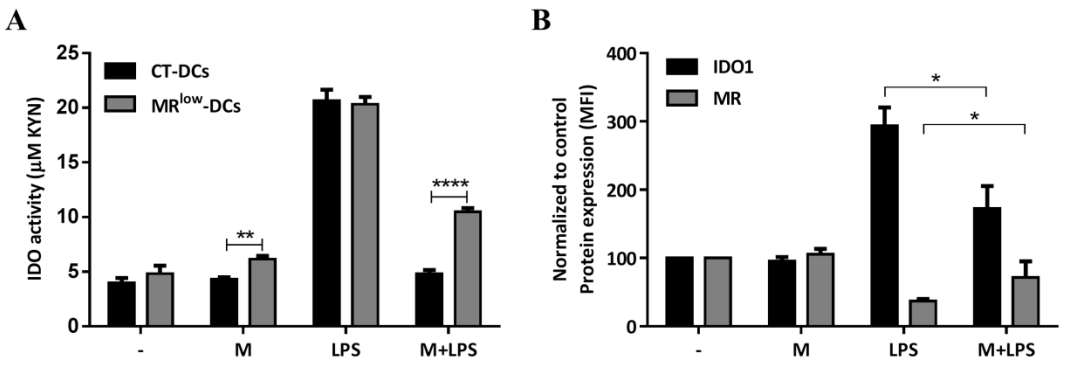

Figure 3.9: Mannose receptor mediates the IDO down-regulation by mannose-based ligands. A. IDO activity in CT and MR^{low}-DCs stimulated with mannan (M) and LPS for 24 hrs (n=3). B. Flow cytometry analysis of MR and IDO1 protein expression in human DCs stimulated or not with mannan and LPS for 24 hrs. MFI values were normalized to control unstimulated sample (n=3).

3.2.5 IDO regulation in human dendritic cells is partially dependent on AhR

In order to study the role of AhR in the induction of IDO in human DCs, AhR^{low}-DCs were generated through gene silencing (**Figure 3.10** and **Figure 3.11**) and analyzed IDO activity after TLR4 stimulation compared with CT-DCs.

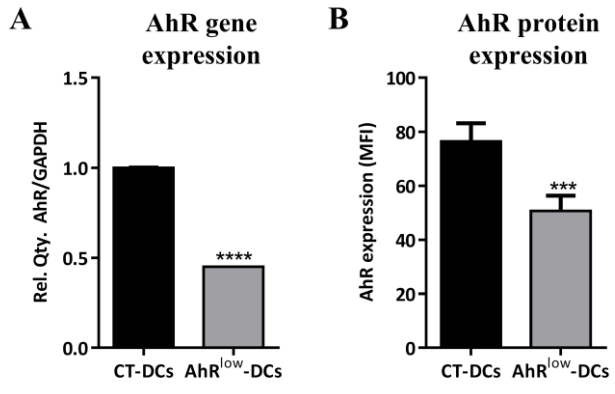

Figure 3.10: AhR down-regulation by gene silencing. A. qRT-PCR analysis of AhR gene expression in CT and AhR^{low}-DCs. Relative expression of AhR was compared with that of GAPDH (n=2). B. Flow cytometry analysis of AhR protein expression in human CT-DCs compared with AhR^{low}-DCs. MFI is shown (n≥3).

The data shows a lack of IDO up-regulation in AhR^{low}-DCs compared with CT-DCs after TLR4 engagement (**Figure 3.12**), which suggests that LPS-mediated IDO induction in human DCs is partially dependent on AhR expression.

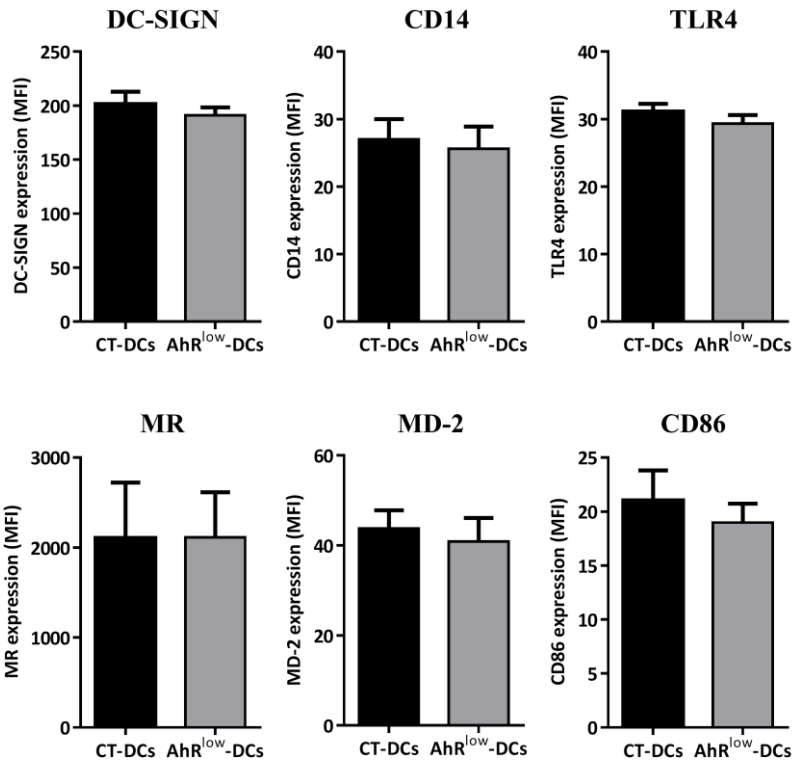

Figure 3.11: AhR down-regulation does not affect the expression of additional relevant molecules. Flow cytometry analysis of MR, DC-SIGN, TLR4, CD14, MD-2 and CD86 protein expression. MFI is shown (n≥2).

Accordingly, it was then evaluated 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 (**Figure 3.13**). This shows that MR engagement can interfere with TLR4 signaling by modulating the AhR-IDO axis in human DCs.

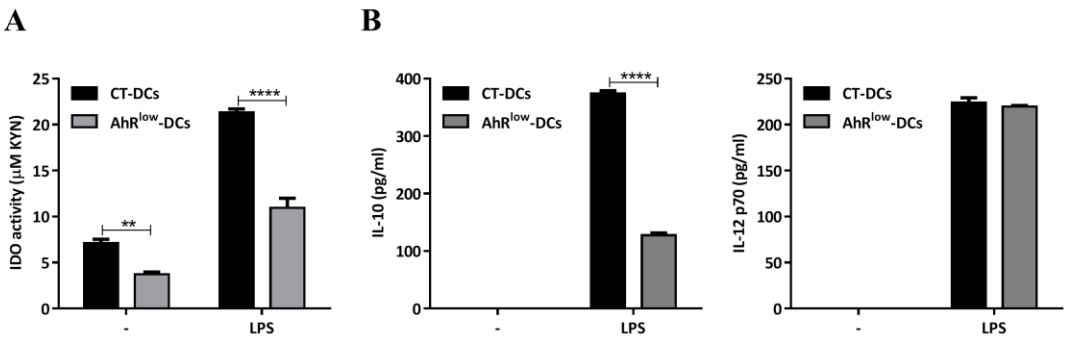

Figure 3.12: IDO regulation in human DCs is partially dependent on AhR. A. IDO activity in CT and AhR^{low}-DCs stimulated or not with LPS for 24 hrs (n=3). B. IL-12p70 and IL-10 production by CT and AhR^{low}-DCs stimulated or not with LPS for 24 hrs (one experiment representative of three).

Furthermore, it was 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 (**Figure 3.12**). 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.

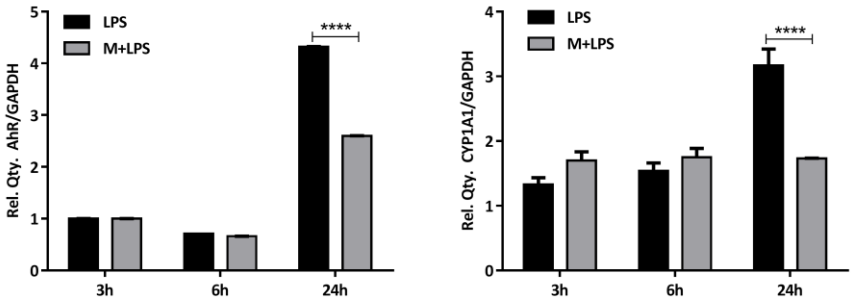

Figure 3.13: Mannose-based ligands down-regulate TLR4-induced AhR expression and AhR activity by human DCs. qRT-PCR analysis of AhR and CYP1A1 gene expression in human DCs stimulated or not with mannan (M) followed by LPS for 24 hrs. Relative expression of AhR and CYP1A1 were compared with that of GAPDH (n=3).

3.2.6 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 (226). On the other hand, IDO induction has been shown to be under the control of the non-canonical NF-κB pathway (150, 169, 227) involving the generation of p52-RelB complexes (226, 228). Accordingly, it was evaluated how MR modulates both the canonical and non-canonical NF-κB pathway in human DCs.

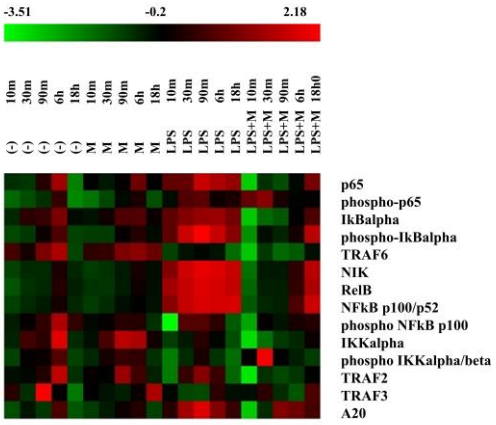

Figure 3.14: The NF-κB pathway is negatively regulated by MR in human DCs. Heat maps representing the relative abundance of proteins upstream the NF-κB signalling pathway using human DCs stimulated with mannan (M) and LPS for different time points.

In order to understand how different components of the NF-κB pathway were regulated upon TLR4 activation (in the presence and absence of mannan) a reverse phase protein array approach was used (217) that enables simultaneous evaluation of protein expression as well as post-translational modifications.

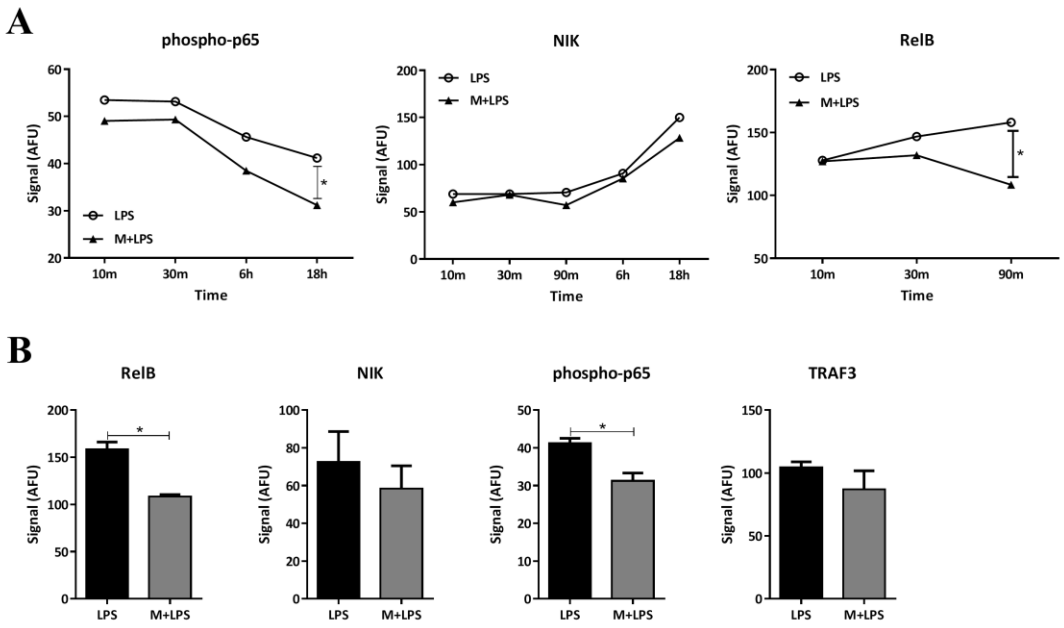

Figure 3.15: The NF-κB pathway is negatively regulated by MR in human DCs. A. Protein expression of phospho-p65, NIK and RelB in human DCs stimulated with mannan followed by LPS. Data are shown as geometric mean of three independent experiments. B. Protein expression of RelB (90 min), NIK (90 min), phospho-p65 (18 hrs) and TRAF3 (18 hrs) in human DCs stimulated with mannan followed by LPS. All fluorescent signals are reported as AFU with β-actin normalisation.

First, a panel of 14 different targets at different time points (10, 30, 90, 360 and 1080 min) was evaluated, 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 (**Figure 3.14**). 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 NF-κB inducing kinase (NIK) accumulation between 6 to 18 hrs (a classical indicator of non-canonical NF-κB activation) (**Figure 3.15**). 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 (**Figure 3.15**). DCs stimulated with mannan and LPS showed a significant decrease in a component of the non-canonical NF-κB pathway namely RelB (**Figure 3.15**), which was further confirmed at gene level by qRT-PCR (**Figure 3.16**). In addition, a decrease was observed in phospho-p65 at late time points (**Figure 3.15**). 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.

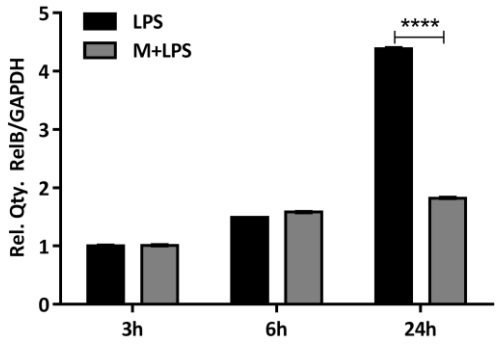

Figure 3.16: The NF-κB pathway is negatively regulated by MR in human DCs. qRT-PCR analysis of RelB gene expression in human DCs stimulated with mannan followed by LPS for 24 hrs. Relative expression of RelB was compared with that of GAPDH (n=2).

3.3 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 Abs 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 (19, 27, 68, 218). Previous observations have shown that TLR4 signaling is crucial for

inducing Th2-mediated inflammation and asthma (65, 89, 90) which is dependent on the levels of LPS exposure (90). 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 (104, 105). In this study it was first established how IDO is regulated in human DCs in response to TLR4 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 (**Figure 3.1**). These data were confirmed after using ultrapure LPS establishing the involvement of TLR4 pathway only (**Figure 3.1**). Two IDO isozymes have been identified namely IDO1 and IDO2 (229), 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 (154, 230, 231). Here it has been shown for the first time that both IDO isozymes are induced after TLR4 engagement (**Figure 3.2**).

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 (68, 102, 103, 108, 110, 115). In the case of MR this is likely to be mediated through down-regulation of IDO activity (102) however the molecular mechanisms of how MR ligation modulates IDO activity remained unclear. In the current study, using siRNA (**Figure 3.7**), it was shown that MR mediates the allergen (and mannan) induced down-regulation of IDO activity in LPS stimulated DCs

(**Figure 3.9**). 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 (**Figure 3.3**). 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 (96, 232).

Furthermore, it was studied how MR engagement might modulate other aspects of DC function. The data showed that mannosylated sugars can particularly down-regulate TLR4-mediated IL-12p70 production, a key cytokine in Th1 polarization (**Figure 3.5**). This shows an antagonistic effect between MR and TLR4 which was in line with previous observations (224, 225, 233, 234). 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 (**Figure 3.5**). In terms of costimulatory molecules, it was found a significant down-regulation in the levels of CD86 in DCs stimulated with mannan (**Figure 3.5**). It is interesting to note that CD86 is one of the B7 family proteins, that has been previously associated with IDO induction (235, 236). Furthermore, it was 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 (**Figure 3.6**). These data clearly suggest that reduction in IDO, through MR, could promote Th2 immune responses (**Figure 3.17**).

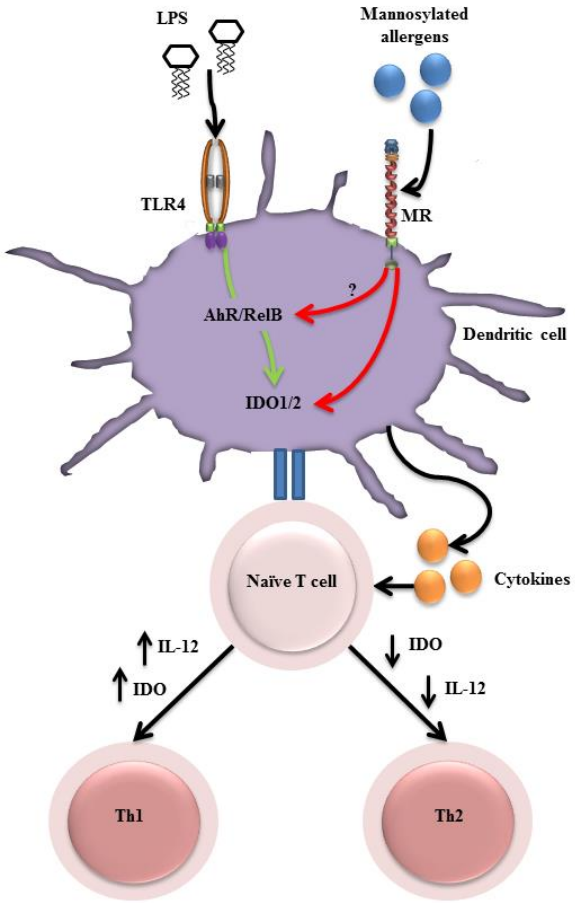

Figure 3.17: MR-mediated IDO down-regulation in human DCs involved the transcription factors AhR and RelB, having an impact on T helper cell polarization.

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 (237). Some studies have shown that IDO can have a protective effect in different models of experimental asthma (167, 182, 183, 188-190). This is in line with clinical studies showing that asymptomatic non-atopic individuals have higher systemic IDO activity than symptomatic atopic individuals (192, 193). 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 (191), which could be due to impaired DC function in IDO deficient DCs (238, 239).

AhR is a ligand-dependent transcription factor and a member of the Per-Arnt-Sim superfamily of proteins, which are involved in the detection of environmental or intracellular changes. The interaction of AhR with ARNT allows it to bind specific enhancer sequences present in target promoters named DREs (200). Previously, AhR has been shown to have a protective role in allergy (201-203, 205). Accordingly, in an attempt to elucidate the mechanism of TLR4 and MR-mediated IDO modulation the potential link between AhR and IDO in human DCs was studied. Here, it was shown that TLR4-mediated induction of IDO is partially dependent on AhR expression (**Figure 3.12**). In addition, it was 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 (**Figure 3.12**). Furthermore, it was demonstrated that MR can down-regulate AhR expression and activity in the presence of LPS in human DCs (**Figure 3.13**), which could explain its effect on IDO expression (**Figure 3.4**) and suppression of Th1 responses (**Figure 3.6**). Interestingly, it has been shown that exogenous AhR ligands like TCDD can impair DC phenotype and function (205-209) suppressing allergic sensitization (202, 205). Furthermore, metabolites produced in the IDO-dependent TRP degradation pathway, such as KYN and kynurenic acid, can activate AhR leading to Treg or Th17 differentiation depending on the immunological context (159, 210, 211, 240). Accordingly, it can be speculated that IDO metabolites might have disparate effects in allergic responses, which will require further investigation.

IDO induction has been shown to be under the control of the non-canonical NF-κB pathway (150, 169, 227). Additionally, the NF-κB together with AhR has been shown

to be pivotal in TLR4 signaling (226, 241-243). Therefore, the role of the NF-κB pathway in IDO regulation by MR was evaluated. Using a protein microarray approach (217) (**Figure 3.14**), the expression of key components of the NF-κB pathway after stimulation with LPS in the presence and absence of mannan was assessed. The data showed that stimulation with mannan and LPS can significantly down-regulate some key components of the non-canonical NF-κB pathway such as RelB, as well as the canonical NF-κB pathway such as phospho-p65 (**Figure 3.15** and **Figure 3.16**). Although a signalling motif has not been identified in MR cytoplasmic domain, members of the NF-κB signaling pathway have been previously implicated in MR-mediated signaling (225, 244). For instance, Man-LAM, most likely through binding to MR, mediates interleukin-1 receptor-associated kinase (IRAK)-M induction which acts as a negative regulator of the NF-κB pathway (225). Interestingly preliminary data also shows that mannan stimulation reduces IRAK-M expression in the presence of LPS in human DCs (data not shown), which is in line with previous observations showing that IRAK-M might protect from complications of asthma, as Th2 cytokines decrease IRAK-M expression in macrophages (245). Collectively these observations highlight the role of the NF-κB pathway in MR mediated IDO regulation in DCs. It is important to note that KYN via the AhR/SOCS2-dependent pathway can induce proteasome-mediated degradation of the adaptor protein TNF receptor-associated factor (TRAF) 6, which might potentially inhibit TLR signalling favouring the non-canonical NF-κB pathway (246). Additionally, an association between AhR and RelB has been suggested (247-249) that might contribute to the stabilization of RelB complexes (250, 251). 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, it has been demonstrated that diverse airborne allergens can down-regulate IDO after TLR4 engagement and this effect was mainly mediated by MR. In addition, it was shown that AhR and RelB are implicated in MR-mediated IDO down-regulation suggesting a new pathway involved in inserting immune regulatory properties of allergens (**Figure 3.17**). 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.

4 Chapter IV: The role of DC-SIGN in modulating IDO and subsequent immune responses

4.1 Introduction

DCs sample the tissue microenvironment for antigens and danger signals using a plethora of PRRs including TLRs and CLRs amongst others. CLRs play a central role in the recognition of glyco-antigens and in shaping both innate and adaptive immune responses to them (67, 68). DC-SIGN, a type-II transmembrane protein receptor, is a CLR that has been shown to recognize different carbohydrate moieties expressed on pathogens (252), allergens (110) and self-molecules (253). It contains a single CRD that binds to mannose branched, as well fucosylated (Lewis antigens) structures (254, 255). Interestingly, the signaling events upon DC-SIGN ligation appear to be dependent on the nature of the antigen. For example DC-SIGN binding to mannose-based antigens, such as Man-LAM (256, 257) or α-glucans (258) from *Mycobacterium tuberculosis,* induce up-regulation of IL-10 production in LPS-activated DCs leading to immunosuppression. Conversely, fucose-expressing extracellular pathogens like *Schistosoma mansoni* and *Helicobacter pylori* can increase IL-10 along with decrease IL-12 production favoring Th2 polarization in a TLR4-dependent manner (113). In the context of allergy, we and others have previously shown that DC-SIGN is able to recognize a number of allergens including Der p 1, Ara h 1 and Can f 1, major allergens from HDM, peanut and dog respectively (108-110). Downstream signaling events after allergen recognition however do not necessarily support Th2 responses as our group have shown to be the case for Der p 1 (a mannosylated allergen) where after knocking down DC-SIGN expression on DCs, T cell responses are biased towards a Th2 profile (110). Remarkably, binding of Der p 1 to a different CLR, namely MR,

biases immune responses towards a Th2 profile (102). Collectively, these data highlight the ability of DC-SIGN in differentially modulating immune responses to glycosylated antigens depending on the nature of their glycosylations.

In the past decade an emerging body of data have demonstrated a key role for the TRP-metabolizing enzyme called IDO in modulating innate and adaptive immune responses (104, 105). IDO activity leads to TRP depletion and production of a series of immune-regulatory metabolites known as kynurenines (KYN). Both phenomena could impact T cell proliferation and differentiation by inducing apoptosis of activated T cells or promoting Treg differentiation (157, 159). DCs are the main source of IDO; they express the highest level of IDO in response to several stimuli such as type-I and type-II IFNs which are the most potent inducers of IDO activity (163, 164, 174). There is also an IFN-independent pathway for induction of IDO which is mainly mediated by TLRs such as TLR4 in myeloid DCs (161) and TLR9 in pDCs (176, 177).

Here, it has been shown that mannosylated sugars through binding to MR are able to down-regulate TLR4-dependent induction of IDO1 and IDO2 expression and IDO activity in human myeloid DCs (259). Therefore, given the ability of DC-SIGN to recognize diverse sugar structures, in this study fucosylated and mannosylated DC-SIGN ligands were used together with gene silencing to investigate the potential role of DC-SIGN in regulating IDO activity in human DCs and how this could affect T cell polarization.

4.2 Results

4.2.1 Fucose-mediated down-regulation of IDO impairs Th1 cell polarization

It was first studied IDO regulation after stimulation with a specific-DC-SIGN ligand. For this purpose human DCs were stimulated with the trisaccharide Lewis-X (Le^X) as a prototypic fucosylated ligand which is solely recognized by DC-SIGN (255, 260). The data show that Le^X was able to significantly down-regulate IDO activity under steady state conditions as well as after TLR4 ligation in human DCs (**Figure 4.1**).

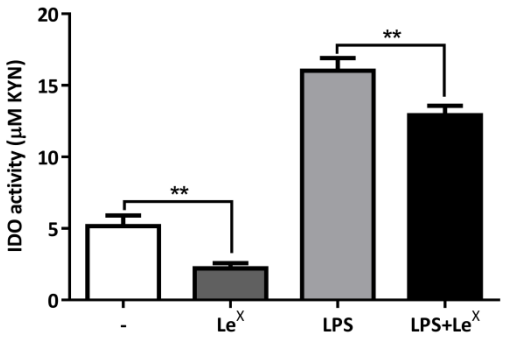

Figure 4.1: Fucose-specific DC-SIGN engagement down-regulates IDO activity in human DCs. IDO activity in DCs stimulated 24 hrs with Lewis-X (Le^X) (50 µg/ml) in the presence and absence of LPS (0.01 µg/ml) (n=3).

It has been shown that MR engagement can down-regulate IDO in DCs and suppresses Th1 polarization (259). Accordingly, the effect of DC-SIGN-mediated IDO modulation on DC over T helper cell polarization was investigated. In order to test this possibility, autologous co-cultures of naïve T cells with DCs primed with Le^X or Le^X+LPS were performed.

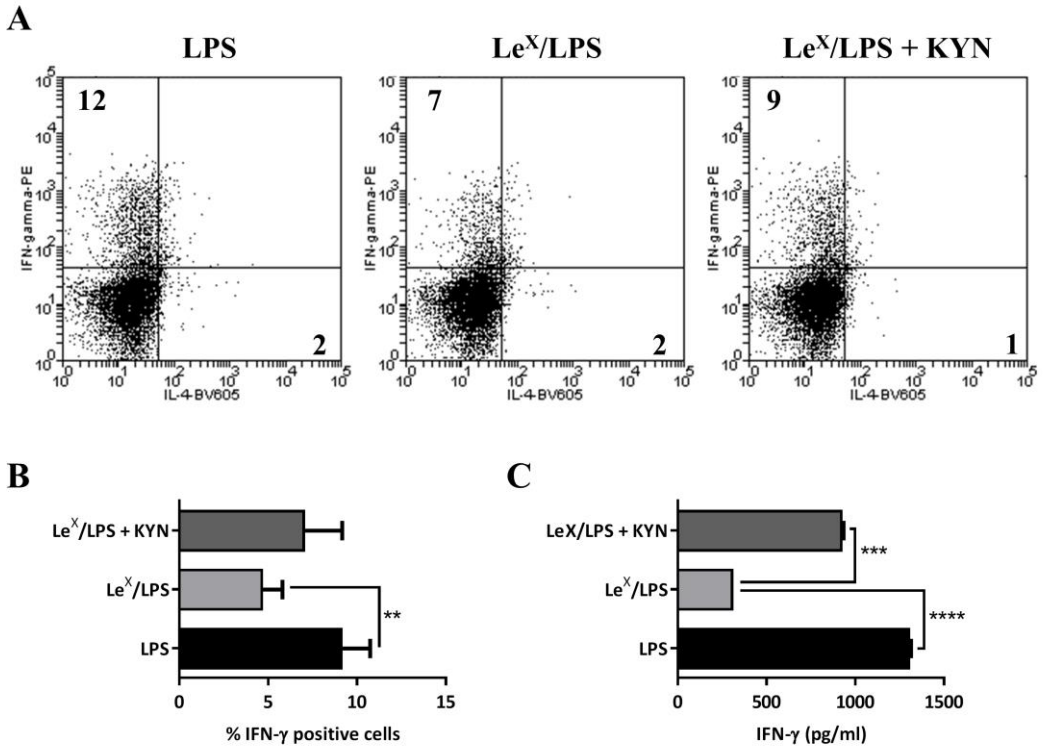

Figure 4.2: Fucose-specific DC-SIGN engagement modulates IDO activity in human DCs affecting T helper polarization. A. IL-4 and IFN-γ intracellular staining of $CD4^+$ gated cells after co-culture (n=3). B-C. Percentage of IFN-γ positive cells and IFN-γ production by T cells in autologous co-cultures respectively (n=3).

The data show a significant down-regulation in IFN-γ production by T cells that were co-cultured with DCs primed with Le^X+LPS compared with LPS only controls (**Figure 4.2**). This reduction in IFN-γ was reflected in both percentage of IFN-γ producing cells as well as the levels of IFN-γ measured in the culture supernatant (**Figure 4.2**). However, no significant differences in IL-4 production were observed (**Figure 4.2**). Furthermore, co-culture experiments in the presence of KYN, one of the main TRP-derived metabolites that mediates most of the immunological effects of IDO (159, 220), were performed. It was observed that IFN-γ production by T cells was partially recovered when cells were co-cultured in the presence of KYN (**Figure**

4.2). Taken together, this data shows that changes in IDO activity after fucose-specific DC-SIGN engagement are involved in modulating the observed changes on T helper cell polarization.

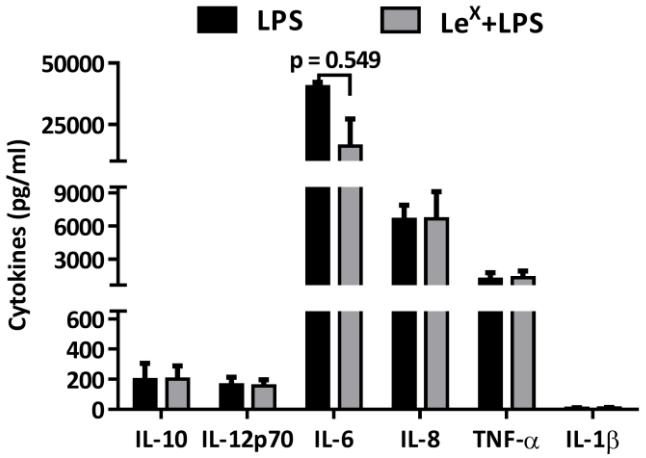

Figure 4.3: Fucose-specific DC-SIGN engagement modulates cytokine production in human DCs. Cytokine production by DCs stimulated 24 hrs with LPS or Le^X and LPS (n=6).

Interestingly, the data also show no significant induction in any of the cytokines tested (IL-10, IL-12p70, IL-6, IL-8, IL-1β and TNF-α) in DCs stimulated with Le^X only (data not shown) or any changes when they were co-stimulated with Le^X and LPS compared with LPS only controls. The only exception was LPS-induced IL-6 production which showed a trend for down-regulation in the presence of Le^X (**Figure 4.3**). Clearly this data does not exclude changes in the production of other DC cytokines.

4.2.2 Fucose-specific DC-SIGN signalling affects the non-canonical NF-κB pathway

We and others have described the key role of the non-canonical NF-κB pathway in IDO regulation (169, 259). Therefore, the involvement of the NF-κB pathway in fucose-specific DC-SIGN-mediated IDO regulation was studied. Reverse phase protein array approach allowed simultaneous analysis of protein expression as well as

post-translational modifications of several components of both the canonical and non-canonical NF-κB pathway. Low expression (green) to high expression (red) heat maps represent the relative abundance of proteins upstream of the NF-κB signalling pathway using β-actin as a housekeeping control (**Figure 4.4**).

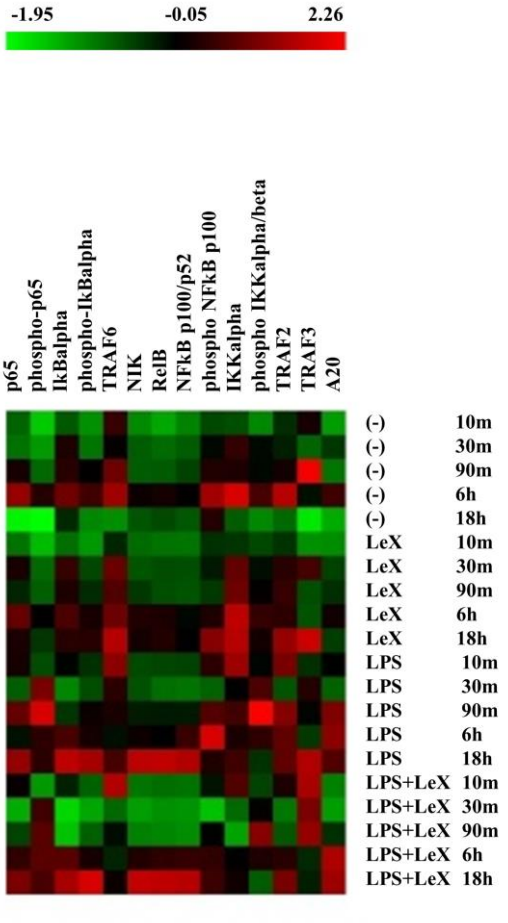

Figure 4.4: Fucose-specific DC-SIGN signalling involves the non-canonical NF-κB pathway in human DCs. Heat maps representing the relative abundance of proteins upstream the NF-κB signalling pathway using DCs stimulated with Le^X and/or LPS for different time points.

Stimulation with Le^X only did not affect the expression of any the molecules quantified in these experiments (data not shown). However, the data shows significant down-regulation in the protein expression of key components of the non-canonical NF-κB such as the protein NIK as well as the adaptor protein TRAF3 in DCs stimulated with Le^X+LPS compared with LPS only controls (**Figure 4.5**). A trend for RelB down-regulation was also observed at early time points (90 min) (**Figure 4.5**). No significant differences were observed in the canonical pathway i.e. p65

phosphorylation (**Figure 4.5**). Collectively these data show that fucose-specific DC-SIGN signaling can potentially affect non-canonical NF-κB activation in the presence of LPS, which could be one of the mechanisms involved in IDO regulation by fucose-based ligands in human DCs.

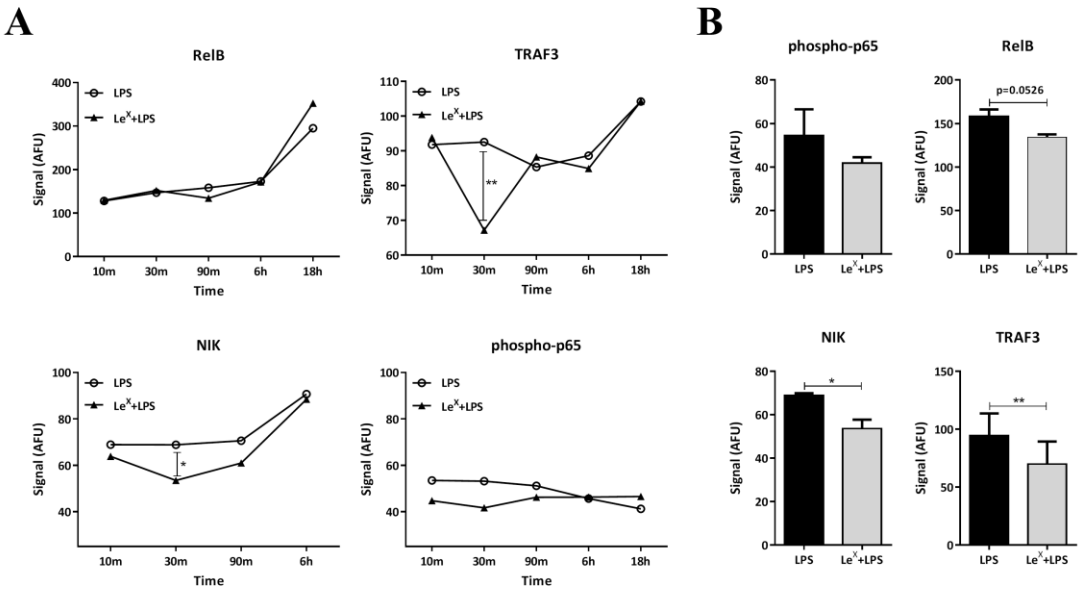

Figure 4.5: Fucose-specific DC-SIGN signalling involves the non-canonical NF-κB pathway in human DCs. A. Protein expression of phospho-p65, NIK, RelB and TRAF3 in DCs stimulated with Le^X and LPS. Data are shown as geometric mean of three independent experiments. B. Protein expression of RelB, NIK, phospho-p65 and TRAF3 in DCs stimulated with Le^X and LPS.

4.2.3 DC-SIGN engagement with mannose and fucose-based ligands differentially modulates IDO activity in human DCs

It has been previously described that mannose and fucose-based ligands have disparate effects on DC-SIGN signaling (113, 252, 257). Accordingly, human DCs were then stimulated with mannan - a polymer of mannose molecules - and its effect on IDO activity was compared to what it was observed with Le^X. The data show that mannan

and Le^X were both able to down-regulate IDO activity under steady state conditions as well as after TLR4 ligation in human DCs (**Figure 4.6**), which is in line with previous data (259).

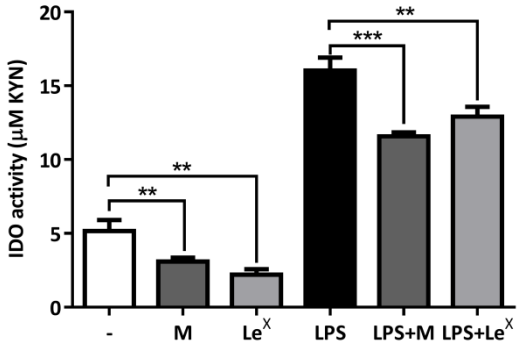

Figure 4.6: Fucose-specific DC-SIGN agonist (Lewis-X) and mannan down-regulate IDO activity in human DCs. IDO activity in DCs stimulated 24 hrs with either mannan (M) (10 μg/ml) or Lewis-X (Le^X) (50 μg/ml) in the presence and absence of LPS (0.01 μg/ml) (n≥3).

Fucose-bearing oligosaccharides are solely recognized by DC-SIGN (255, 260), however in addition to DC-SIGN mannosylated sugars can interact with other CLRs including MR (223, 224). Consequently, to elucidate the role of DC-SIGN and MR in IDO modulation by mannan siRNA was used to knock-down DC-SIGN and MR respectively, as it has previously described (102, 110, 259). Using this approach DC-SIGN expression was inhibited by an average of 50% at gene level (**Figure 4.7**) and 62% at protein level (**Figure 4.7**) with no effect on MR or TLR4 expression (**Figure 4.8**). Additionally, no effect on the maturation status of the cells was observed as evidenced by no changes in MHC-II expression (**Figure 4.8**). MR^{low}-DCs were also generated by reducing MR expression an average of 80% (**Figure 4.7**) and no significant effects on DC-SIGN, TLR4 and MHC-II expression were observed (**Figure 4.8**).

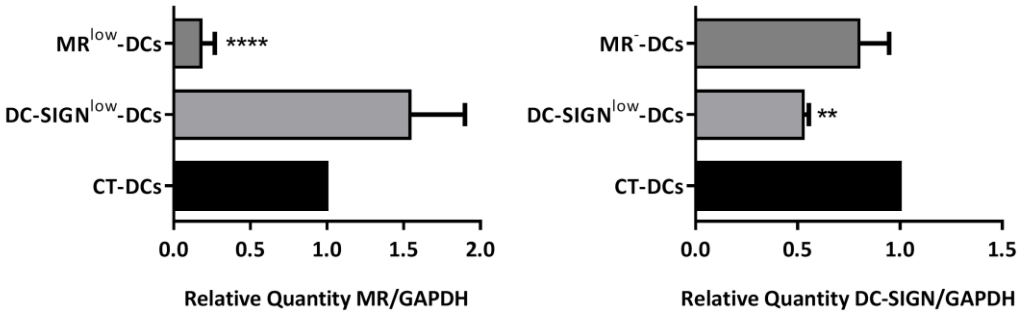

Figure 4.7: DC-SIGN and MR specific knock-down using gene silencing. qRT-PCR analysis of DC-SIGN and MR gene expression in CT, DC-SIGN^{low} and MR^{low}-DCs. Relative expression of DC-SIGN and MR were compared with that of GAPDH (n=2).

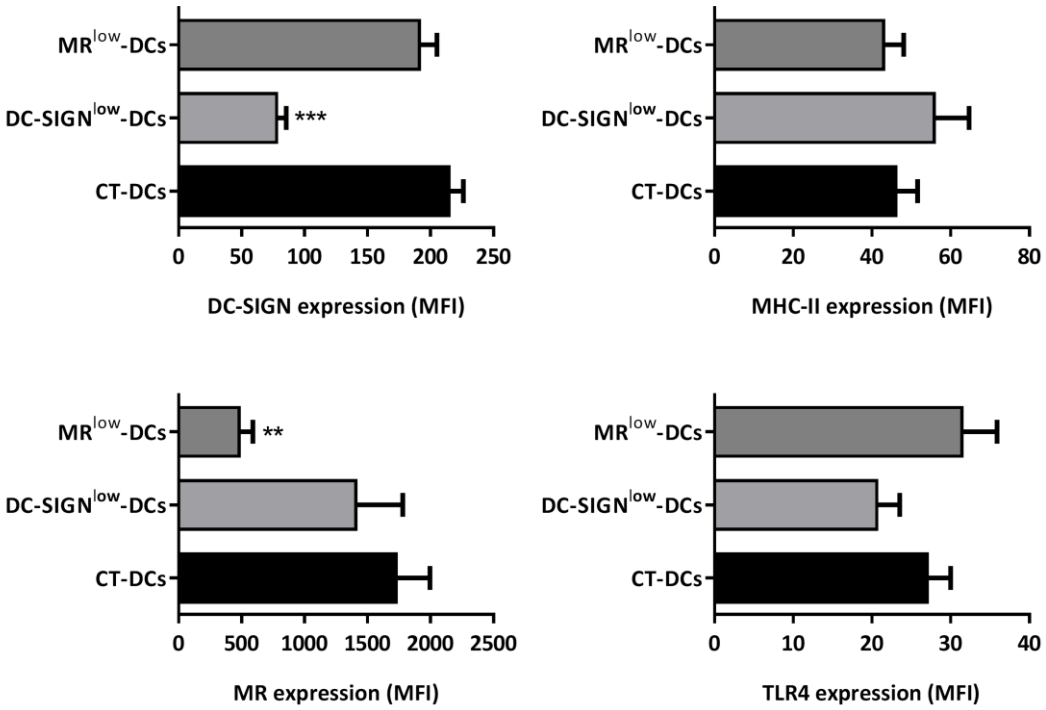

Figure 4.8: DC-SIGN and MR specific knock-down using gene silencing. Flow cytometry analysis of DC-SIGN, MR, TLR4 and MHC-II protein expression in CT, DC-SIGN^{low} and MR^{low}-DCs (n=5).

IDO regulation in CT compared to DC-SIGN^{low} as well as MR^{low}-DCs was then investigated. Surprisingly, it was found a significant further reduction in IDO activity in the presence of mannan in DC-SIGN^{low}-DCs compared with CT-DCs, which suggest that mannose-based ligands could potentially up-regulate IDO activity after DC-SIGN engagement in human DCs (**Figure 4.9**). In addition, the data show a significant increase in IDO activity in the presence of mannan in MR^{low}-DCs compared with CT-DCs, suggesting that mannose-based ligands can down-regulate IDO activity after MR engagement in human DCs (**Figure 4.9**), which is supported by our recent publication (259). Remarkably, the basal levels of IDO were also reduced after DC-SIGN down-regulation, which again is opposite to what happens after MR knock-down (**Figure 4.9**). In addition, the data shows a lack of IDO up-regulation in DC-SIGN^{low}-DCs compared with CT-DCs after TLR4 engagement (**Figure 4.9**), which was not associated with an effect on TLR4 expression (**Figure 4.8**).

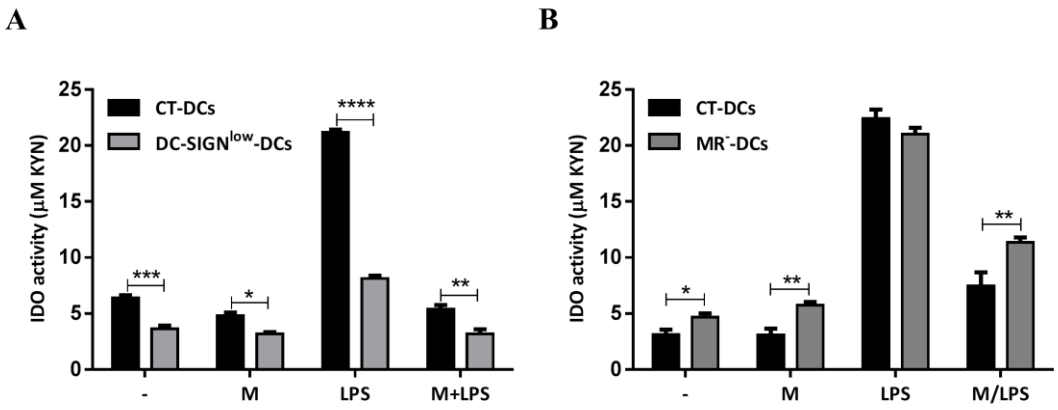

Figure 4.9: DC-SIGN and MR differentially regulate IDO activity in response to mannan in human DCs. A. IDO activity in CT and DC-SIGN^{low}-DCs stimulated with mannan (M) and LPS. B. IDO activity in CT and MR^{low}-DCs stimulated with mannan and LPS (n=3).

These data underline an opposite role for DC-SIGN and MR in IDO regulation in human DCs. Further studies should be focused on understanding the mechanisms of IDO up-regulation after DC-SIGN engagement by mannosylated sugars and its effect on T helper cell polarization.

4.3 Discussion

CLRs are a superfamily of soluble and membrane-associated proteins characterized by the presence of a CRD, which enables them to recognize different carbohydrate structures on antigens (67, 68). DC-SIGN belongs to the Ca^{2+} dependent subfamily of CLRs with a single CRD in its extracellular portion and an iITAM in its cytoplasmic domain. DC-SIGN is primarily expressed by immature DCs and it is involved in various DC functions including differentiation, migration, antigen capture and T cell priming (254). DC-SIGN can interact with endogenous molecules such as the intracellular adhesion molecule 3 (ICAM3) expressed by resting T cells that supports immune responses (253). In addition, it can mediate the response against exogenous ligands derived from pathogens such as viruses, bacteria, fungi and parasites as well as allergens inducing intracellular cascades, depending on the nature of the antigen (108-110, 252, 261).

IDO is an inducible enzyme that catalyzes the rate-limiting step in TRP metabolism and plays a critical role in immune tolerance and immunosuppression (104, 105). Initially implicated as a counter-regulatory mechanism in maternal-fetal tolerance, IDO has been found to be crucial in balancing inflammation and tolerance in diverse pathological conditions including allergy, autoimmunity, cancer, chronic infection and transplantation (262-266). By promoting local metabolic changes, IDO can impact cellular and systemic responses to inflammatory and immunological signals (104,

105). In this study the role of DC-SIGN in IDO regulation was investigated and its implications in modulating immune responses. Here it has been demonstrated for the first time that DC-SIGN has a dual role in IDO regulation. Mannosylated sugars can potentially increase IDO activity in the presence of LPS. Conversely, fucose-bearing antigens can down-regulate TLR4-mediated IDO induction in human DCs (**Figure 4.6** and **Figure 4.9**). Additionally, it was shown that, unlike DC-SIGN, MR ligation with mannan can down-regulate TLR4-induction of IDO in human DCs (**Figure 4.6**). This is in line with recent data by our group showing that MR-mediated IDO down-regulation in human DCs involved a cross-talk between the transcription factors AhR - involved in mediating the toxic effects of diverse xenobiotics - and RelB, a member of the non-canonical NF-κB family (259) (**Figure 4.5**). Thus, it can be hypothesized that the higher expression of MR than DC-SIGN in human Mo-DCs (267) might account for the overall response in the presence of mannan leading to IDO reduction. In addition, other factors such as affinity and ligand complexity might be influencing receptor ligation. In this context, it has been shown that DC-SIGN interacts more strongly with mannose type N-glycans carrying 5-9 mannose residues than single mannose (260, 268). In addition, it has been suggested that MR recognizes single terminal mannose residues, while DC-SIGN interacts with more complex mannose residues in specific conformations (223).

The impact of IDO modulation by fucose-specific DC-SIGN engagement was also investigated on DC cytokine profile and down-stream T cell responses. Previous observations have shown that fucose ligands can alter TLR4-mediated cytokine production by DCs favouring Th2 immune responses (113, 252). The data clearly shows that DCs priming with Le^X followed by co-culture with T cells in the presence of LPS impairs TLR4-mediated Th1 cell polarization. A significant reduction in IFN-γ

production by T cells was observed with no effect on IL-4 production (**Figure 4.2**). In order to establish the role of IDO activity in T cell polarization in these experiments, co-cultures using DCs primed with Le^X+LPS or LPS only and autologous naïve T cells were performed in the presence of KYN, one of the main metabolites produced in the IDO-mediated TRP catabolizing pathway (159, 220). The data demonstrated that IDO mediates the effect of fucosylated primed-DC on T helper cell polarization in the presence of LPS, as IFN-γ production was partially re-established when KYN was added to the co-culture (**Figure 4.2**). This data highlight the role of DC-SIGN in modulating immune responses through regulation of IDO levels. Furthermore, it was shown that fucose-specific DC-SIGN ligation can potentially modulates TLR4 signalling as a trend for IL-6 down-regulation was observed in human DCs. However, neither IL-10 nor IL-12p70 production exhibited differences (**Figure 4.3**). Due to the ability of DC-SIGN to modulate IL-6 production it could be interesting to study its role in Th17 differentiation. Some previous studies have suggested that DC-SIGN might modulate the development of Th17 responses (269, 270). Interestingly, IDO and TRP catabolism have been shown to alter the balance between Th17 and Treg in different pathological conditions (176, 271-274).

Finally, some insights into the signalling pathway involved in fucose-specific DC-SIGN-mediated IDO modulation were obtained. The analysis was focused on the NF-κB pathway, as it has been shown to participate in downstream signalling mediated by DC-SIGN (113, 257) as well as IDO regulation (169, 259). Initially, protein expression of a panel of 14 different targets within the NF-κB pathway was analysed, including members of the NF-κB family, IκB family, IκB kinase (IKK) family, TRAF protein family as well as NF-κB regulators (**Figure 4.5**). It is known that p65 undergoes post-translational modifications at several sites involving phosphorylation

as well as acetylation (275). Phosphorylation of p65 on Ser536 is required for LPS-dependent NF-κB activation (276). The data shows no significant differences in p65 phosphorylation on Ser536 after stimulation of DCs with Le^X only and together with LPS compared with unstimulated and LPS only controls respectively (**Figure 4.5**). On the contrary, a significant reduction in the expression of some components upstream of the non-canonical NF-κB pathway was observed, such as NIK and TRAF3 (**Figure 4.5**). A trend for RelB down-regulation was also observed (**Figure 4.5**). This is in line with previous data showing the key role of the non-canonical NF-κB pathway in maintaining IDO levels after CD40 and TLR4 ligation (169, 259). Under resting conditions TRAF3 interacts with a TRAF2-cIAP complex to direct polyubiquitylation and proteosomal degradation of NIK. After stimulation, TRAF2-dependent degradation of TRAF3 and subsequent accumulation of NIK activates IKKα to induce p100 phosphorylation culminating in the generation of transcriptionally active p52-RelB complexes (226, 277). Strikingly, the data shows that fucose-specific DC-SIGN signalling reduces NIK protein and TRAF3 expression which can inhibit and facilitate non-canonical NF-κB activation respectively and in that context acting in an opposite manner. RelB nuclear shuttling and transcriptional activity are mechanisms that regulate non-canonical NF-κB activation and ultimately gene expression (278-281). Thus, nuclear translocation of specific NF-κB complexes should be analysed in order to confirm the effect of DC-SIGN on the NF-κB pathway. Previous data have shown that recognition of fucose-expressing extracellular pathogens like *Helycobacter pylori* and *Schistosoma mansoni* by DC-SIGN activate the atypical IκB family member Bcl3 repressing TLR-induced pro-inflammatory cytokine expression, while enhancing IL-10 and Th2-attracting chemokine expression (113, 252). Consequently, due to the complex heterogenicity of the NF-κB family members and regulatory proteins, future

studies should aim at elucidating the specific dimers that are translocated to the nucleus, their DNA-binding activity and ultimately their ability to modulate gene expression, particularly IDO expression.

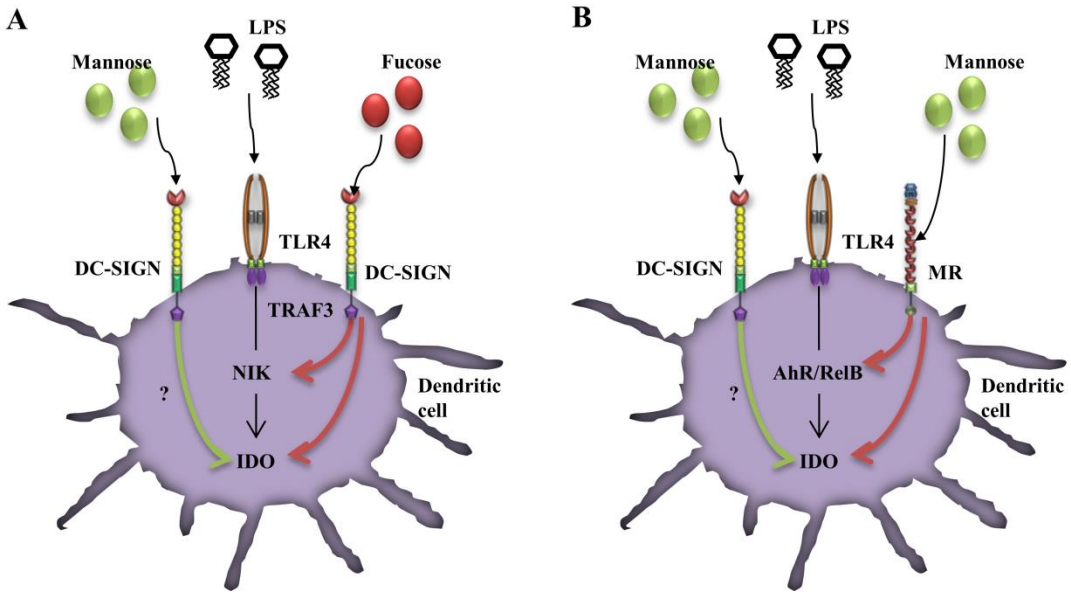

Figure 4.10: DC-SIGN and MR can modulate TLR4-mediated induction of IDO in human DCs. A. Carbohydrate-specific regulation of IDO activity by DC-SIGN in human DCs. B. Antagonistic role of MR and DC-SIGN in regulating IDO levels in response to mannosylated molecules in human DCs.

DC-SIGN is constitutively associated with a signalosome complex consisting of the scaffold proteins LSP1, KSR1 and CNK that mediates intracellular activation (252). Besides the NF-κB pathway, activation of additional intracellular signalling such as MEK/ERK1/2 and PI3K/Akt, have been shown after DC-SIGN engagement with Abs and immunomodulatory proteins including allergens (108, 282, 283). Given the robustness of the reverse phase protein microarray platform, it could be interesting to analyse the activity of additional signalling molecules, such as the ones linked to the

MAPK and PI3K signalling pathways, under the conditions tested above to clarify their role in IDO modulation.

Unlike fucose, mannose-expressing extracellular pathogens such as *Mycobacterium tuberculosis*, *M. leprae*, *Candida albicans*, measles virus and HIV induce the recruitment of the signalosome complex and the activation of the Raf-1-acetylation-depedent signalling pathway of the NF-κB subunit p65 (252, 257, 258) after interaction with DC-SIGN. In addition, a large-scale gene expression profile has shown that DC-SIGN engagement with HIV induces activation of LARG and RHOA, which might act as upstream activators of Raf-1 (284). Therefore, the involvement of the Raf-1 signalling pathway in DC-SIGN-mediated IDO modulation by mannosylated antigens remains to be established.

In summary the data describes a carbohydrate specific role for DC-SIGN in IDO modulation in human DCs. Fucose-bearing antigens can down-regulate TLR4-induced IDO activity affecting Th1 polarization, while highly mannosylated molecules are able to induce IDO activity acting in an opposite manner to MR (**Figure 4.10**). In addition, the data provide new insights into the mechanisms involved in DC-SIGN-dependent modulation of TLR4 signalling, with the non-canonical NF-κB pathway being one of the targets downstream of DC-SIGN. Future studies should aim at elucidating the exact mechanism of DC-SIGN mediated IDO regulation and the involvement of some key molecules linked with the IDO pathway, such as the AhR.

5 Chapter V: The role of TLR4 engagement in modulating IDO

5.1 Introduction

Inflammation is a complex pathophysiological state orchestrated primarily by innate immune cells in response to infection and/or tissue damage. This phenomenon is indispensable for protecting the host from invading pathogens, however, overproduction of inflammatory molecules can cause tissue damage and has been implicated in the onset of pathological hyper-inflammatory responses, including allergy, atherosclerosis, sepsis and a number of autoimmune diseases (285, 286). Innate immune cells have developed mechanisms to counteract over-inflammatory responses and reduce their negative effects by restoring homeostasis. IDO is an immune-regulatory enzyme, mainly expressed in DCs, that can modulate adaptive immune responses by promoting immune-suppression and tolerance.

Under inflammatory conditions, IFN-γ can induce high levels of IDO which, through TRP depletion and production of TRP metabolites can help to resolve inflammation and restore tissue homeostasis (104, 287). Additionally, there is an IFN-γ-independent pathway for induction of IDO which is mainly mediated by TLRs such as TLR4 in myeloid DCs (161, 175) and TLR9 in pDCs (176, 177). This up-regulation of IDO by TLR ligands involves pathways that converge in non-canonical NF-κB (176, 177). Interestingly, the ligand-dependent transcription factor AhR - originally implicated in the mechanisms behind the toxicity mediated by xenobiotics - has been closely linked with IDO in modulation of immune responses (200). Particularly, it has been shown that an interaction between KYN, main metabolite produced in the IDO-mediated TRP-catabolizing pathway, and AhR can modulate DC immunogenicity and induce

Tregs (159, 220). More recently, the AhR-IDO axis has been shown to be involved in endotoxin tolerance in mice (288).

DCs are professional APCs and key regulators of adaptive immunity. They possess high functional plasticity influenced by diverse external stimuli which allow them to orchestrate adaptive immune responses by either promoting or suppressing T-cell responses. Antigen recognition and uptake in the periphery followed by migration to secondary lymphoid organs along with expression of co-stimulatory molecules and secretion of cytokines are the main features that enable DCs to stimulate naïve T cells or under specific microenvironmental conditions induce tolerance (68, 218). LPS activates the TLR4 pathway that induces the release of pro-inflammatory cytokines and the induction of co-stimulatory molecules necessary to activate immune responses. Engagement of TLR4 and specific signaling adaptor molecules such as MyD88 and MyD88-adaptor-like protein (MAL) or TRIF and TRIF-related adaptor molecule (TRAM) stimulate downstream signaling pathways that involve interaction of kinases and TRAFs ultimately leading to the activation of different transcription factors. One important family of transcription factors activated downstream the TLR4 signaling pathway is the NF-κB pathway, however other transcription factors such as CREB and AP1 are also important (226, 289). Conversely, LPS has been also shown to induce tolerance after repeated stimulation leading to immune-suppression but the mechanism of such tolerance is still not fully understood (290-293).

Here the effect of LPS-conditioning on human DCs in the expression of the immuno-modulatory molecules IDO and AhR as well as cytokine production was studied. Additionally, the expression pattern and post-translational modifications of key proteins along the canonical and non-canonical NF-κB pathway were investigated in

order to better understand their role in TLR4-mediated IDO modulation and endotoxin tolerance in human DCs.

5.2 Results

5.2.1 LPS conditioning induces high levels of both IDO isoforms and AhR in human DCs

It has been previously shown that TLR4 signaling can induce IDO in human DCs (161). In particular, IDO1 and IDO2 were both induced after single stimulation with LPS in human DCs (259). In this study it was aimed to investigate IDO regulation after TLR4 ligation in LPS-conditioned human DCs as an *in vitro* model of endotoxin tolerance. IDO activity as well as IDO1 and IDO2 expression was evaluated in human LPS conditioned DCs compared with DCs stimulated once with LPS (unprimed controls). IDO activity was significantly induced in unprimed DCs after LPS stimulation compared with unstimulated DCs (media only). Moreover, it was found a significant induction in IDO activity in LPS conditioned DCs compared with unstimulated or single LPS stimulation controls (**Figure 5.1**). Strikingly, unlike DCs, monocytes did not induce high levels of IDO after subsequent stimulation with LPS showing that, this is a particular feature of DCs (**Figure 5.1**).

The contribution of both IDO isozymes named IDO1 and IDO2 in IDO activity was then evaluated by assessing their gene expression. The data shows that single stimulation with LPS induce IDO1 as well as IDO2 gene expression in human DCs in a time-dependent manner with a peak at 24 hrs. Conversely, LPS-primed DCs induced much higher levels of both IDO isoforms at early time points (3 hrs) (**Figure 5.1**). This data is in contrast with what has been previously shown in mouse splenic DC, where no induction of IDO2 was reported (294).

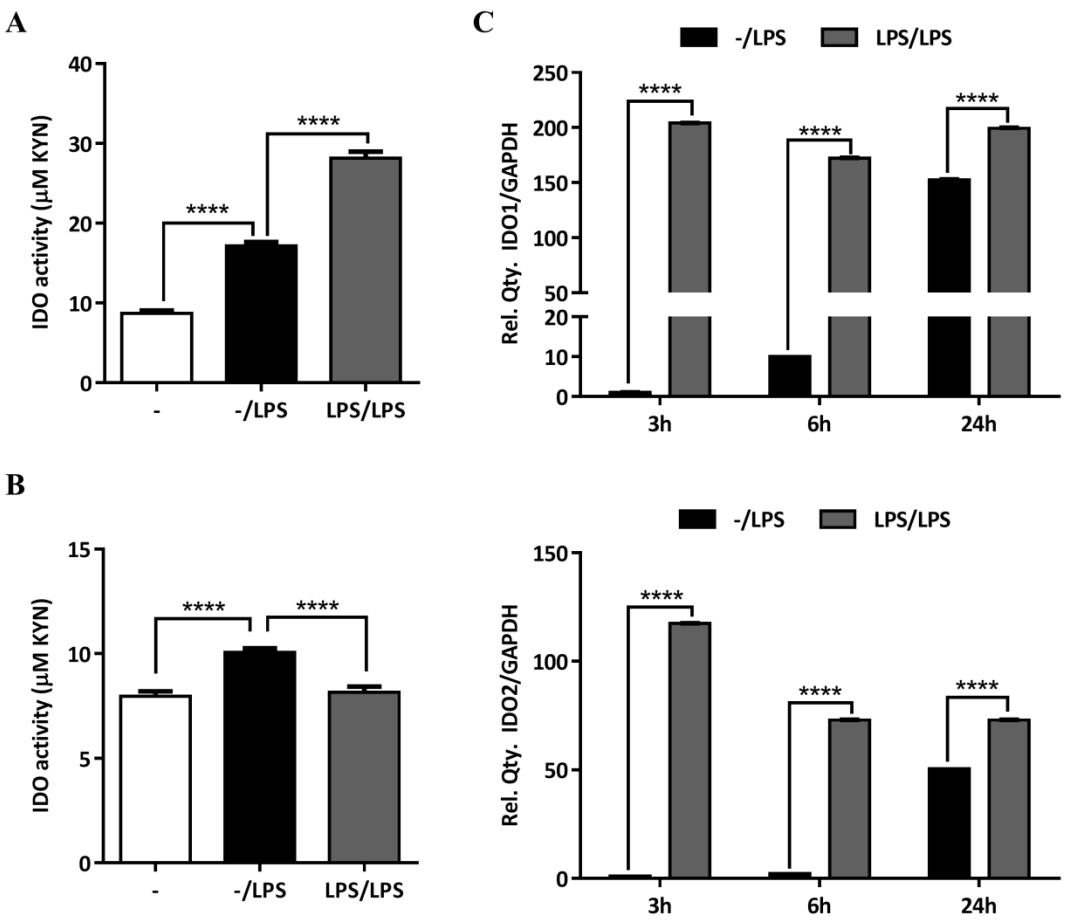

Figure 5.1: IDO1 and IDO2 are induced in LPS-conditioned human DCs but not monocytes. A. IDO activity in human DCs stimulated either once (unprimed DCs) or twice (LPS-primed DCs) with LPS (0.01 µg/ml) for 24 hrs (n=6). B. IDO activity in human monocytes stimulated either once or twice with LPS (0.01 µg/ml) for 24 hrs (n=4). C. qRT-PCR analysis of IDO1 and IDO2 gene expression in unprimed versus LPS-primed DCs after re-stimulation with LPS. Relative expression of IDO1 and IDO2 was compared with that of GAPDH (n=2).

The ligand-dependent transcription factor AhR has been shown to mediate some of the immuno-modulatory functions of IDO metabolites and regulate IDO1 signaling in mouse DCs (159, 220, 288). Here the data show that upon LPS stimulation AhR gene expression and activity, assessed by measuring the expression of one of its target genes named CYP1A1, are highly induced in LPS-conditioned DCs compared with

unprimed controls. The kinetics of AhR induction was similar to the one observed for IDO1 and IDO2, with high levels after 24 hrs in unprimed controls while early induction at 3 hrs was observed in LPS-primed DCs (**Figure 5.2**). This data underline the potential role of the AhR as well as both IDO isoforms in the induction of endotoxin tolerance in human DCs.

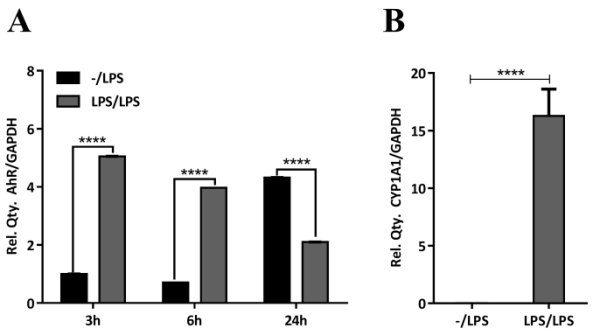

Figure 5.2: AhR is induced in LPS-conditioned human DCs. A. qRT-PCR analysis of AhR gene expression in unprimed versus LPS-primed DCs after re-stimulation with LPS (n=2). B. qRT-PCR analysis of CYP1A1 gene expression in unprimed versus LPS-primed DCs after re-stimulation with LPS (n=2). Relative expression of AhR and CYP1A1 was compared with that of GAPDH.

5.2.2 LPS conditioning induce an anti-inflammatory profile in human DCs

One of the main features of LPS conditioned cells is their ability to differentially modulate the production of pro and anti-inflammatory cytokines after re-challenge with LPS (290, 293). Accordingly, the levels of pro-inflammatory cytokines such as IL-12p70, IL-6, IL-8, TNF-α and IL-1β, as well as the anti-inflammatory cytokine IL-10 were evaluated in human LPS-conditioned DCs compared with unprimed controls. A significant induction in most of the cytokines tested including IL-10, IL-6, IL-8, IL-12p70 and TNF-α was observed in DCs stimulated with LPS once with no differences in the levels of IL-1β, a cytokine mainly produced by macrophages as an inactive precursor that require activated caspase-1 to generate its biological active compound

(295). Interestingly, no significant differences were observed between LPS-conditioned and unprimed DCs in all pro-inflammatory cytokines tested. However, a significant induction in IL-10 was observed in LPS-conditioned DCs compared with single stimulated controls (**Figure 5.3**) after LPS stimulation. All these data show that LPS-conditioned human DCs adopt a particular phenotype characterized by an increased expression in immuno-regulatory mediators such as IDO1, IDO2, AhR and IL-10.

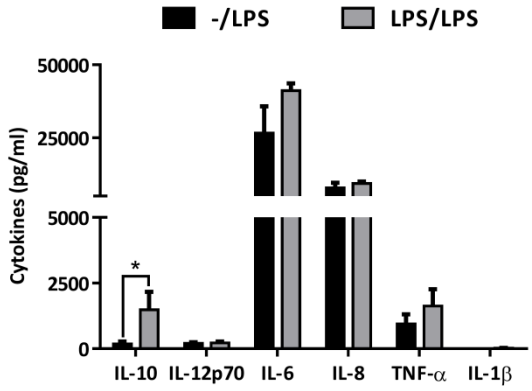

Figure 5.3: LPS-conditioning modulates cytokine production in human DCs. Cytokine production by unprimed and LPS-primed DCs after re-stimulation with LPS (n=5).

5.2.3 LPS conditioning favours the non-canonical NF-κB pathway in human DCs

The NF-κB pathway has been previously linked with the induction of endotoxin tolerance (290, 293, 296) as well as IDO modulation (150, 169). Therefore, the levels of several components of the NF-κB pathway were evaluated in an attempt to understand the molecular mechanisms behind IDO modulation in LPS-conditioned DCs.

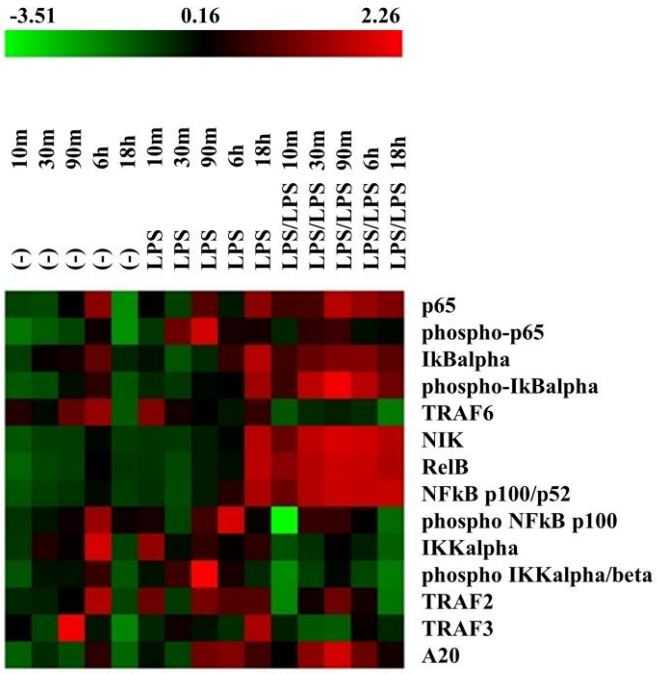

Figure 5.4: LPS-conditioning favours the non-canonical NF-κB pathway in human DCs. Heat maps representing the relative abundance of proteins upstream the NF-κB signalling pathway using unprimed and LPS-primed DCs re-stimulated with LPS for different time points.

A reverse phase protein array approach was used (217, 259) to evaluate simultaneously protein expression as well as post-translational modifications in LPS-conditioned DCs using unprimed DCs as controls. A panel of 14 different targets were evaluated including members of the NF-κB family, IκB family, IKK family, TRAF protein family as well as NF-κB regulators 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 (**Figure 5.4**). It was found a significant increase in the expression of key components of the non-canonical NF-κB pathway (i.e. RelB and NIK) at early time points (30 min) in LPS-conditioned DCs compared with unprimed controls (**Figure 5.5**). Additionally, a significant reduction in the activity of the canonical pathway was observed in LPS-conditioned DCs compared with single stimulated controls. This was reflected in the levels of p65 phosphorylation at late time points (6 hrs) (**Figure 5.5**).

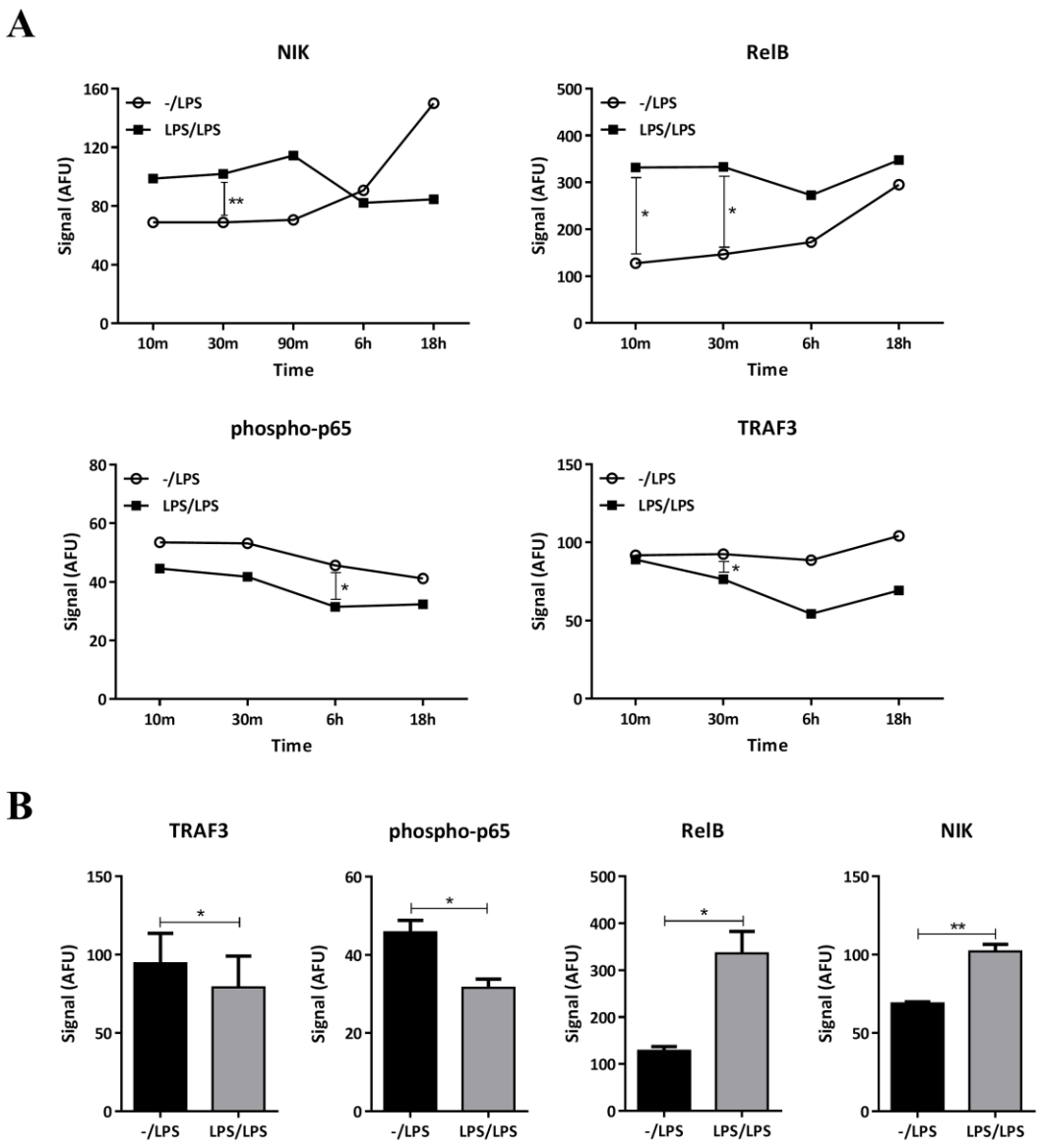

Figure 5.5: LPS-conditioning favours the non-canonical NF-κB pathway in human DCs. A. Protein expression of phospho-p65, NIK, RelB and TRAF3 in unprimed and LPS-primed DCs re-stimulated with LPS. Data are shown as geometric mean of three independent experiments. B. Protein expression of RelB, NIK, phospho-p65 and TRAF3 in unprimed and LPS-primed DCs re-stimulated with LPS.

Furthermore, the data shows a significant down-regulation in the levels of TRAF3, a negative regulator of the non-canonical pathway (**Figure 5.5**). Collectively these data show that DCs stimulated once with LPS favours the canonical NF-κB pathway, as

evidenced by an increase in phospho-p65 at early time points, while an induction in the non-canonical pathway was observed at late time points. Conversely, LPS-conditioning biased the activation in favour of the non-canonical pathway, evidenced by an increase in NIK and RelB as well as a decrease in TRAF3 protein levels. This highlights the potential role of the NF-κB pathway in mediating LPS-induced reprogramming in human DCs through IDO.

5.3 Discussion

Uncontrolled or sustained inflammation has been implicated in the pathogenesis of several conditions including metabolic disorders, neurodegenerative diseases, allergy and cancer (297-299). Despite many clinical research efforts no effective treatments have been developed to combat systemic inflammatory responses such as in sepsis (300). Therefore, better understanding of the mechanisms of inflammation and immune-suppression are essential for developing more effective therapeutic strategies. Previous studies have shown that degradation of TRP and high IDO activity are associated with the development of sepsis in bacteremic patients (301-303). In this context, IDO has been suggested to promote LPS-induced endotoxin shock in mice by changing the balance between IL-12 and IL-10 production in favor of IL-12 (304). Conversely, it has been shown that IDO1 mediates protection of mice from endotoxin shock in LPS-conditioned DCs (294). More recently, TRP metabolism and the ligand-dependent transcription factor AhR have been shown to mediate tolerance to infection *in vivo* (288). Accordingly, the role of IDO pathway in human endotoxemia and more importantly endotoxin tolerance has been controversial and remains elusive.

The resolution of inflammatory responses is mediated by innate immune cells in the later phase of inflammation to avoid tissue damage and promote healing and tissue

repair (305). Endotoxin tolerance is a phenomenon characterized by unresponsiveness to a second exposure to LPS following an initial encounter with endotoxin that affects most of the TLR4-expressing innate immune cells (290-293). DCs are sentinels and sensors of the immune system linking innate with adaptive immunity by inducing T cell activation or tolerance (68, 218). In addition, DCs are the major producers of IDO in the immune system (104, 287). Here IDO modulation was investigated in LPS conditioned human DCs upon second exposure to LPS. The role of the NF-κB pathway in regulating IDO levels was also studied under these conditions. It was shown that both IDO isoforms as well as IDO activity are highly induced in LPS-conditioned DCs compared with unprimed controls (**Figure 5.1**). In addition, a similar pattern for AhR expression as well as activity was found (**Figure 5.2**). Furthermore, an increased in the anti-inflammatory cytokine IL-10 was found in LPS-conditioned DCs compared with single stimulated controls (**Figure 5.3**). Hence, these data suggest that LPS primed DCs undergo a reprogramming with an induction in anti-inflammatory mediators such as IDO1, IDO2, AhR and IL-10. The differences observed in this model in terms of pro-inflammatory cytokines compared with previous studies in mouse DCs are most likely due to specie-specific characteristics, which have been previously suggested by other authors too (306). In line with this, it has been also found for the first time an induction in IDO2 gene expression in LPS-conditioned human DCs, which is in contrast to what has been previously shown in mouse splenic DCs (294). In addition, differences in LPS concentrations can also account for the dissimilar readouts, which suggest that LPS-mediated reprogramming of DCs is complex and is tightly regulated by different environmental factors.

Mechanistically, it has been shown that the transcription factor Twist-2, a negative regulator of the TLR4 pathway, can modulate the production of pro-inflammatory

cytokines like IL-12, IL-6 and TNF-α under endotoxin tolerance conditions in mouse myeloid DCs (307). In addition, a similar role for the TLR4 adaptor protein IRAK-1 has been suggested in human DCs (308). The NF-κB pathway exhibits great plasticity in inflammatory TLR signaling and also plays a central role in the resolution of inflammation (309). TLR4 signaling can induce both the canonical and non-canonical NF-κB pathway. The canonical pathway starts with IKKβ-dependent phosphorylation and proteosomal degradation of IκB resulting in the release of the p65-p50 dimers. Subsequent p65 phosphorylation and nuclear translocation leads to the induction of pro-inflammatory cytokine expression that activates the immune response. In contrast, the non-canonical pathway involves IKKα-mediated phosphorylation of p100 associated with RelB, which leads to p100 processing and the generation of the p52-RelB complexes (226). Switch in NF-κB function from an inflammatory (MyD88-mediated) to an anti-inflammatory (TRIF-dependent) has been observed in the course of inflammation and in the context of endotoxin tolerance (290, 293, 310). Overexpression of inhibitory p50 homodimers and decreased levels of transcriptional active p65-p50 heterodimers have been observed in endotoxin tolerant monocytes and macrophages (311, 312). In addition, p50 $^{-/-}$ murine macrophages fail to develop endotoxin tolerance (306, 313). Members of the non-canonical pathway have also been implicated in endotoxin tolerance. Particularly, overexpression of RelB has been found in endotoxin tolerant human monocytes (314, 315). Accordingly, the attention was focused in studying the role of both arms of the NF-κB pathway in LPS-mediated reprogramming of human DCs (**Figure 5.4**). It was found an induction in the non-canonical NF-κB pathway in LPS-conditioned DCs after TLR4 engagement. Overexpression of RelB along with NIK protein as well as down-regulation in TRAF3 and phospho-p65 was found in LPS-conditioned DCs compared with unprimed

controls (**Figure 5.5**). This data provides new insight into the mechanisms of LPS-mediated reprogramming in human DCs and endotoxin tolerance.

The hygiene hypothesis, which describes the protective influence of repeated microbial exposure in early life on the development of allergy and asthma, can be described as an induced tolerogenic state to allergens (8). This phenomenon of tolerance to allergens can be controlled by mechanisms similar to those induced in an endotoxin tolerance state. Therefore, it could be interesting to explore the role that the IDO pathway might potentially play in protecting against developing allergic responses (259).

In conclusion, this data highlights the role of IDO in LPS-induced DC reprogramming. LPS-conditioning in human DCs induces gene reprogramming with induction of anti-inflammatory mediators such as IDO, AhR and IL-10. This was also associated with an induction of the non-canonical NF-κB signaling pathway. These data can help to further understand the mechanisms behind LPS modulation of DC phenotype and function under steady-state and inflammatory conditions.

6 Chapter VI: The role of TLR9 engagement in modulating IDO

6.1 Introduction

TLRs are PRRs that play a critical role in innate immunity by recognizing conserved PAMPs in microorganisms as well as endogenous danger signals. TLR9 is an intracellular receptor that recognizes unmethylated bacterial cytosine-phosphate guanosine (CpG) motifs leading to pro-inflammatory cytokine production. Particularly, TLR9-mediated recognition of CpG by pDCs results in potent type-I IFN production (316). Moreover, synthetic ODN containing CpG motifs have been shown to induce activation and cytokine production in diverse immune cells, such as B cells, NK cells, monocytes, macrophages and myeloid DCs through TLR9-dependent mechanisms (317-320). Accordingly, three major types of CpG DNA have been identified based on structural characteristics and activity on pDCs and B cells named CpG-A, CpG-B and CpG-C (321, 322). CpG-A is characterized by a phosphodiester backbone CpG motif within a hairpin-forming palindrome region and phosphorothioate-modified poly G stretches at the 5' and 3' end. The poly G tails form intermolecular tetrads that result in high molecular weight ordered structures. Those structures confer enhanced stability and increased endosomal uptake contributing to the production of large amounts of IFN-α and the maturation of pDCs (323).

It has been shown that not only the plasmacytoid subset of DCs can respond to TLR9 agonists. Other DC types, including myeloid peripheral blood DCs (324, 325) as well as Mo-DCs (326-328), are strongly activated by CpG motifs to promote Th1-like immune responses. In addition, monocytes can acquire a DC-like phenotype that preferentially activates $CD8^+$ T cells and induce Th1 immunity after CpG stimulation

(317, 329). However, several studies have suggested that CpG does not activate human Mo-DCs (324, 330-334).

Besides their pro-inflammatory functions, CpG-ODN TLR9 agonists have been associated with immunosuppressive activities for the treatment of conditions such as allergy and autoimmunity (335). This regulatory function has been attributed to their induction of the immunoregulatory enzyme IDO. In particular, mouse splenic $CD19^+$ pDCs (176, 336, 337), as well as human pDCs (177), have been shown to upregulate IDO after CpG stimulation through type-I IFN signaling inducing Treg differentiation. IDO being a central molecule in modulating immune responses, it is crucial to understand TLR-mediated IDO regulation primarily in human DCs. This is highly relevant during infection where immune cells can come across an array of PAMPs, resulting in sequential or simultaneous activation of TLRs.

Here CpG-mediated IDO regulation and cytokine production in human myeloid DCs was studied. It has been demonstrated that CpG-A down-regulates IDO activity as well as TLR4-induced IDO activity in myeloid DCs, including peripheral blood DCs and Mo-DCs. In addition, it was found that TLR9 stimulation can also interfere with TLR4-mediated cytokine production in Mo-DCs.

6.2 Results

6.2.1 CpG-A down-regulates IDO activity in human myeloid DCs without affecting cell viability

CpG has been previously shown to induce IDO in pDCs (176, 177, 336, 337), however its role in IDO modulation in myeloid DCs has not been explored yet. In the present study, IDO modulation by CpG motifs has been analysed in human DCs. The

analysis was focused on CpG-A, which has been shown to be the main inducer of cytokine production and IDO in pDCs (338). The data shows that CpG-A can down-regulate IDO activity in Mo-DCs in a dose-dependent manner (**Figure 6.1**). It has been shown that LPS can induce IDO expression and activity in human DCs (259). Accordingly, the cross-talk between TLR9 and TLR4 in modulating IDO activity in monocyte-derived as well as peripheral myeloid DCs was investigated. It was found a significant down-regulation in IDO activity in DCs stimulated with LPS and CpG compared with single LPS controls (**Figure 6.1**). This data suggests that TLR9 engagement can interfere with TLR4 signalling in modulating IDO activity in myeloid DCs. In addition, it was demonstrated that IDO reduction was not associated with impaired cell viability by quantifying the levels of annexin-V as well as propidium iodide (PI) in DCs stimulated with CpG. No significant differences were observed between CpG stimulated and unstimulated controls. However, a significant increase in early apoptotic (annexin-V-FITC$^+$ PI$^-$) and late apoptotic or necrotic (annexin-V-FITC$^+$ PI$^+$) cells was observed with the positive control formaldehyde (**Figure 6.2**).

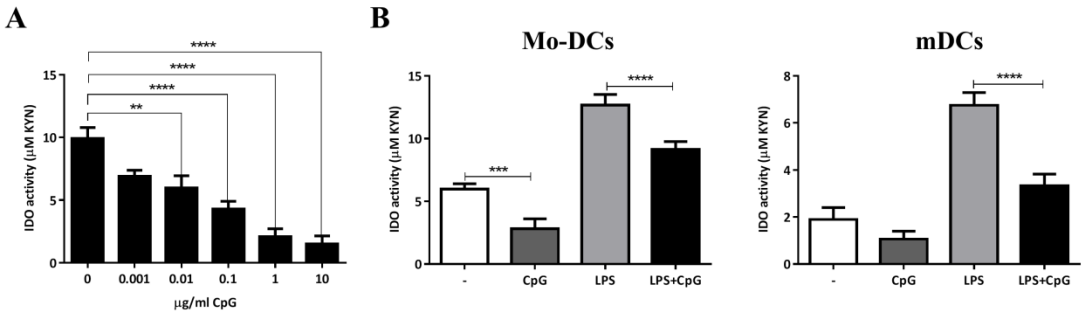

Figure 6.1: CpG suppresses IDO activity in human myeloid DCs. A. IDO activity in human Mo-DCs stimulated 24 hrs with increasing concentrations of CpG (n=4). B. IDO activity in human Mo-DCs (n=5) and mDCs (n=2) stimulated 24 hrs with CpG (1 µg/ml) in the presence and absence of LPS (0.01 µg/ml). Cells unstimulated and stimulated with LPS only were used as controls.

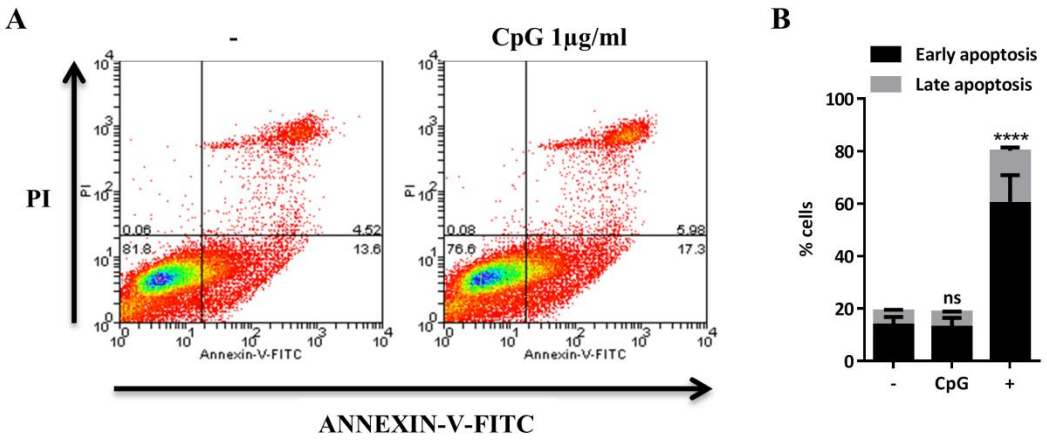

Figure 6.2: TLR9 stimulation of human DCs does not affect cell viability. A. Flow cytometry analysis of annexin-V-FITC and PI staining in human Mo-DCs stimulated 24 hrs with CpG (1 µg/ml) (one experiment representative of three). B. Summary of three independent experiments showing early (annexin-V-FITC^{+}PI^{-}) and late (annexin-V-FITC^{+}PI^{+}) apoptotic cells. Formaldehyde 4% was used as positive control.

6.2.2 CpG-A interfere in LPS-induced cytokine production in DCs

The role of CpG motifs in inducing pro-inflammatory cytokine production in pDCs has been well established (316), however, its effect in myeloid DCs has been rather controversial (324, 330-334). Accordingly, pro and anti-inflammatory cytokine production were analysed in Mo-DCs exposed to TLR9 and TLR4 agonists. The levels of six different cytokines were measured (IL-6, IL-8, IL-10, IL-12p70, IL-1β, TNF-α) at different time points (1, 3, 6 and 24 hrs) in Mo-DCs stimulated with LPS and CpG. CpG on its own does not induce any of the cytokines tested for a period of 24 hrs. However, it was found a significant induction in pro-inflammatory cytokines, including IL-8, TNF-α, IL-6 and IL-1β in Mo-DCs co-stimulated with LPS and CpG compared with LPS only controls (**Figure 6.3**). Additionally, it was observed a significant down-regulation in the anti-inflammatory cytokine IL-10 (**Figure 6.3**). All

these data suggest that TLR9 engagement on Mo-DCs can regulate TLR4-induced IDO and cytokine production favouring a pro-inflammatory phenotype.

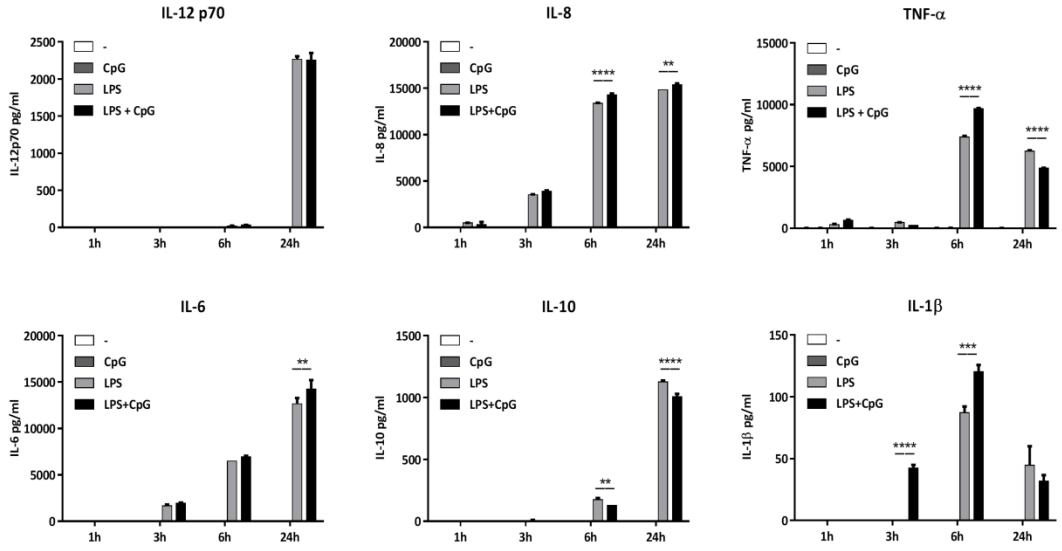

Figure 6.3: TLR9 stimulation potentiates a pro-inflammatory phenotype in response to TLR4 stimulation in human DCs. Cytokine profile of human Mo-DCs stimulated for different time points (1, 3, 6 and 24 hrs) with CpG (1 µg/ml) in the presence and absence of LPS (0.01 µg/ml). Cells unstimulated and stimulated with LPS only were used as controls (one experiment representative of three).

6.3 Discussion

IDO is the rate-limiting enzyme in the TRP-metabolizing pathway that leads to generation of several immune-regulatory metabolites known as KYN with multiple implications in health and disease (339, 340). In infectious disease states, IDO has been shown to have disparate roles (341). IDO can suppresses the replication of certain bacteria and parasite as well as prevent viral spread (342-344), a mechanism that involves TRP depletion in an IFN-γ dependent manner. However, at the same time IDO can act on host cells to suppress immune reactions by inducing either apoptosis or tolerance (157, 159). In this context, pathogens have developed

mechanisms to take over the immunosuppressive effects of IDO and use them to their advantage to dampen immune responses (345, 346). Particularly, HIV can inhibit T cell proliferation by inducing IDO in pDCs (346), besides uropathogenic *E.coli* can induce IDO on epithelial cells attenuating innate immune responses (345). TLR9 engagement on pDCs has been previously shown to induce IDO (176, 177, 336, 337). In addition, it has been suggested that TLR7 and TLR8 stimulation can impair IDO expression in Mo-DCs (347). Accordingly, understanding TLR-mediated regulation of IDO is of vital importance to combat infectious diseases. In this study, it has been shown for the first time that CpG DNA, particularly CpG-A, reduces IDO activity in human myeloid DCs. In addition, TLR4-induced IDO activity was down-regulated in the presence of ODN containing CpG motifs (**Figure 6.1**).

As a heme-containing enzyme, IDO has been demonstrated to be regulated by alterations in cellular redox status. Particularly, nitric oxide (NO) as well as peroxynitrite has been both shown to inhibit IDO activity induced by IFN-γ in human PBMCs and monocyte-derived macrophages (348-350). NO can inhibit IDO reversibly by binding to the active site heme to trap the enzyme as an inactive nitrosyl-Fe(II) enzyme adduct with TRP bound and O_2 displaced (350). Conversely, peroxynitrite induces direct nitration and subsequent inactivation of IDO (349). In addition, antioxidants can exert a post-translational regulation of IDO by limiting the availability of heme for the formation of the holoenzyme (351). Interestingly, NO production has been shown to accelerate degradation of IDO protein by the proteasome (352). On the other hand, it has been demonstrated that activation of TLR9 signalling in mouse macrophages by CpG induces NO synthase expression and subsequent NO production in a mechanism dependent on cytosolic phospholipase A2 (cPLA2) activity via p38 and Akt pathways (353, 354). All these data suggest that

reactive oxygen species production could account for TLR9-mediated IDO activity down-regulation in myeloid DCs. Interestingly, it has been shown that TLR co-stimulation can induce high levels of reactive oxygen species, which then lead to increased ATP externalization, inflammasome activation and IL-1β secretion (355).

During infection, immune cells are likely to encounter a range of TLR ligands, which might result in sequential or simultaneous activation of TLRs. Accordingly, the type of TLR agonist encountered, the timing as well as concentration could determine the nature of the inflammatory response elicited towards a particular pathogen. Indeed, signalling crosstalk between TLRs can result in complementary, synergistic (i.e. potentiation) or antagonistic (i.e. tolerance) effects that modulate innate and adaptive immune responses (356). The mechanisms that give rise to tolerance have been widely studied, while the potentiation between TLR signalling pathways has been little explored. Particularly, it has been shown that stimulation of mouse macrophages with both polyinositic-polycytidylic acid (polyI:C) and CpG induced more than additive levels of pro-inflammatory cytokines including IL-12p40, IL-6 and TNF-α (357). However, only low-level synergy was observed when TLR2 and TLR9 ligands were combined (358, 359). Here, the data show that TLR9 engagement can modulate TLR4-mediated cytokine production in myeloid DCs. In particular, it was found an increase in IL-8, TNF-α, IL-6 and IL-1β production along with a decrease in IL-10 production in Mo-DCs stimulated with LPS and CpG compared with LPS only controls (**Figure 6.3**). Interestingly, the data showed no differences in IL-12p70 production (**Figure 6.3**), which is in contrast to previous observations that suggested that TLR9 signalling can exert a tight regulation over IL-12 production through type-I IFNs secretion by pDCs (360). Previous reports have described that sequential TLR4 and TLR9 activation amplifies the inflammatory response, including TNF-α

production, of mouse macrophages. This effect was associated with Erk1/2, JNK and p38 MAPK activation (361). Accordingly, it would be interesting to study the involvement of those pathways in the crosstalk between TLR4 and TLR9 in human myeloid DCs and their impact on T helper cell differentiation.

To sum up, a cross-talk between TLR4 and TLR9 has been demonstrated in human DCs. Particularly, it was shown that TLR9 engagement with CpG-A can down-regulate IDO activity as well as TLR4-induced IDO activity in human myeloid DCs. Finally, it was found a differential effect on anti-inflammatory and pro-inflammatory cytokines in favour of the latest in Mo-DCs stimulated with CpG and LPS compared with LPS single controls. This data highlights the potential role of TLR9 in modulating TLR4 signalling, which might have implications in several pathological conditions such as allergy, autoimmune diseases and infections. Further studies should aim to understand the molecular mechanism behind TLR4 and TLR9 crosstalk in the context of IDO and their role in the modulation of DC immunogenicity and immune responses.

7 Chapter VII: General Discussion

The clarification of the regulatory mechanisms underlying the workings of the immune system could help us to better understand different pathologies. In mammals, amino acid metabolism could have evolved as a result of the need to control pathogen invasion to regulate their own immune response. In that way, catabolic enzymes have acquired new functions to control both innate and adaptive immune responses (362). The immunomodulatory enzyme IDO has been demonstrated to be involved in different processes in health and disease (262-266), however its role in allergic diseases still remains to be established. Therefore, the major objective of this project was to clarify the role of IDO in modulating allergic responses. The study was focused on the initial steps of allergen recognition, particularly on how different PRRs, including TLRs and CLRs, can modulate IDO production by DC and how this impacts Th2-mediated immune responses.

Initially, the pattern of IDO expression in human Mo-DCs was analysed by looking at gene and protein expression as well as IDO enzymatic activity. Diverse TLR and CLR agonists were tested, of which TLR4 engagement was found to act as the main IDO inducer, while MR and TLR9 agonists were shown to reduce TLR4-induced IDO in DCs. Interestingly, DC-SIGN exhibited a dual role in IDO modulation depending on the nature of the antigen. This differential regulation in IDO levels could have different implications in pathological conditions. Many allergens are highly glycosylated molecules and have been shown to interact with different CLRs on DCs, particularly MR and DC-SIGN (71, 102, 103, 110). Therefore, MR and DC-SIGN might compete and/or antagonize each other in allergen recognition and in the intracellular events that lead to IDO modulation. Mo-DCs have higher levels of MR

than DC-SIGN (267), which could explain the overall IDO reduction observed in DCs stimulated with mannan or mannosylated allergens. However, additional factors might influence receptor ligation, such as ligand affinity and its complexity (223, 260, 268). It is interesting to note that DCs isolated from asthmatic patients exhibit reduced expression of DC-SIGN (112), which is in contrast to the high levels of MR expression reported in DCs from atopic individuals (98, 99). In addition, it has been shown that some Th2 cytokines such as IL-4 and IL-13 can up-regulate MR expression in macrophages and DCs, which support the MR's ability to facilitate Th2-driven immune responses (363-366). Furthermore, the data show an induction of IDO in MR^{low}-DCs compared with CT-DCs, which demonstrates that MR is responsible for IDO reduction in the presence of mannan, but also suggests that another receptor might be involved in IDO modulation in human DCs. On the contrary, stimulating DC-$SIGN^{low}$-DCs with mannan results in IDO down-regulation compared to CT-DCs, suggesting that DC-SIGN upon recognition of mannosylated sugars on allergens can induce IDO levels antagonizing MR. Interestingly, DCs stimulated with DC-SIGN-specific fucosylated molecules lead to IDO reduction. This data demonstrated that DC-SIGN have a dual role in IDO regulation depending on the antigen's sugar moieties, with mannosylated sugars inducing IDO, while fucosylated sugars reducing IDO.

TLR signalling pathways, particularly TLR4, as well as LPS exposure have been shown to be pivotal in inducing Th2-mediated immunity and asthma (65, 89-91). In addition, previous reports have described a TLR-dependent pathway for the induction of IDO, mainly mediated by TLR3 and TLR4 in Mo-DCs (161, 175) and TLR9 in pDCs (176, 177). Here it was found that stimulation of human DCs with a single or repeated dose of LPS induce high levels of IDO expression as well as its enzymatic

activity. Intriguingly, TLR9 engagement with CpG-A can reduce IDO activity and TLR4-mediated IDO induction in human DCs. This data are relevant to infectious diseases, where immune cells are likely to encounter a range of TLR ligands that might result in sequential or simultaneous activation of TLRs.

In addition to IDO, other factors such as cytokine production and expression of co-stimulatory molecules influence DC ability to induce immune responses (73). Hence, it was analysed how IDO-modulation by CLR and TLRs can affect DC phenotype and function. A comprehensive panel of various pro- and anti-inflammatory cytokines were tested. The data show specific cytokine features. Mannosylated molecules, including allergens, were able to specifically down-regulate TLR4-mediated IL-12p70 production in human DC. However, fucosylated antigens showed a reduction in IL-6 production after LPS stimulation, which could impact Th17 immune responses (269, 270). Besides the effects on cytokine production, mannan in the presence of LPS was able to significantly reduce CD86 expression in human DC compared with single LPS controls. Despite these differences, pre-stimulation of DCs with either mannan or Le^X leads to a reduction in Th1 polarization in autologous DC-T cell co-cultures in the presence of LPS. This was reflected in the level of IFN-γ production by T cells as well as the number of IFN-γ positive cell and it was overturned in the presence of one the IDO metabolites KYN. Therefore, modulation in IDO levels on DCs by mannosylated and/or fucosylated allergens in the presence of LPS will eventually create a microenvironment inductive of Th2 polarization.

Previously, it has been shown that CLRs are able to modulate TLR signaling, particularly TLR4 signaling. Particularly, MR has been shown to be involved in Man-LAM inhibition of IL-12 production after challenge with LPS (224, 225). *In vitro* studies with macrophages have also suggested a cross-talk between MR and TLRs

after stimulation with mannosylated bacterial components leading to a synergistic (244, 367) or antagonistic (224, 225, 233) effects in terms of cytokine production with TLR2 and TLR4 respectively. On the other hand, in line with what it has been described here, DC-SIGN has been shown to have a dual response determined by the type of antigen, in particular its sugar moieties. Mannose-based antigens such as Man-LAM (256, 257) or α-glucans (258) from *Mycobacterium tuberculosis* can up-regulate IL-10 production in LPS-activated DCs leading to immunosuppression, while fucose-expressing extracellular pathogens like *Schistosoma mansoni* and *Helicobacter pylori* can increase IL-10 along with decrease IL-12 production favoring Th2 polarization in a TLR4-dependent way (113). However, some contradictory data have shown that fucosylated glycolipids from *Schistosoma mansoni* (261) can upregulate pro-inflammatory cytokines to facilitate Th1 immunity. Furthermore, interactions with other receptors have been proposed (234, 368). All these data suggest that different antigens upon DC-SIGN/MR engagement can have disparate effects on DCs and that IDO might account for these effects. Many allergens are multi-glycosylated molecules, each one of them has a different glycosylation pattern. Therefore, their oligosaccharide levels and spatial structure will determine their binding to specific CLRs on the DC surface. Additionally, the different levels of expression and affinity of CLRs to particular sugar moieties on allergens will determine the outcome of the immune response.

Dectin receptors have also been implicated in allergic diseases. Particularly, dectin-2, being able to recognize high mannose and α-mannan structures, has been shown to act as a receptor for HDM extracts leading to cysteinyl leukotriene and proinflammatory cytokine generation, and Th2 immunity (115, 116, 369). Dectin-2 can induce the activation of NF-κB pathway through a Syk- and CARD9 dependent pathway (370).

In addition to MR and DC-SIGN, Dectin-2 can also act as a receptor of Man-LAM inducing pro and anti-inflammatory cytokines, which contribute to host immunity against mycobacterial infection (371). Accordingly, it would be interesting to study how Dectin receptors as well as the Syk-CARD9 pathway can both interfere in IDO regulation by either using specific inhibitors and/or knock-down models.

Furthermore, the data show that DCs exposed twice to subsequent doses of LPS, besides inducing higher levels of IDO compared with single stimulated controls, exhibit an induction in IL-10 production. Previously, IDO has been associated with the expression of the anti-inflammatory cytokine IL-10 (165, 166). Moreover, TLR9 engagement in the presence of LPS leads to a pro-inflammatory phenotype on DCs with induction in IL-8, IL-6, TNF-α and IL-1β and reduction in IL-10 production. As mentioned before, these data could have implications in tolerance - a condition characterized by a refractory state to LPS exposure after an initial encounter to endotoxin - as well as infectious diseases where simultaneous stimulation with several TLR ligands might take place. Further studies are needed to analyse their impact on T helper cell polarization.

In an attempt to understand the molecular mechanisms of IDO regulation, the role of the ligand-dependent transcription factor AhR was analysed, which has been previously linked with IDO modulation (204, 220) and suggested to have a protective role in experimental models of allergy (201-203, 205). Mannan was shown to reduce AhR induction in human DCs after LPS challenge. This was reflected on AhR gene expression as well as AhR activity, as revealed in the levels of expression of one of its target genes i.e. CYP1A1. On the contrary, DCs exposed to repeated doses of LPS induced higher levels of AhR and AhR activity than single stimulated controls. This data highlights the opposite role of TLR4 and MR on the modulation of the AhR-IDO

axis in human DCs. The key role of AhR in IDO induction was further confirmed by analysing IDO activity in AhR^{low}-DCs, where AhR gene has been knocked down using gene silencing. AhR^{low}-DCs lacked the ability of IDO induction after LPS stimulation. In addition, the basal levels of IDO were reduced in AhR^{low}-DCs compared with CT-DCs. This underlines the importance of AhR in regulating the basal levels of IDO as well as upon TLR4 engagement in human DCs. In this context, it would be interesting to analyse how DC-SIGN can interfere in the TLR4-AhR axis particularly AhR expression. AhR has been extensively studied by the pharmacology/toxicology community for its role in mediating the toxicity of xenobiotics including TCDD (200). Xenobiotics can act as allergens and elicit hypersensitivity responses. Additionally, they can modulate hypersensitivity responses to other allergens such as pollen or HDM by acting as adjuvants and enhancing the development of hypersensitivity (372). Accordingly, it would be also interesting to study how specific AhR ligands, such as TCDD, can affect MR and DC-SIGN expression and their intracellular signalling in human DCs.

NF-κB transcription factors are critical regulators in immunity and inflammation. NF-κB signalling pathway together with the MAPK both have been involved in CLR, in particular the DC-SIGN signalling pathway (283). DC-SIGN is constitutively associated with a signalosome complex consisting of the scaffold proteins LSP1, KSR1 and CNK (252). Mannose-expressing extracellular pathogens such as *Mycobacterium tuberculosis*, *M. leprae*, *Candida albicans*, measles virus and HIV after interaction with DC-SIGN induce the recruitment of the complex and the activation of the Raf-1-acetylation-depedent signalling pathway of the NF-κB subunit p65 (252, 257, 258). In addition, a large-scale gene expression profile showed that DC-SIGN engagement with HIV induces activation of LARG and RHOA, which

might act as upstream activators of Raf-1 (284). Conversely, recognition of fucose-expressing extracellular pathogens like *Helycobacter pylori* and *Schistosoma mansoni* by DC-SIGN induces the dissociation of the complex favouring Th2 responses via activation of atypical NF-κB family member Bcl3 (113, 252). Some allergens have also been found to induce signalling pathways in DCs through DC-SIGN. Particularly, Arah1, main allergen from peanut, have been shown to induce ERK1/2 phosphorylation (108). Similar findings were observed after stimulation of DC-SIGN with specific Abs (282). However, BG-60, the main allergen from BGP, induces not only ERK but Raf-1 activation (109). MR does not have any signalling motif in its cytoplasmic domain, however some studies involving the interaction between pathogens and macrophages have suggested a role in intracellular pathway activation. It has been demonstrated that *Pseudomonas aeruginosa* activates NF-κB and MAPK pathway though MR together with TLR2 (244). In addition, MR has been shown to mediate the activation of the Rho GTPase Cdc42 and RhoB to induce bacterial phagocytosis (373), a feature shared with DC-SIGN in the context of HIV infection (374). Man-LAM, potentially through MR, mediates IRAK-M induction that negatively regulates TLR signalling (225). It is also interesting to note that IRAK-M might protect from complications of asthma, as Th2 cytokines decrease IRAK-M expression in macrophages (245). The interaction between MR and Man-LAM can also induces peroxisome proliferator-activated receptor (PPAR)γ activation preventing phagosome-lysosome fusion, which promotes the intracellular survival of the bacteria (375, 376). It was previously shown that the Th2 cytokine IL-13 can induce MR through PPARγ signaling pathway in murine macrophages (363). Similar findings have been found in a model of *Trypanosoma cruzi* infection, where MR ligation and subsequent recycling induces arginase activity and bias the NO/urea balance towards

urea production thereby favoring intracellular parasite growth (377). Recently, it has been established that MR has a role in the response to *Leishmania infantum* infection, where MR can promotes the assembly of the NADPH oxidase subunits to the membrane through arachidonic acid mobilization, which is essential in ROS production (378).

Here the study was focused on the NF-κB pathway by using a reverse protein microarray approach. A comprehensive analysis of several components of the canonical and non-canonical pathway was assessed by measuring its protein expression as well as post-translational modifications. Particularly, the data show that mannosylated structures can negatively regulate both arms of the NF-κB pathway in human DCs, which was reflected in reduced p65 phosphorylation as well as reduced expression of RelB, some of the main components of the canonical and non-canonical pathway respectively. This was further confirmed by looking at RelB gene expression by qPCR. Conversely, DCs stimulated with fucosylated molecules were shown to interfere in the non-canonical pathway alone, by reducing NIK protein as well as TRAF3 expression. Both molecules can act in an opposite way by facilitating or blocking the non-canonical NF-κB pathway, respectively (226, 277). Finally, it was shown that exposure to a subsequent dose of LPS induced the non-canonical pathway by increasing the expression of RelB and NIK. On the contrary, negative regulators of the non-canonical pathway, such as TRAF3, as well as phospho-p65 were reduced. This data showed the differential role exerted by TLR4 on the NF-κB pathway under different conditions. This further highlights the pivotal role of CLR and TLR interactions in human DCs in modulating IDO, potentially through the NF-κB pathway. Nuclear shuttling and transcriptional activity of specific NF-κB family members are critical in regulating gene expression. Due to the complex heterogeneity

of the NF-κB family members and regulatory proteins, future studies should be focused on elucidating the specific dimers that are translocated to the nucleus, their DNA-binding activity and ultimately their ability to modulate gene expression particularly IDO expression.

More recently, it has been shown that KYN, via the AhR/SOCS2-dependent pathway, induce proteasome-mediated degradation of TRAF6, which might potentially inhibit TLR signalling favouring non-canonical NF-κB pathway (246). In the context of endotoxin tolerance, the AhR-complex-associated Src kinase activity promotes IDO1 phosphorylation and signalling ability protecting mice against infection (288). It is interesting to note that MR and DC-SIGN have also been associated with the activation and/or interaction with the Src tyrosine kinases (257, 368, 374, 379, 380). Additionally, an association between AhR and RelB has been suggested (247-249) that might contribute to the stabilization of RelB complexes (250, 251). 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 with after MR engagement. Future studies should aim at elucidating the potential physical association between AhR and RelB in regulating IDO levels by analysing the occurrence of AhR-RelB dimers in human DCs by immunoprecipitation and subsequent mass spectrometry to identify potential additional molecules involved in such interaction.

The MR has been linked with the activation of diverse intracellular molecules including the Rho GTPases - in the context of vesicular trafficking - as well as kinases and transcription factors (225, 363, 373, 375, 376). In particular, MR can induces PPARγ activation in human macrophages and DCs (363, 375, 376, 381). The PPARs are members of the nuclear receptor superfamily of ligand-inducible transcription factors that control expression of networks of genes involved in adipogenesis, lipid

metabolism and inflammation (382-384). In DCs, PPARγ regulates lipid metabolism and transport as well as various processes, including antigen uptake, maturation, activation, migration, cytokine production and antigen presentation (381, 385-394). Mo-DCs express high levels of PPARγ during differentiation, which makes them a good model to study PPARγ function (384, 386, 395-397). Human Mo-DCs pre-treated with rosiglitazone, a synthetic ligand for PPARγ, upon LPS stimulation expressed less inducible IL-12 subunit and other Th1-specific inflammatory cytokines and chemokines, suggesting that maturation of DCs in the presence of PPARγ ligands might promote Th2 over Th1 T-cell responses (385, 396). Interestingly, Th2 cytokines such as IL-13 and IL-4 can induce PPARγ activity through MR (363, 381). In addition, PPARγ ligand treatment resulted in increased MR expression in mouse DCs, which consequently led to enhanced MR-mediated endocytosis of soluble ovalbumin antigen (398). These data underline a link between the MR and PPARγ signalling in DC function and the modulation of immune responses. Strikingly, PPARγ ligands were shown to interfere with NF-κB signalling including RelB down-regulation (384, 388, 399-402). Additionally, the non-canonical NF-κB pathway through RelB has been shown to inhibit PPARγ expression, which suggests an antagonistic effect between them (403, 404). Finally, AhR ligands have also been shown to inhibit lipid synthesis by affecting PPARγ expression (405-407). This data highlights the potential role of MR in affecting the AhR-RelB pathways by inducing PPARγ activity, which can be further explored in the future.

Finally, it has been found that DC-SIGN can also interfere with type-I IFNs (284, 408) by inhibition of PP1 phosphatases (408), which might have implications in IDO regulation, as IFNs are one of the main inducers of IDO expression (162, 164). In addition, STAT1, a member of a family of transcription factors involved in the

signalling pathway of IFNs, might interact with AhR and regulate LPS-induced inflammatory responses in macrophages (409).

Understanding the early events of allergen recognition that leads to allergen sensitization will pave the way to more effective therapeutic strategies against allergic diseases. Here it has been shown the key role of IDO in modulating immune responses to glycosylated molecules, including allergens. DC recognition of diverse sugar structures on allergens by CLRs and its interaction with TLR4 can affect DC phenotype - by modulating co-stimulatory molecules and cytokine production - and DC function by affecting their ability to induce immune responses in an IDO-dependent manner. In addition, the data suggest that both arms of the NF-κB pathway could account for the mechanisms behind IDO regulation in human DCs. Further studies should be focused on a better understanding of these interactions at a molecular level and to study the implications of IDO regulation by CLR-TLR cross-talk using *in vivo* models.

8 Chapter VIII: References

1. Parkin, J., and Cohen, B. 2001. An overview of the immune system. *Lancet* 357:1777-1789.
2. Chaplin, D.D. 2010. Overview of the immune response. *The Journal of allergy and clinical immunology* 125:S3-23.
3. Bonilla, F.A., and Oettgen, H.C. 2010. Adaptive immunity. *The Journal of allergy and clinical immunology* 125:S33-40.
4. Wenzel, S.E. 2012. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nature medicine* 18:716-725.
5. Seumois, G., Chavez, L., Gerasimova, A., Lienhard, M., Omran, N., Kalinke, L., Vedanayagam, M., Ganesan, A.P., Chawla, A., Djukanovic, R., et al. 2014. Epigenomic analysis of primary human T cells reveals enhancers associated with TH2 memory cell differentiation and asthma susceptibility. *Nature immunology* 15:777-788.
6. Zhang, Y., Moffatt, M.F., and Cookson, W.O. 2012. Genetic and genomic approaches to asthma: new insights for the origins. *Current opinion in pulmonary medicine* 18:6-13.
7. Kim, S.R., and Rhee, Y.K. 2010. Overlap Between Asthma and COPD: Where the Two Diseases Converge. *Allergy, asthma & immunology research* 2:209-214.
8. Liu, A.H. 2015. Revisiting the hygiene hypothesis for allergy and asthma. *The Journal of allergy and clinical immunology* 136:860-865.
9. Holt, P.G. 2015. The mechanism or mechanisms driving atopic asthma initiation: The infant respiratory microbiome moves to center stage. *The Journal of allergy and clinical immunology* 136:15-22.
10. Julia, V., Macia, L., and Dombrowicz, D. 2015. The impact of diet on asthma and allergic diseases. *Nature reviews. Immunology* 15:308-322.
11. Uzzaman, A., and Cho, S.H. 2012. Chapter 28: Classification of hypersensitivity reactions. *Allergy and asthma proceedings : the official journal of regional and state allergy societies* 33 Suppl 1:S96-99.
12. Rajan, T.V. 2003. The Gell-Coombs classification of hypersensitivity reactions: a re-interpretation. *Trends in immunology* 24:376-379.
13. Descotes, J., and Choquet-Kastylevsky, G. 2001. Gell and Coombs's classification: is it still valid? *Toxicology* 158:43-49.
14. Holgate, S.T., and Polosa, R. 2008. Treatment strategies for allergy and asthma. *Nature reviews. Immunology* 8:218-230.
15. Kim, H.Y., DeKruyff, R.H., and Umetsu, D.T. 2010. The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nature immunology* 11:577-584.
16. Paul, W.E., and Zhu, J. 2010. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol* 10:225-235.
17. Deckers, J., Branco Madeira, F., and Hammad, H. 2013. Innate immune cells in asthma. *Trends in immunology*.
18. Fahy, J.V. 2015. Type 2 inflammation in asthma--present in most, absent in many. *Nature reviews. Immunology* 15:57-65.
19. Lambrecht, B.N., and Hammad, H. 2015. The immunology of asthma. *Nature immunology* 16:45-56.

20. Christianson, C.A., Goplen, N.P., Zafar, I., Irvin, C., Good, J.T., Jr., Rollins, D.R., Gorentla, B., Liu, W., Gorska, M.M., Chu, H., et al. 2015. Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. *The Journal of allergy and clinical immunology* 136:59-68 e14.
21. Artis, D., and Spits, H. 2015. The biology of innate lymphoid cells. *Nature* 517:293-301.
22. Bartemes, K.R., Iijima, K., Kobayashi, T., Kephart, G.M., McKenzie, A.N., and Kita, H. 2012. IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *Journal of immunology* 188:1503-1513.
23. Halim, T.Y., Steer, C.A., Matha, L., Gold, M.J., Martinez-Gonzalez, I., McNagny, K.M., McKenzie, A.N., and Takei, F. 2014. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 40:425-435.
24. Halim, T.Y., Krauss, R.H., Sun, A.C., and Takei, F. 2012. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* 36:451-463.
25. Chang, Y.J., Kim, H.Y., Albacker, L.A., Baumgarth, N., McKenzie, A.N., Smith, D.E., Dekruyff, R.H., and Umetsu, D.T. 2011. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nature immunology* 12:631-638.
26. Hong, J.Y., Bentley, J.K., Chung, Y., Lei, J., Steenrod, J.M., Chen, Q., Sajjan, U.S., and Hershenson, M.B. 2014. Neonatal rhinovirus induces mucous metaplasia and airways hyperresponsiveness through IL-25 and type 2 innate lymphoid cells. *The Journal of allergy and clinical immunology* 134:429-439.
27. Salazar, F., and Ghaemmaghami, A.M. 2013. Allergen Recognition by Innate Immune Cells: Critical Role of Dendritic and Epithelial Cells. *Frontiers in immunology* 4:356.
28. Willart, M., and Hammad, H. 2011. Lung dendritic cell-epithelial cell crosstalk in Th2 responses to allergens. *Current opinion in immunology* 23:772-777.
29. Hammad, H., and Lambrecht, B.N. 2015. Barrier Epithelial Cells and the Control of Type 2 Immunity. *Immunity* 43:29-40.
30. Lee, S.E., Jeong, S.K., and Lee, S.H. 2010. Protease and protease-activated receptor-2 signaling in the pathogenesis of atopic dermatitis. *Yonsei medical journal* 51:808-822.
31. Arora, P., Ricks, T.K., and Trejo, J. 2007. Protease-activated receptor signalling, endocytic sorting and dysregulation in cancer. *Journal of cell science* 120:921-928.
32. Herbert, C.A., King, C.M., Ring, P.C., Holgate, S.T., Stewart, G.A., Thompson, P.J., and Robinson, C. 1995. Augmentation of permeability in the bronchial epithelium by the house dust mite allergen Der p1. *American journal of respiratory cell and molecular biology* 12:369-378.
33. Roche, N., Chinet, T.C., Belouchi, N.E., Julie, C., and Huchon, G.J. 2000. Dermatophagoides pteronyssinus and bioelectric properties of airway epithelium: role of cysteine proteases. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 16:309-315.
34. Wan, H., Winton, H.L., Soeller, C., Gruenert, D.C., Thompson, P.J., Cannell, M.B., Stewart, G.A., Garrod, D.R., and Robinson, C. 2000. Quantitative

structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 30:685-698.

35. Wan, H., Winton, H.L., Soeller, C., Taylor, G.W., Gruenert, D.C., Thompson, P.J., Cannell, M.B., Stewart, G.A., Garrod, D.R., and Robinson, C. 2001. The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of Dermatophagoides pteronyssinus. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 31:279-294.
36. Tai, H.Y., Tam, M.F., Chou, H., Peng, H.J., Su, S.N., Perng, D.W., and Shen, H.D. 2006. Pen ch 13 allergen induces secretion of mediators and degradation of occludin protein of human lung epithelial cells. *Allergy* 61:382-388.
37. Runswick, S., Mitchell, T., Davies, P., Robinson, C., and Garrod, D.R. 2007. Pollen proteolytic enzymes degrade tight junctions. *Respirology* 12:834-842.
38. Vinhas, R., Cortes, L., Cardoso, I., Mendes, V.M., Manadas, B., Todo-Bom, A., Pires, E., and Verissimo, P. 2011. Pollen proteases compromise the airway epithelial barrier through degradation of transmembrane adhesion proteins and lung bioactive peptides. *Allergy* 66:1088-1098.
39. Tomee, J.F., van Weissenbruch, R., de Monchy, J.G., and Kauffman, H.F. 1998. Interactions between inhalant allergen extracts and airway epithelial cells: effect on cytokine production and cell detachment. *The Journal of allergy and clinical immunology* 102:75-85.
40. King, C., Brennan, S., Thompson, P.J., and Stewart, G.A. 1998. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *Journal of immunology* 161:3645-3651.
41. Kauffman, H.F., Tomee, J.F., van de Riet, M.A., Timmerman, A.J., and Borger, P. 2000. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. *The Journal of allergy and clinical immunology* 105:1185-1193.
42. Yu, H.S., Angkasekwinai, P., Chang, S.H., Chung, Y., and Dong, C. 2010. Protease allergens induce the expression of IL-25 via Erk and p38 MAPK pathway. *Journal of Korean medical science* 25:829-834.
43. Kamijo, S., Takeda, H., Tokura, T., Suzuki, M., Inui, K., Hara, M., Matsuda, H., Matsuda, A., Oboki, K., Ohno, T., et al. 2013. IL-33-Mediated Innate Response and Adaptive Immune Cells Contribute to Maximum Responses of Protease Allergen-Induced Allergic Airway Inflammation. *Journal of immunology* 190:4489-4499.
44. Sun, G., Stacey, M.A., Schmidt, M., Mori, L., and Mattoli, S. 2001. Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. *Journal of immunology* 167:1014-1021.
45. Asokananthan, N., Graham, P.T., Stewart, D.J., Bakker, A.J., Eidne, K.A., Thompson, P.J., and Stewart, G.A. 2002. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *Journal of immunology* 169:4572-4578.
46. Page, K., Strunk, V.S., and Hershenson, M.B. 2003. Cockroach proteases increase IL-8 expression in human bronchial epithelial cells via activation of protease-activated receptor (PAR)-2 and extracellular-signal-regulated kinase. *The Journal of allergy and clinical immunology* 112:1112-1118.

47. Chiu, L.L., Perng, D.W., Yu, C.H., Su, S.N., and Chow, L.P. 2007. Mold allergen, pen C 13, induces IL-8 expression in human airway epithelial cells by activating protease-activated receptor 1 and 2. *Journal of immunology* 178:5237-5244.
48. Page, K., Ledford, J.R., Zhou, P., Dienger, K., and Wills-Karp, M. 2010. Mucosal sensitization to German cockroach involves protease-activated receptor-2. *Respiratory research* 11:62.
49. Day, S.B., Ledford, J.R., Zhou, P., Lewkowich, I.P., and Page, K. 2012. German cockroach proteases and protease-activated receptor-2 regulate chemokine production and dendritic cell recruitment. *Journal of innate immunity* 4:100-110.
50. Page, K., Hughes, V.S., Bennett, G.W., and Wong, H.R. 2006. German cockroach proteases regulate matrix metalloproteinase-9 in human bronchial epithelial cells. *Allergy* 61:988-995.
51. Ebeling, C., Lam, T., Gordon, J.R., Hollenberg, M.D., and Vliagoftis, H. 2007. Proteinase-activated receptor-2 promotes allergic sensitization to an inhaled antigen through a TNF-mediated pathway. *Journal of immunology* 179:2910-2917.
52. Heijink, I.H., van Oosterhout, A., and Kapus, A. 2010. Epidermal growth factor receptor signalling contributes to house dust mite-induced epithelial barrier dysfunction. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 36:1016-1026.
53. Shin, S.H., Lee, Y.H., and Jeon, C.H. 2006. Protease-dependent activation of nasal polyp epithelial cells by airborne fungi leads to migration of eosinophils and neutrophils. *Acta oto-laryngologica* 126:1286-1294.
54. Takeda, K., Kaisho, T., and Akira, S. 2003. Toll-like receptors. *Annual review of immunology* 21:335-376.
55. Dabbagh, K., Dahl, M.E., Stepick-Biek, P., and Lewis, D.B. 2002. Toll-like receptor 4 is required for optimal development of Th2 immune responses: role of dendritic cells. *Journal of immunology* 168:4524-4530.
56. Hammad, H., Chieppa, M., Perros, F., Willart, M.A., Germain, R.N., and Lambrecht, B.N. 2009. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 15:410-416.
57. Phipps, S., Lam, C.E., Kaiko, G.E., Foo, S.Y., Collison, A., Mattes, J., Barry, J., Davidson, S., Oreo, K., Smith, L., et al. 2009. Toll/IL-1 signaling is critical for house dust mite-specific helper T cell type 2 and type 17 [corrected] responses. *Am J Respir Crit Care Med* 179:883-893.
58. Tan, A.M., Chen, H.C., Pochard, P., Eisenbarth, S.C., Herrick, C.A., and Bottomly, H.K. 2010. TLR4 signaling in stromal cells is critical for the initiation of allergic Th2 responses to inhaled antigen. *J Immunol* 184:3535-3544.
59. Noulin, N., Quesniaux, V.F., Schnyder-Candrian, S., Schnyder, B., Maillet, I., Robert, T., Vargaftig, B.B., Ryffel, B., and Couillin, I. 2005. Both hemopoietic and resident cells are required for MyD88-dependent pulmonary inflammatory response to inhaled endotoxin. *Journal of immunology* 175:6861-6869.
60. Piggott, D.A., Eisenbarth, S.C., Xu, L., Constant, S.L., Huleatt, J.W., Herrick, C.A., and Bottomly, K. 2005. MyD88-dependent induction of allergic Th2 responses to intranasal antigen. *J Clin Invest* 115:459-467.
61. Poynter, M.E., Cloots, R., van Woerkom, T., Butnor, K.J., Vacek, P., Taatjes, D.J., Irvin, C.G., and Janssen-Heininger, Y.M. 2004. NF-kappa B activation in

airways modulates allergic inflammation but not hyperresponsiveness. *Journal of immunology* 173:7003-7009.

62. Skerrett, S.J., Liggitt, H.D., Hajjar, A.M., Ernst, R.K., Miller, S.I., and Wilson, C.B. 2004. Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin. *American journal of physiology. Lung cellular and molecular physiology* 287:L143-152.
63. Sheller, J.R., Polosukhin, V.V., Mitchell, D., Cheng, D.S., Peebles, R.S., and Blackwell, T.S. 2009. Nuclear factor kappa B induction in airway epithelium increases lung inflammation in allergen-challenged mice. *Experimental lung research* 35:883-895.
64. Ather, J.L., Hodgkins, S.R., Janssen-Heininger, Y.M., and Poynter, M.E. 2011. Airway epithelial NF-kappaB activation promotes allergic sensitization to an innocuous inhaled antigen. *American journal of respiratory cell and molecular biology* 44:631-638.
65. Eisenbarth, S.C., Piggott, D.A., Huleatt, J.W., Visintin, I., Herrick, C.A., and Bottomly, K. 2002. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *The Journal of experimental medicine* 196:1645-1651.
66. Li, D.Q., Zhang, L., Pflugfelder, S.C., De Paiva, C.S., Zhang, X., Zhao, G., Zheng, X., Su, Z., and Qu, Y. 2011. Short ragweed pollen triggers allergic inflammation through Toll-like receptor 4-dependent thymic stromal lymphopoietin/OX40 ligand/OX40 signaling pathways. *J Allergy Clin Immunol* 128:1318-1325 e1312.
67. Sancho, D., and Reis e Sousa, C. 2012. Signaling by myeloid C-type lectin receptors in immunity and homeostasis. *Annual review of immunology* 30:491-529.
68. Salazar, F., Sewell, H.F., Shakib, F., and Ghaemmaghami, A.M. 2013. The role of lectins in allergic sensitization and allergic disease. *The Journal of allergy and clinical immunology* 132:27-36.
69. Nathan, A.T., Peterson, E.A., Chakir, J., and Wills-Karp, M. 2009. Innate immune responses of airway epithelium to house dust mite are mediated through beta-glucan-dependent pathways. *The Journal of allergy and clinical immunology* 123:612-618.
70. Brown, G.D., Herre, J., Williams, D.L., Willment, J.A., Marshall, A.S., and Gordon, S. 2003. Dectin-1 mediates the biological effects of beta-glucans. *The Journal of experimental medicine* 197:1119-1124.
71. Al-Ghouleh, A., Johal, R., Sharquie, I.K., Emara, M., Harrington, H., Shakib, F., and Ghaemmaghami, A.M. 2012. The glycosylation pattern of common allergens: the recognition and uptake of Der p 1 by epithelial and dendritic cells is carbohydrate dependent. *PLoS One* 7:e33929.
72. Mercer, N., Guzman, L., Cueto Rua, E., Drut, R., Ahmed, H., Vasta, G.R., Toscano, M.A., Rabinovich, G.A., and Docena, G.H. 2009. Duodenal intraepithelial lymphocytes of children with cow milk allergy preferentially bind the glycan-binding protein galectin-3. *International journal of immunopathology and pharmacology* 22:207-217.
73. Banchereau, J., and Steinman, R.M. 1998. Dendritic cells and the control of immunity. *Nature* 392:245-252.
74. Gill, M.A. 2012. The role of dendritic cells in asthma. *The Journal of allergy and clinical immunology* 129:889-901.

75. Hammad, H., Plantinga, M., Deswarte, K., Pouliot, P., Willart, M.A., Kool, M., Muskens, F., and Lambrecht, B.N. 2010. Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med* 207:2097-2111.
76. Plantinga, M., Guilliams, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., Vanhoutte, L., Neyt, K., Killeen, N., Malissen, B., et al. 2013. Conventional and Monocyte-Derived CD11b(+) Dendritic Cells Initiate and Maintain T Helper 2 Cell-Mediated Immunity to House Dust Mite Allergen. *Immunity*.
77. van Rijt, L.S., Jung, S., Kleinjan, A., Vos, N., Willart, M., Duez, C., Hoogsteden, H.C., and Lambrecht, B.N. 2005. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *The Journal of experimental medicine* 201:981-991.
78. van Rijt, L.S., Vos, N., Willart, M., Muskens, F., Tak, P.P., van der Horst, C., Hoogsteden, H.C., and Lambrecht, B.N. 2011. Persistent activation of dendritic cells after resolution of allergic airway inflammation breaks tolerance to inhaled allergens in mice. *American journal of respiratory and critical care medicine* 184:303-311.
79. Hammad, H., and Lambrecht, B.N. 2008. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nature reviews. Immunology* 8:193-204.
80. Sung, S.S., Fu, S.M., Rose, C.E., Jr., Gaskin, F., Ju, S.T., and Beaty, S.R. 2006. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. *Journal of immunology* 176:2161-2172.
81. Guo, S., Nighot, M., Al-Sadi, R., Alhmoud, T., Nighot, P., and Ma, T.Y. 2015. Lipopolysaccharide Regulation of Intestinal Tight Junction Permeability Is Mediated by TLR4 Signal Transduction Pathway Activation of FAK and MyD88. *Journal of immunology*.
82. Lambrecht, B.N., and Hammad, H. 2014. Allergens and the airway epithelium response: Gateway to allergic sensitization. *The Journal of allergy and clinical immunology* 134:499-507.
83. Besnard, A.G., Togbe, D., Guillou, N., Erard, F., Quesniaux, V., and Ryffel, B. 2011. IL-33-activated dendritic cells are critical for allergic airway inflammation. *European journal of immunology* 41:1675-1686.
84. Noti, M., Wojno, E.D., Kim, B.S., Siracusa, M.C., Giacomin, P.R., Nair, M.G., Benitez, A.J., Ruymann, K.R., Muir, A.B., Hill, D.A., et al. 2013. Thymic stromal lymphopoietin-elicited basophil responses promote eosinophilic esophagitis. *Nature medicine* 19:1005-1013.
85. Motomura, Y., Morita, H., Moro, K., Nakae, S., Artis, D., Endo, T.A., Kuroki, Y., Ohara, O., Koyasu, S., and Kubo, M. 2014. Basophil-derived interleukin-4 controls the function of natural helper cells, a member of ILC2s, in lung inflammation. *Immunity* 40:758-771.
86. Brubaker, S.W., Bonham, K.S., Zanoni, I., and Kagan, J.C. 2015. Innate immune pattern recognition: a cell biological perspective. *Annual review of immunology* 33:257-290.
87. Chiou, Y.L., and Lin, C.Y. 2009. Der p2 activates airway smooth muscle cells in a TLR2/MyD88-dependent manner to induce an inflammatory response. *Journal of cellular physiology* 220:311-318.

88. Trompette, A., Divanovic, S., Visintin, A., Blanchard, C., Hegde, R.S., Madan, R., Thorne, P.S., Wills-Karp, M., Gioannini, T.L., Weiss, J.P., et al. 2009. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457:585-588.
89. Liu, A.H. 2002. Endotoxin exposure in allergy and asthma: reconciling a paradox. *J Allergy Clin Immunol* 109:379-392.
90. Kim, Y.K., Oh, S.Y., Jeon, S.G., Park, H.W., Lee, S.Y., Chun, E.Y., Bang, B., Lee, H.S., Oh, M.H., Kim, Y.S., et al. 2007. Airway exposure levels of lipopolysaccharide determine type 1 versus type 2 experimental asthma. *Journal of immunology* 178:5375-5382.
91. Lauw, F.N., ten Hove, T., Dekkers, P.E., de Jonge, E., van Deventer, S.J., and van Der Poll, T. 2000. Reduced Th1, but not Th2, cytokine production by lymphocytes after in vivo exposure of healthy subjects to endotoxin. *Infection and immunity* 68:1014-1018.
92. Wilson, R.H., Maruoka, S., Whitehead, G.S., Foley, J.F., Flake, G.P., Sever, M.L., Zeldin, D.C., Kraft, M., Garantziotis, S., Nakano, H., et al. 2012. The Toll-like receptor 5 ligand flagellin promotes asthma by priming allergic responses to indoor allergens. *Nature medicine* 18:1705-1710.
93. Millien, V.O., Lu, W., Shaw, J., Yuan, X., Mak, G., Roberts, L., Song, L.Z., Knight, J.M., Creighton, C.J., Luong, A., et al. 2013. Cleavage of fibrinogen by proteinases elicits allergic responses through Toll-like receptor 4. *Science* 341:792-796.
94. Schmidt, M., Raghavan, B., Muller, V., Vogl, T., Fejer, G., Tchaptchet, S., Keck, S., Kalis, C., Nielsen, P.J., Galanos, C., et al. 2010. Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nature immunology* 11:814-819.
95. Raghavan, B., Martin, S.F., Esser, P.R., Goebeler, M., and Schmidt, M. 2012. Metal allergens nickel and cobalt facilitate TLR4 homodimerization independently of MD2. *EMBO reports* 13:1109-1115.
96. Herre, J., Gronlund, H., Brooks, H., Hopkins, L., Waggoner, L., Murton, B., Gangloff, M., Opaleye, O., Chilvers, E.R., Fitzgerald, K., et al. 2013. Allergens as Immunomodulatory Proteins: The Cat Dander Protein Fel d 1 Enhances TLR Activation by Lipid Ligands. *Journal of immunology* 191:1529-1535.
97. Taylor, P.R., Gordon, S., and Martinez-Pomares, L. 2005. The mannose receptor: linking homeostasis and immunity through sugar recognition. *Trends in immunology* 26:104-110.
98. Deslee, G., Charbonnier, A.S., Hammad, H., Angyalosi, G., Tillie-Leblond, I., Mantovani, A., Tonnel, A.B., and Pestel, J. 2002. Involvement of the mannose receptor in the uptake of Der p 1, a major mite allergen, by human dendritic cells. *J Allergy Clin Immunol* 110:763-770.
99. Kayserova, J., Zentsova-Jaresova, I., Budinsky, V., Rozkova, D., Kopecka, J., Vernerova, E., Pohunek, P., Skalicka, V., Spisek, R., and Sediva, A. 2012. Selective increase in blood dendritic cell antigen-3-positive dendritic cells in bronchoalveolar lavage fluid in allergic patients. *Scandinavian journal of immunology* 75:305-313.
100. Hattori, T., Konno, S., Hizawa, N., Isada, A., Takahashi, A., Shimizu, K., Gao, P., Beaty, T.H., Barnes, K.C., Huang, S.K., et al. 2009. Genetic variants in the mannose receptor gene (MRC1) are associated with asthma in two independent populations. *Immunogenetics* 61:731-738.

101. Li, X., Fleis, R.I., Shubitowski, D.M., Ramadas, R.A., and Ewart, S.L. 2007. Fine mapping of murine asthma quantitative trait loci and analyses of Ptgs1 and Mrc1 as positional candidate genes. *DNA sequence : the journal of DNA sequencing and mapping* 18:190-195.
102. Royer, P.J., Emara, M., Yang, C., Al-Ghouleh, A., Tighe, P., Jones, N., Sewell, H.F., Shakib, F., Martinez-Pomares, L., and Ghaemmaghami, A.M. 2010. 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. *J Immunol* 185:1522-1531.
103. Emara, M., Royer, P.J., Abbas, Z., Sewell, H.F., Mohamed, G.G., Singh, S., Peel, S., Fox, J., Shakib, F., Martinez-Pomares, L., et al. 2011. Recognition of the major cat allergen Fel d 1 through the cysteine-rich domain of the mannose receptor determines its allergenicity. *J Biol Chem* 286:13033-13040.
104. Munn, D.H., and Mellor, A.L. 2012. Indoleamine 2,3 dioxygenase and metabolic control of immune responses. *Trends in immunology* 34:137-143.
105. Orabona, C., Pallotta, M.T., and Grohmann, U. 2012. Different partners, opposite outcomes: A new perspective of IDO's immunobiology. *Molecular medicine* 18:834-842.
106. Everts, B., Hussaarts, L., Driessen, N.N., Meevissen, M.H., Schramm, G., van der Ham, A.J., van der Hoeven, B., Scholzen, T., Burgdorf, S., Mohrs, M., et al. 2012. Schistosome-derived omega-1 drives Th2 polarization by suppressing protein synthesis following internalization by the mannose receptor. *The Journal of experimental medicine* 209:1753-1767.
107. Zhou, T., Chen, Y., Hao, L., and Zhang, Y. 2006. DC-SIGN and immunoregulation. *Cellular & molecular immunology* 3:279-283.
108. Shreffler, W.G., Castro, R.R., Kucuk, Z.Y., Charlop-Powers, Z., Grishina, G., Yoo, S., Burks, A.W., and Sampson, H.A. 2006. The major glycoprotein allergen from Arachis hypogaea, Ara h 1, is a ligand of dendritic cell-specific ICAM-grabbing nonintegrin and acts as a Th2 adjuvant in vitro. *J Immunol* 177:3677-3685.
109. Hsu, S.C., Chen, C.H., Tsai, S.H., Kawasaki, H., Hung, C.H., Chu, Y.T., Chang, H.W., Zhou, Y., Fu, J., Plunkett, B., et al. 2010. Functional interaction of common allergens and a C-type lectin receptor, dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), on human dendritic cells. *J Biol Chem* 285:7903-7910.
110. Emara, M., Royer, P.J., Mahdavi, J., Shakib, F., and Ghaemmaghami, A.M. 2012. Retagging Identifies Dendritic Cell-specific Intercellular Adhesion Molecule-3 (ICAM3)-grabbing Non-integrin (DC-SIGN) Protein as a Novel Receptor for a Major Allergen from House Dust Mite. *J Biol Chem* 287:5756-5763.
111. Furmonaviciene, R., Ghaemmaghami, A.M., Boyd, S.E., Jones, N.S., Bailey, K., Willis, A.C., Sewell, H.F., Mitchell, D.A., and Shakib, F. 2007. The protease allergen Der p 1 cleaves cell surface DC-SIGN and DC-SIGNR: experimental analysis of in silico substrate identification and implications in allergic responses. *Clin Exp Allergy* 37:231-242.
112. Huang, H.J., Lin, Y.L., Liu, C.F., Kao, H.F., and Wang, J.Y. 2011. Mite allergen decreases DC-SIGN expression and modulates human dendritic cell differentiation and function in allergic asthma. *Mucosal immunology* 4:519-527.

113. Gringhuis, S.I., Kaptein, T.M., Wevers, B.A., Mesman, A.W., and Geijtenbeek, T.B. 2014. Fucose-specific DC-SIGN signalling directs T helper cell type-2 responses via IKKepsilon- and CYLD-dependent Bcl3 activation. *Nature communications* 5:3898.
114. Sato, K., Yang, X.L., Yudate, T., Chung, J.S., Wu, J., Luby-Phelps, K., Kimberly, R.P., Underhill, D., Cruz, P.D., Jr., and Ariizumi, K. 2006. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. *J Biol Chem* 281:38854-38866.
115. Barrett, N.A., Maekawa, A., Rahman, O.M., Austen, K.F., and Kanaoka, Y. 2009. Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. *Journal of immunology* 182:1119-1128.
116. Barrett, N.A., Rahman, O.M., Fernandez, J.M., Parsons, M.W., Xing, W., Austen, K.F., and Kanaoka, Y. 2011. Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes. *The Journal of experimental medicine* 208:593-604.
117. Liu, F.T., Yang, R.Y., and Hsu, D.K. 2012. Galectins in acute and chronic inflammation. *Annals of the New York Academy of Sciences* 1253:80-91.
118. Chen, H.Y., Sharma, B.B., Yu, L., Zuberi, R., Weng, I.C., Kawakami, Y., Kawakami, T., Hsu, D.K., and Liu, F.T. 2006. Role of galectin-3 in mast cell functions: galectin-3-deficient mast cells exhibit impaired mediator release and defective JNK expression. *Journal of immunology* 177:4991-4997.
119. Hsu, D.K., Chernyavsky, A.I., Chen, H.Y., Yu, L., Grando, S.A., and Liu, F.T. 2009. Endogenous galectin-3 is localized in membrane lipid rafts and regulates migration of dendritic cells. *The Journal of investigative dermatology* 129:573-583.
120. Ge, X.N., Bahaie, N.S., Kang, B.N., Hosseinkhani, M.R., Ha, S.G., Frenzel, E.M., Liu, F.T., Rao, S.P., and Sriramarao, P. 2010. Allergen-induced airway remodeling is impaired in galectin-3-deficient mice. *Journal of immunology* 185:1205-1214.
121. Zuberi, R.I., Hsu, D.K., Kalayci, O., Chen, H.Y., Sheldon, H.K., Yu, L., Apgar, J.R., Kawakami, T., Lilly, C.M., and Liu, F.T. 2004. Critical role for galectin-3 in airway inflammation and bronchial hyperresponsiveness in a murine model of asthma. *The American journal of pathology* 165:2045-2053.
122. Saegusa, J., Hsu, D.K., Chen, H.Y., Yu, L., Fermin, A., Fung, M.A., and Liu, F.T. 2009. Galectin-3 is critical for the development of the allergic inflammatory response in a mouse model of atopic dermatitis. *The American journal of pathology* 174:922-931.
123. Niki, T., Tsutsui, S., Hirose, S., Aradono, S., Sugimoto, Y., Takeshita, K., Nishi, N., and Hirashima, M. 2009. Galectin-9 is a high affinity IgE-binding lectin with anti-allergic effect by blocking IgE-antigen complex formation. *The Journal of biological chemistry* 284:32344-32352.
124. Katoh, S., Ishii, N., Nobumoto, A., Takeshita, K., Dai, S.Y., Shinonaga, R., Niki, T., Nishi, N., Tominaga, A., Yamauchi, A., et al. 2007. Galectin-9 inhibits CD44-hyaluronan interaction and suppresses a murine model of allergic asthma. *American journal of respiratory and critical care medicine* 176:27-35.
125. Yamamoto, H., Kashio, Y., Shoji, H., Shinonaga, R., Yoshimura, T., Nishi, N., Nabe, T., Nakamura, T., Kohno, S., and Hirashima, M. 2007. Involvement of

galectin-9 in guinea pig allergic airway inflammation. *International archives of allergy and immunology* 143 Suppl 1:95-105.

126. Sziksz, E., Kozma, G.T., Pallinger, E., Komlosi, Z.I., Adori, C., Kovacs, L., Szebeni, B., Rusai, K., Losonczy, G., Szabo, A., et al. 2010. Galectin-9 in allergic airway inflammation and hyper-responsiveness in mice. *International archives of allergy and immunology* 151:308-317.
127. Kishore, U., Greenhough, T.J., Waters, P., Shrive, A.K., Ghai, R., Kamran, M.F., Bernal, A.L., Reid, K.B., Madan, T., and Chakraborty, T. 2006. Surfactant proteins SP-A and SP-D: structure, function and receptors. *Molecular immunology* 43:1293-1315.
128. Hickling, T.P., Clark, H., Malhotra, R., and Sim, R.B. 2004. Collectins and their role in lung immunity. *Journal of leukocyte biology* 75:27-33.
129. Malhotra, R., Haurum, J., Thiel, S., Jensenius, J.C., and Sim, R.B. 1993. Pollen grains bind to lung alveolar type II cells (A549) via lung surfactant protein A (SP-A). *Bioscience reports* 13:79-90.
130. Erpenbeck, V.J., Malherbe, D.C., Sommer, S., Schmiedl, A., Steinhilber, W., Ghio, A.J., Krug, N., Wright, J.R., and Hohlfeld, J.M. 2005. Surfactant protein D increases phagocytosis and aggregation of pollen-allergen starch granules. *American journal of physiology. Lung cellular and molecular physiology* 288:L692-698.
131. Wang, J.Y., Kishore, U., Lim, B.L., Strong, P., and Reid, K.B. 1996. Interaction of human lung surfactant proteins A and D with mite (Dermatophagoides pteronyssinus) allergens. *Clinical and experimental immunology* 106:367-373.
132. Wang, J.Y., Shieh, C.C., You, P.F., Lei, H.Y., and Reid, K.B. 1998. Inhibitory effect of pulmonary surfactant proteins A and D on allergen-induced lymphocyte proliferation and histamine release in children with asthma. *American journal of respiratory and critical care medicine* 158:510-518.
133. Madan, T., Kishore, U., Shah, A., Eggleton, P., Strong, P., Wang, J.Y., Aggrawal, S.S., Sarma, P.U., and Reid, K.B. 1997. Lung surfactant proteins A and D can inhibit specific IgE binding to the allergens of Aspergillus fumigatus and block allergen-induced histamine release from human basophils. *Clinical and experimental immunology* 110:241-249.
134. Deb, R., Shakib, F., Reid, K., and Clark, H. 2007. Major house dust mite allergens Dermatophagoides pteronyssinus 1 and Dermatophagoides farinae 1 degrade and inactivate lung surfactant proteins A and D. *The Journal of biological chemistry* 282:36808-36819.
135. Madan, T., Reid, K.B., Singh, M., Sarma, P.U., and Kishore, U. 2005. Susceptibility of mice genetically deficient in the surfactant protein (SP)-A or SP-D gene to pulmonary hypersensitivity induced by antigens and allergens of Aspergillus fumigatus. *Journal of immunology* 174:6943-6954.
136. Schaub, B., Westlake, R.M., He, H., Arestides, R., Haley, K.J., Campo, M., Velasco, G., Bellou, A., Hawgood, S., Poulain, F.R., et al. 2004. Surfactant protein D deficiency influences allergic immune responses. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 34:1819-1826.
137. Brinker, K.G., Garner, H., and Wright, J.R. 2003. Surfactant protein A modulates the differentiation of murine bone marrow-derived dendritic cells. *American journal of physiology. Lung cellular and molecular physiology* 284:L232-241.

138. Brinker, K.G., Martin, E., Borron, P., Mostaghel, E., Doyle, C., Harding, C.V., and Wright, J.R. 2001. Surfactant protein D enhances bacterial antigen presentation by bone marrow-derived dendritic cells. *American journal of physiology. Lung cellular and molecular physiology* 281:L1453-1463.
139. Wilsher, M.L., Hughes, D.A., and Haslam, P.L. 1988. Immunoregulatory properties of pulmonary surfactant: effect of lung lining fluid on proliferation of human blood lymphocytes. *Thorax* 43:354-359.
140. Borron, P.J., Crouch, E.C., Lewis, J.F., Wright, J.R., Possmayer, F., and Fraher, L.J. 1998. Recombinant rat surfactant-associated protein D inhibits human T lymphocyte proliferation and IL-2 production. *J Immunol* 161:4599-4603.
141. Kishor, U., Madan, T., Sarma, P.U., Singh, M., Urban, B.C., and Reid, K.B. 2002. Protective roles of pulmonary surfactant proteins, SP-A and SP-D, against lung allergy and infection caused by Aspergillus fumigatus. *Immunobiology* 205:610-618.
142. Homer, R.J., Zheng, T., Chupp, G., He, S., Zhu, Z., Chen, Q., Ma, B., Hite, R.D., Gobran, L.I., Rooney, S.A., et al. 2002. Pulmonary type II cell hypertrophy and pulmonary lipoproteinosis are features of chronic IL-13 exposure. *American journal of physiology. Lung cellular and molecular physiology* 283:L52-59.
143. Kaur, S., Gupta, V.K., Shah, A., Thiel, S., Sarma, P.U., and Madan, T. 2005. Plasma mannan-binding lectin levels and activity are increased in allergic patients. *The Journal of allergy and clinical immunology* 116:1381-1383.
144. Kaur, S., Gupta, V.K., Shah, A., Thiel, S., Sarma, P.U., and Madan, T. 2006. Elevated levels of mannan-binding lectin [corrected] (MBL) and eosinophilia in patients of bronchial asthma with allergic rhinitis and allergic bronchopulmonary aspergillosis associate with a novel intronic polymorphism in MBL. *Clinical and experimental immunology* 143:414-419.
145. Kaur, S., Thiel, S., Sarma, P.U., and Madan, T. 2006. Mannan-binding lectin in asthma and allergy. *Current allergy and asthma reports* 6:377-383.
146. Li, J., Jiang, H., Wen, W., Zheng, J., and Xu, G. 2010. The dendritic cell mannose receptor mediates allergen internalization and maturation involving notch 1 signalling. *Clinical and experimental immunology* 162:251-261.
147. Kojima, K., Arikawa, T., Saita, N., Goto, E., Tsumura, S., Tanaka, R., Masunaga, A., Niki, T., Oomizu, S., Hirashima, M., et al. 2011. Galectin-9 attenuates acute lung injury by expanding CD14- plasmacytoid dendritic cell-like macrophages. *American journal of respiratory and critical care medicine* 184:328-339.
148. Munn, D.H., Zhou, M., Attwood, J.T., Bondarev, I., Conway, S.J., Marshall, B., Brown, C., and Mellor, A.L. 1998. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science* 281:1191-1193.
149. Mellor, A.L., and Munn, D.H. 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 4:762-774.
150. Puccetti, P., and Grohmann, U. 2007. IDO and regulatory T cells: a role for reverse signalling and non-canonical NF-kappaB activation. *Nat Rev Immunol* 7:817-823.
151. Moffett, J.R., and Namboodiri, M.A. 2003. Tryptophan and the immune response. *Immunology and cell biology* 81:247-265.
152. Terness, P., Chuang, J.J., and Opelz, G. 2006. The immunoregulatory role of IDO-producing human dendritic cells revisited. *Trends Immunol* 27:68-73.

153. Ball, H.J., Sanchez-Perez, A., Weiser, S., Austin, C.J., Astelbauer, F., Miu, J., McQuillan, J.A., Stocker, R., Jermiin, L.S., and Hunt, N.H. 2007. Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice. *Gene* 396:203-213.
154. Hansen, A.M., Ball, H.J., Mitchell, A.J., Miu, J., Takikawa, O., and Hunt, N.H. 2004. Increased expression of indoleamine 2,3-dioxygenase in murine malaria infection is predominantly localised to the vascular endothelium. *International journal for parasitology* 34:1309-1319.
155. Yuasa, H.J., Ball, H.J., Ho, Y.F., Austin, C.J., Whittington, C.M., Belov, K., Maghzal, G.J., Jermiin, L.S., and Hunt, N.H. 2009. Characterization and evolution of vertebrate indoleamine 2, 3-dioxygenases IDOs from monotremes and marsupials. *Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology* 153:137-144.
156. Metz, R., Duhadaway, J.B., Kamasani, U., Laury-Kleintop, L., Muller, A.J., and Prendergast, G.C. 2007. Novel tryptophan catabolic enzyme IDO2 is the preferred biochemical target of the antitumor indoleamine 2,3-dioxygenase inhibitory compound D-1-methyl-tryptophan. *Cancer research* 67:7082-7087.
157. Munn, D.H., Sharma, M.D., Baban, B., Harding, H.P., Zhang, Y., Ron, D., and Mellor, A.L. 2005. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. *Immunity* 22:633-642.
158. Grohmann, U., Fallarino, F., and Puccetti, P. 2003. Tolerance, DCs and tryptophan: much ado about IDO. *Trends in immunology* 24:242-248.
159. Mezrich, J.D., Fechner, J.H., Zhang, X., Johnson, B.P., Burlingham, W.J., and Bradfield, C.A. 2010. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J Immunol* 185:3190-3198.
160. Chung, D.J., Rossi, M., Romano, E., Ghith, J., Yuan, J., Munn, D.H., and Young, J.W. 2009. Indoleamine 2,3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells. *Blood* 114:555-563.
161. Von Bubnoff, D., Scheler, M., Wilms, H., Fimmers, R., and Bieber, T. 2011. Identification of IDO-positive and IDO-negative human dendritic cells after activation by various proinflammatory stimuli. *Journal of immunology* 186:6701-6709.
162. Hassanain, H.H., Chon, S.Y., and Gupta, S.L. 1993. Differential regulation of human indoleamine 2,3-dioxygenase gene expression by interferons-gamma and -alpha. Analysis of the regulatory region of the gene and identification of an interferon-gamma-inducible DNA-binding factor. *The Journal of biological chemistry* 268:5077-5084.
163. Robinson, C.M., Shirey, K.A., and Carlin, J.M. 2003. Synergistic transcriptional activation of indoleamine dioxygenase by IFN-gamma and tumor necrosis factor-alpha. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 23:413-421.
164. Puccetti, P. 2007. On watching the watchers: IDO and type I/II IFN. *European journal of immunology* 37:876-879.
165. Munn, D.H., Sharma, M.D., Lee, J.R., Jhaver, K.G., Johnson, T.S., Keskin, D.B., Marshall, B., Chandler, P., Antonia, S.J., Burgess, R., et al. 2002.

Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. *Science* 297:1867-1870.

166. Romani, L., and Puccetti, P. 2006. Protective tolerance to fungi: the role of IL-10 and tryptophan catabolism. *Trends in microbiology* 14:183-189.
167. Grohmann, U., Volpi, C., Fallarino, F., Bozza, S., Bianchi, R., Vacca, C., Orabona, C., Belladonna, M.L., Ayroldi, E., Nocentini, G., et al. 2007. Reverse signaling through GITR ligand enables dexamethasone to activate IDO in allergy. *Nature medicine* 13:579-586.
168. Fallarino, F., Orabona, C., Vacca, C., Bianchi, R., Gizzi, S., Asselin-Paturel, C., Fioretti, M.C., Trinchieri, G., Grohmann, U., and Puccetti, P. 2005. Ligand and cytokine dependence of the immunosuppressive pathway of tryptophan catabolism in plasmacytoid dendritic cells. *International immunology* 17:1429-1438.
169. Tas, S.W., Vervoordeldonk, M.J., Hajji, N., Schuitemaker, J.H., van der Sluijs, K.F., May, M.J., Ghosh, S., Kapsenberg, M.L., Tak, P.P., and de Jong, E.C. 2007. Noncanonical NF-kappaB signaling in dendritic cells is required for indoleamine 2,3-dioxygenase (IDO) induction and immune regulation. *Blood* 110:1540-1549.
170. Grohmann, U., Orabona, C., Fallarino, F., Vacca, C., Calcinaro, F., Falorni, A., Candeloro, P., Belladonna, M.L., Bianchi, R., Fioretti, M.C., et al. 2002. CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nature immunology* 3:1097-1101.
171. Mellor, A.L., Baban, B., Chandler, P., Marshall, B., Jhaver, K., Hansen, A., Koni, P.A., Iwashima, M., and Munn, D.H. 2003. Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. *Journal of immunology* 171:1652-1655.
172. Fallarino, F., Asselin-Paturel, C., Vacca, C., Bianchi, R., Gizzi, S., Fioretti, M.C., Trinchieri, G., Grohmann, U., and Puccetti, P. 2004. Murine plasmacytoid dendritic cells initiate the immunosuppressive pathway of tryptophan catabolism in response to CD200 receptor engagement. *Journal of immunology* 173:3748-3754.
173. Seo, S.K., Choi, J.H., Kim, Y.H., Kang, W.J., Park, H.Y., Suh, J.H., Choi, B.K., Vinay, D.S., and Kwon, B.S. 2004. 4-1BB-mediated immunotherapy of rheumatoid arthritis. *Nature medicine* 10:1088-1094.
174. Fallarino, F., Grohmann, U., and Puccetti, P. 2012. Indoleamine 2,3-dioxygenase: From catalyst to signaling function. *European journal of immunology* 42:1932-1937.
175. Hwu, P., Du, M.X., Lapointe, R., Do, M., Taylor, M.W., and Young, H.A. 2000. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. *Journal of immunology* 164:3596-3599.
176. Baban, B., Chandler, P.R., Sharma, M.D., Pihkala, J., Koni, P.A., Munn, D.H., and Mellor, A.L. 2009. IDO activates regulatory T cells and blocks their conversion into Th17-like T cells. *Journal of immunology* 183:2475-2483.
177. Chen, W., Liang, X., Peterson, A.J., Munn, D.H., and Blazar, B.R. 2008. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. *Journal of immunology* 181:5396-5404.

178. Braun, D., Longman, R.S., and Albert, M.L. 2005. A two-step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic-cell maturation. *Blood* 106:2375-2381.
179. Belladonna, M.L., Orabona, C., Grohmann, U., and Puccetti, P. 2009. TGF-beta and kynurenines as the key to infectious tolerance. *Trends Mol Med* 15:41-49.
180. Belladonna, M.L., Volpi, C., Bianchi, R., Vacca, C., Orabona, C., Pallotta, M.T., Boon, L., Gizzi, S., Fioretti, M.C., Grohmann, U., et al. 2008. Cutting edge: Autocrine TGF-beta sustains default tolerogenesis by IDO-competent dendritic cells. *J Immunol* 181:5194-5198.
181. Pallotta, M.T., Orabona, C., Volpi, C., Vacca, C., Belladonna, M.L., Bianchi, R., Servillo, G., Brunacci, C., Calvitti, M., Bicciato, S., et al. 2011. Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells. *Nature immunology* 12:870-878.
182. Montagnoli, C., Fallarino, F., Gaziano, R., Bozza, S., Bellocchio, S., Zelante, T., Kurup, W.P., Pitzurra, L., Puccetti, P., and Romani, L. 2006. Immunity and tolerance to Aspergillus involve functionally distinct regulatory T cells and tryptophan catabolism. *Journal of immunology* 176:1712-1723.
183. Hayashi, T., Beck, L., Rossetto, C., Gong, X., Takikawa, O., Takabayashi, K., Broide, D.H., Carson, D.A., and Raz, E. 2004. Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. *The Journal of clinical investigation* 114:270-279.
184. Padrid, P.A., Mathur, M., Li, X., Herrmann, K., Qin, Y., Cattamanchi, A., Weinstock, J., Elliott, D., Sperling, A.I., and Bluestone, J.A. 1998. CTLA4Ig inhibits airway eosinophilia and hyperresponsiveness by regulating the development of Th1/Th2 subsets in a murine model of asthma. *American journal of respiratory cell and molecular biology* 18:453-462.
185. Romani, L., Bistoni, F., Perruccio, K., Montagnoli, C., Gaziano, R., Bozza, S., Bonifazi, P., Bistoni, G., Rasi, G., Velardi, A., et al. 2006. Thymosin alpha1 activates dendritic cell tryptophan catabolism and establishes a regulatory environment for balance of inflammation and tolerance. *Blood* 108:2265-2274.
186. Horner, A.A., Van Uden, J.H., Zubeldia, J.M., Broide, D., and Raz, E. 2001. DNA-based immunotherapeutics for the treatment of allergic disease. *Immunological reviews* 179:102-118.
187. Hayashi, T., and Raz, E. 2006. TLR9-based immunotherapy for allergic disease. *The American journal of medicine* 119:897 e891-896.
188. Hayashi, T., Mo, J.H., Gong, X., Rossetto, C., Jang, A., Beck, L., Elliott, G.I., Kufareva, I., Abagyan, R., Broide, D.H., et al. 2007. 3-Hydroxyanthranilic acid inhibits PDK1 activation and suppresses experimental asthma by inducing T cell apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* 104:18619-18624.
189. Taher, Y.A., Piavaux, B.J., Gras, R., van Esch, B.C., Hofman, G.A., Bloksma, N., Henricks, P.A., and van Oosterhout, A.J. 2008. Indoleamine 2,3-dioxygenase-dependent tryptophan metabolites contribute to tolerance induction during allergen immunotherapy in a mouse model. *The Journal of allergy and clinical immunology* 121:983-991 e982.
190. Odemuyiwa, S.O., Ebeling, C., Duta, V., Abel, M., Puttagunta, L., Cravetchi, O., Majaesic, C., Vliagoftis, H., and Moqbel, R. 2009. Tryptophan catabolites regulate mucosal sensitization to ovalbumin in respiratory airways. *Allergy* 64:488-492.

191. Xu, H., Oriss, T.B., Fei, M., Henry, A.C., Melgert, B.N., Chen, L., Mellor, A.L., Munn, D.H., Irvin, C.G., Ray, P., et al. 2008. Indoleamine 2,3-dioxygenase in lung dendritic cells promotes Th2 responses and allergic inflammation. *Proceedings of the National Academy of Sciences of the United States of America* 105:6690-6695.
192. von Bubnoff, D., Fimmers, R., Bogdanow, M., Matz, H., Koch, S., and Bieber, T. 2004. Asymptomatic atopy is associated with increased indoleamine 2,3-dioxygenase activity and interleukin-10 production during seasonal allergen exposure. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 34:1056-1063.
193. Raitala, A., Karjalainen, J., Oja, S.S., Kosunen, T.U., and Hurme, M. 2006. Indoleamine 2,3-dioxygenase (IDO) activity is lower in atopic than in non-atopic individuals and is enhanced by environmental factors protecting from atopy. *Molecular immunology* 43:1054-1056.
194. Maneechotesuwan, K., Wamanuttajinda, V., Kasetsinsombat, K., Huabprasert, S., Yaikwawong, M., Barnes, P.J., and Wongkajornsilp, A. 2009. Der p 1 suppresses indoleamine 2, 3-dioxygenase in dendritic cells from house dust mite-sensitive patients with asthma. *The Journal of allergy and clinical immunology* 123:239-248.
195. Stockinger, B., Hirota, K., Duarte, J., and Veldhoen, M. 2011. External influences on the immune system via activation of the aryl hydrocarbon receptor. *Seminars in immunology* 23:99-105.
196. Veldhoen, M., and Duarte, J.H. 2010. The aryl hydrocarbon receptor: fine-tuning the immune-response. *Current opinion in immunology* 22:747-752.
197. Nguyen, N.T., Kimura, A., Nakahama, T., Chinen, I., Masuda, K., Nohara, K., Fujii-Kuriyama, Y., and Kishimoto, T. 2010. Aryl hydrocarbon receptor negatively regulates dendritic cell immunogenicity via a kynurenine-dependent mechanism. *Proc Natl Acad Sci U S A* 107:19961-19966.
198. Veldhoen, M., Hirota, K., Westendorf, A.M., Buer, J., Dumoutier, L., Renauld, J.C., and Stockinger, B. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453:106-109.
199. Vogel, C.F., Goth, S.R., Dong, B., Pessah, I.N., and Matsumura, F. 2008. Aryl hydrocarbon receptor signaling mediates expression of indoleamine 2,3-dioxygenase. *Biochem Biophys Res Commun* 375:331-335.
200. Stockinger, B., Di Meglio, P., Gialitakis, M., and Duarte, J.H. 2014. The aryl hydrocarbon receptor: multitasking in the immune system. *Annual review of immunology* 32:403-432.
201. Tarkowski, M., Kur, B., Nocun, M., and Sitarek, K. 2010. Perinatal exposure of mice to TCDD decreases allergic sensitisation through inhibition of IL-4 production rather than T regulatory cell-mediated suppression. *Int J Occup Med Environ Health* 23:75-83.
202. Schulz, V.J., Smit, J.J., Willemsen, K.J., Fiechter, D., Hassing, I., Bleumink, R., Boon, L., van den Berg, M., van Duursen, M.B., and Pieters, R.H. 2011. Activation of the aryl hydrocarbon receptor suppresses sensitization in a mouse peanut allergy model. *Toxicol Sci* 123:491-500.
203. Jeong, K.T., Hwang, S.J., Oh, G.S., and Park, J.H. 2012. FICZ, a tryptophan photoproduct, suppresses pulmonary eosinophilia and Th2-type cytokine production in a mouse model of ovalbumin-induced allergic asthma. *International immunopharmacology* 13:377-385.

204. Jux, B., Kadow, S., and Esser, C. 2009. Langerhans cell maturation and contact hypersensitivity are impaired in aryl hydrocarbon receptor-null mice. *Journal of immunology* 182:6709-6717.
205. Schulz, V.J., van Roest, M., Bol-Schoenmakers, M., van Duursen, M.B., van den Berg, M., Pieters, R.H., and Smit, J.J. 2013. Aryl hydrocarbon receptor activation affects the dendritic cell phenotype and function during allergic sensitization. *Immunobiology* 218:1055-1062.
206. Jin, G.B., Moore, A.J., Head, J.L., Neumiller, J.J., and Lawrence, B.P. 2010. Aryl hydrocarbon receptor activation reduces dendritic cell function during influenza virus infection. *Toxicological sciences : an official journal of the Society of Toxicology* 116:514-522.
207. Platzer, B., Richter, S., Kneidinger, D., Waltenberger, D., Woisetschlager, M., and Strobl, H. 2009. Aryl hydrocarbon receptor activation inhibits in vitro differentiation of human monocytes and Langerhans dendritic cells. *Journal of immunology* 183:66-74.
208. Bankoti, J., Rase, B., Simones, T., and Shepherd, D.M. 2010. Functional and phenotypic effects of AhR activation in inflammatory dendritic cells. *Toxicol Appl Pharmacol* 246:18-28.
209. Simones, T., and Shepherd, D.M. 2011. Consequences of AhR activation in steady-state dendritic cells. *Toxicol Sci* 119:293-307.
210. Litzenburger, U.M., Opitz, C.A., Sahm, F., Rauschenbach, K.J., Trump, S., Winter, M., Ott, M., Ochs, K., Lutz, C., Liu, X., et al. 2014. Constitutive IDO expression in human cancer is sustained by an autocrine signaling loop involving IL-6, STAT3 and the AHR. *Oncotarget* 5:1038-1051.
211. DiNatale, B.C., Murray, I.A., Schroeder, J.C., Flaveny, C.A., Lahoti, T.S., Laurenzana, E.M., Omiecinski, C.J., and Perdew, G.H. 2010. Kynurenic acid is a potent endogenous aryl hydrocarbon receptor ligand that synergistically induces interleukin-6 in the presence of inflammatory signaling. *Toxicol Sci* 115:89-97.
212. Song, J., Clagett-Dame, M., Peterson, R.E., Hahn, M.E., Westler, W.M., Sicinski, R.R., and DeLuca, H.F. 2002. A ligand for the aryl hydrocarbon receptor isolated from lung. *Proceedings of the National Academy of Sciences of the United States of America* 99:14694-14699.
213. Garcia-Nieto, S., Johal, R.K., Shakesheff, K.M., Emara, M., Royer, P.J., Chau, D.Y., Shakib, F., and Ghaemmaghami, A.M. 2010. Laminin and fibronectin treatment leads to generation of dendritic cells with superior endocytic capacity. *PLoS One* 5:e10123.
214. Sharquie, I.K., Al-Ghouleh, A., Fitton, P., Clark, M.R., Armour, K.L., Sewell, H.F., Shakib, F., and Ghaemmaghami, A.M. 2013. An investigation into IgE-facilitated allergen recognition and presentation by human dendritic cells. *BMC Immunol* 14:54.
215. Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402-408.
216. Wong, M.L., and Medrano, J.F. 2005. Real-time PCR for mRNA quantitation. *BioTechniques* 39:75-85.
217. Negm, O.H., Mannsperger, H.A., McDermott, E.M., Drewe, E., Powell, R.J., Todd, I., Fairclough, L.C., and Tighe, P.J. 2014. A pro-inflammatory signalome is constitutively activated by C33Y mutant TNF receptor 1 in TNF

receptor-associated periodic syndrome (TRAPS). *European journal of immunology* 44:2096-2110.

218. Walsh, K.P., and Mills, K.H. 2013. Dendritic cells and other innate determinants of T helper cell polarisation. *Trends in immunology* 34:521-530.
219. Martinez-Pomares, L. 2012. The mannose receptor. *Journal of leukocyte biology* 92:1177-1186.
220. Nguyen, N.T., Kimura, A., Nakahama, T., Chinen, I., Masuda, K., Nohara, K., Fujii-Kuriyama, Y., and Kishimoto, T. 2010. Aryl hydrocarbon receptor negatively regulates dendritic cell immunogenicity via a kynurenine-dependent mechanism. *Proceedings of the National Academy of Sciences of the United States of America* 107:19961-19966.
221. Terness, P., Chuang, J.J., Bauer, T., Jiga, L., and Opelz, G. 2005. Regulation of human auto- and alloreactive T cells by indoleamine 2,3-dioxygenase (IDO)-producing dendritic cells: too much ado about IDO? *Blood* 105:2480-2486.
222. Jurgens, B., Hainz, U., Fuchs, D., Felzmann, T., and Heitger, A. 2009. Interferon-gamma-triggered indoleamine 2,3-dioxygenase competence in human monocyte-derived dendritic cells induces regulatory activity in allogeneic T cells. *Blood* 114:3235-3243.
223. Cambi, A., Netea, M.G., Mora-Montes, H.M., Gow, N.A., Hato, S.V., Lowman, D.W., Kullberg, B.J., Torensma, R., Williams, D.L., and Figdor, C.G. 2008. Dendritic cell interaction with Candida albicans critically depends on N-linked mannan. *The Journal of biological chemistry* 283:20590-20599.
224. Nigou, J., Zelle-Rieser, C., Gilleron, M., Thurnher, M., and Puzo, G. 2001. Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *Journal of immunology* 166:7477-7485.
225. Pathak, S.K., Basu, S., Bhattacharyya, A., Pathak, S., Kundu, M., and Basu, J. 2005. Mycobacterium tuberculosis lipoarabinomannan-mediated IRAK-M induction negatively regulates Toll-like receptor-dependent interleukin-12 p40 production in macrophages. *The Journal of biological chemistry* 280:42794-42800.
226. Oeckinghaus, A., Hayden, M.S., and Ghosh, S. 2011. Crosstalk in NF-kappaB signaling pathways. *Nature immunology* 12:695-708.
227. Manches, O., Fernandez, M.V., Plumas, J., Chaperot, L., and Bhardwaj, N. 2012. Activation of the noncanonical NF-kappaB pathway by HIV controls a dendritic cell immunoregulatory phenotype. *Proceedings of the National Academy of Sciences of the United States of America* 109:14122-14127.
228. Sun, S.C. 2011. Non-canonical NF-kappaB signaling pathway. *Cell research* 21:71-85.
229. Ball, H.J., Jusof, F.F., Bakmiwewa, S.M., Hunt, N.H., and Yuasa, H.J. 2014. Tryptophan-catabolizing enzymes - party of three. *Frontiers in immunology* 5:485.
230. Metz, R., Smith, C., Duhadaway, J.B., Chandler, P., Baban, B., Merlo, L.M., Pigott, E., Keough, M.P., Rust, S., Mellor, A.L., et al. 2014. IDO2 is critical for IDO1-mediated T-cell regulation and exerts a non-redundant function in inflammation. *International immunology*.
231. Merlo, L.M., Pigott, E., Duhadaway, J.B., Grabler, S., Metz, R., Prendergast, G.C., and Mandik-Nayak, L. 2014. IDO2 Is a Critical Mediator of

Autoantibody Production and Inflammatory Pathogenesis in a Mouse Model of Autoimmune Arthritis. *Journal of immunology*.

232. Hentges, F., Leonard, C., Arumugam, K., and Hilger, C. 2014. Immune responses to inhalant Mammalian allergens. *Frontiers in immunology* 5:234.
233. Zhang, J., Tachado, S.D., Patel, N., Zhu, J., Imrich, A., Manfruelli, P., Cushion, M., Kinane, T.B., and Koziel, H. 2005. Negative regulatory role of mannose receptors on human alveolar macrophage proinflammatory cytokine release in vitro. *J Leukoc Biol* 78:665-674.
234. Klaver, E.J., Kuijk, L.M., Laan, L.C., Kringel, H., van Vliet, S.J., Bouma, G., Cummings, R.D., Kraal, G., and van Die, I. 2013. Trichuris suis-induced modulation of human dendritic cell function is glycan-mediated. *International journal for parasitology* 43:191-200.
235. Koorella, C., Nair, J.R., Murray, M.E., Carlson, L.M., Watkins, S.K., and Lee, K.P. 2014. Novel regulation of CD80/CD86-induced phosphatidylinositol 3-kinase signaling by NOTCH1 protein in interleukin-6 and indoleamine 2,3-dioxygenase production by dendritic cells. *The Journal of biological chemistry* 289:7747-7762.
236. Onodera, T., Jang, M.H., Guo, Z., Yamasaki, M., Hirata, T., Bai, Z., Tsuji, N.M., Nagakubo, D., Yoshie, O., Sakaguchi, S., et al. 2009. Constitutive expression of IDO by dendritic cells of mesenteric lymph nodes: functional involvement of the CTLA-4/B7 and CCL22/CCR4 interactions. *Journal of immunology* 183:5608-5614.
237. Xu, H., Zhang, G.X., Ciric, B., and Rostami, A. 2008. IDO: a double-edged sword for T(H)1/T(H)2 regulation. *Immunology letters* 121:1-6.
238. Hill, M., Tanguy-Royer, S., Royer, P., Chauveau, C., Asghar, K., Tesson, L., Lavainne, F., Remy, S., Brion, R., Hubert, F.X., et al. 2007. IDO expands human CD4+CD25high regulatory T cells by promoting maturation of LPS-treated dendritic cells. *European journal of immunology* 37:3054-3062.
239. Hwang, S.L., Chung, N.P., Chan, J.K., and Lin, C.L. 2005. Indoleamine 2, 3-dioxygenase (IDO) is essential for dendritic cell activation and chemotactic responsiveness to chemokines. *Cell research* 15:167-175.
240. Veldhoen, M., Hirota, K., Christensen, J., O'Garra, A., and Stockinger, B. 2009. Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. *The Journal of experimental medicine* 206:43-49.
241. Kawai, T., and Akira, S. 2007. Signaling to NF-kappaB by Toll-like receptors. *Trends in molecular medicine* 13:460-469.
242. Vogel, C.F., Kahn, E.M., Leung, P.S., Gershwin, M.E., Chang, W.L., Wu, D., Haarmann-Stemmann, T., Hoffmann, A., and Denison, M.S. 2013. Cross-talk between Aryl Hydrocarbon Receptor and the inflammatory response: a Role for NF-kappaB. *The Journal of biological chemistry*.
243. Tian, Y. 2009. Ah receptor and NF-kappaB interplay on the stage of epigenome. *Biochemical pharmacology* 77:670-680.
244. Xaplanteri, P., Lagoumintzis, G., Dimitracopoulos, G., and Paliogianni, F. 2009. Synergistic regulation of Pseudomonas aeruginosa-induced cytokine production in human monocytes by mannose receptor and TLR2. *European journal of immunology* 39:730-740.
245. Scotton, C.J., Martinez, F.O., Smelt, M.J., Sironi, M., Locati, M., Mantovani, A., and Sozzani, S. 2005. Transcriptional profiling reveals complex regulation

of the monocyte IL-1 beta system by IL-13. *Journal of immunology* 174:834-845.

246. McBerry, C., Gonzalez, R.M., Shryock, N., Dias, A., and Aliberti, J. 2012. SOCS2-induced proteasome-dependent TRAF6 degradation: a common anti-inflammatory pathway for control of innate immune responses. *PLoS One* 7:e38384.
247. Vogel, C.F., Sciullo, E., Li, W., Wong, P., Lazennec, G., and Matsumura, F. 2007. RelB, a new partner of aryl hydrocarbon receptor-mediated transcription. *Molecular endocrinology* 21:2941-2955.
248. Vogel, C.F., and Matsumura, F. 2009. A new cross-talk between the aryl hydrocarbon receptor and RelB, a member of the NF-kappaB family. *Biochemical pharmacology* 77:734-745.
249. Vogel, C.F., Wu, D., Goth, S.R., Baek, J., Lollies, A., Domhardt, R., Grindel, A., and Pessah, I.N. 2013. Aryl hydrocarbon receptor signaling regulates NF-kappaB RelB activation during dendritic-cell differentiation. *Immunology and cell biology* 91:568-575.
250. Thatcher, T.H., Maggirwar, S.B., Baglole, C.J., Lakatos, H.F., Gasiewicz, T.A., Phipps, R.P., and Sime, P.J. 2007. Aryl hydrocarbon receptor-deficient mice develop heightened inflammatory responses to cigarette smoke and endotoxin associated with rapid loss of the nuclear factor-kappaB component RelB. *The American journal of pathology* 170:855-864.
251. Baglole, C.J., Maggirwar, S.B., Gasiewicz, T.A., Thatcher, T.H., Phipps, R.P., and Sime, P.J. 2008. The aryl hydrocarbon receptor attenuates tobacco smoke-induced cyclooxygenase-2 and prostaglandin production in lung fibroblasts through regulation of the NF-kappaB family member RelB. *The Journal of biological chemistry* 283:28944-28957.
252. Gringhuis, S.I., den Dunnen, J., Litjens, M., van der Vlist, M., and Geijtenbeek, T.B. 2009. Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to Mycobacterium tuberculosis, HIV-1 and Helicobacter pylori. *Nature immunology* 10:1081-1088.
253. Geijtenbeek, T.B., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Adema, G.J., van Kooyk, Y., and Figdor, C.G. 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* 100:575-585.
254. Svajger, U., Anderluh, M., Jeras, M., and Obermajer, N. 2010. C-type lectin DC-SIGN: an adhesion, signalling and antigen-uptake molecule that guides dendritic cells in immunity. *Cellular signalling* 22:1397-1405.
255. Pederson, K., Mitchell, D.A., and Prestegard, J.H. 2014. Structural characterization of the DC-SIGN-Lewis(X) complex. *Biochemistry* 53:5700-5709.
256. Geijtenbeek, T.B., Van Vliet, S.J., Koppel, E.A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C.M., Appelmelk, B., and Van Kooyk, Y. 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. *The Journal of experimental medicine* 197:7-17.
257. Gringhuis, S.I., den Dunnen, J., Litjens, M., van Het Hof, B., van Kooyk, Y., and Geijtenbeek, T.B. 2007. C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. *Immunity* 26:605-616.
258. Geurtsen, J., Chedammi, S., Mesters, J., Cot, M., Driessen, N.N., Sambou, T., Kakutani, R., Ummels, R., Maaskant, J., Takata, H., et al. 2009. Identification

of mycobacterial alpha-glucan as a novel ligand for DC-SIGN: involvement of mycobacterial capsular polysaccharides in host immune modulation. *Journal of immunology* 183:5221-5231.

259. Salazar, F., Hall, L., Negm, O.H., Awuah, D., Tighe, P.J., Shakib, F., and Ghaemmaghami, A.M. 2015. The mannose receptor negatively modulates the Toll-like receptor 4-aryl hydrocarbon receptor-indoleamine 2,3-dioxygenase axis in dendritic cells affecting T helper cell polarization. *The Journal of allergy and clinical immunology*. doi: 10.1016/j.jaci.2015.10.033.
260. van Liempt, E., Bank, C.M., Mehta, P., Garcia-Vallejo, J.J., Kawar, Z.S., Geyer, R., Alvarez, R.A., Cummings, R.D., Kooyk, Y., and van Die, I. 2006. Specificity of DC-SIGN for mannose- and fucose-containing glycans. *FEBS letters* 580:6123-6131.
261. van Stijn, C.M., Meyer, S., van den Broek, M., Bruijns, S.C., van Kooyk, Y., Geyer, R., and van Die, I. 2010. Schistosoma mansoni worm glycolipids induce an inflammatory phenotype in human dendritic cells by cooperation of TLR4 and DC-SIGN. *Molecular immunology* 47:1544-1552.
262. von Bubnoff, D., and Bieber, T. 2012. The indoleamine 2,3-dioxygenase (IDO) pathway controls allergy. *Allergy* 67:718-725.
263. Nguyen, N.T., Nakahama, T., Le, D.H., Van Son, L., Chu, H.H., and Kishimoto, T. 2014. Aryl hydrocarbon receptor and kynurenine: recent advances in autoimmune disease research. *Frontiers in immunology* 5:551.
264. Smith, C., Chang, M.Y., Parker, K.H., Beury, D.W., DuHadaway, J.B., Flick, H.E., Boulden, J., Sutanto-Ward, E., Soler, A.P., Laury-Kleintop, L.D., et al. 2012. IDO is a nodal pathogenic driver of lung cancer and metastasis development. *Cancer discovery* 2:722-735.
265. Zhang, Y.J., Reddy, M.C., Ioerger, T.R., Rothchild, A.C., Dartois, V., Schuster, B.M., Trauner, A., Wallis, D., Galaviz, S., Huttenhower, C., et al. 2013. Tryptophan biosynthesis protects mycobacteria from CD4 T-cell-mediated killing. *Cell* 155:1296-1308.
266. Lob, S., and Konigsrainer, A. 2009. Role of IDO in organ transplantation: promises and difficulties. *International reviews of immunology* 28:185-206.
267. Horlock, C., Shakib, F., Mahdavi, J., Jones, N.S., Sewell, H.F., and Ghaemmaghami, A.M. 2007. Analysis of proteomic profiles and functional properties of human peripheral blood myeloid dendritic cells, monocyte-derived dendritic cells and the dendritic cell-like KG-1 cells reveals distinct characteristics. *Genome biology* 8:R30.
268. Feinberg, H., Castelli, R., Drickamer, K., Seeberger, P.H., and Weis, W.I. 2007. Multiple modes of binding enhance the affinity of DC-SIGN for high mannose N-linked glycans found on viral glycoproteins. *The Journal of biological chemistry* 282:4202-4209.
269. Zenaro, E., Donini, M., and Dusi, S. 2009. Induction of Th1/Th17 immune response by Mycobacterium tuberculosis: role of dectin-1, Mannose Receptor, and DC-SIGN. *Journal of leukocyte biology* 86:1393-1401.
270. Ponichtera, H.E., Shainheit, M.G., Liu, B.C., Raychowdhury, R., Larkin, B.M., Russo, J.M., Salantes, D.B., Lai, C.Q., Parnell, L.D., Yun, T.J., et al. 2014. CD209a Expression on Dendritic Cells Is Critical for the Development of Pathogenic Th17 Cell Responses in Murine Schistosomiasis. *Journal of immunology* 192:4655-4665.
271. Favre, D., Mold, J., Hunt, P.W., Kanwar, B., Loke, P., Seu, L., Barbour, J.D., Lowe, M.M., Jayawardene, A., Aweeka, F., et al. 2010. Tryptophan catabolism

by indoleamine 2,3-dioxygenase 1 alters the balance of TH17 to regulatory T cells in HIV disease. *Science translational medicine* 2:32ra36.

272. Sharma, M.D., Hou, D.Y., Liu, Y., Koni, P.A., Metz, R., Chandler, P., Mellor, A.L., He, Y., and Munn, D.H. 2009. Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. *Blood* 113:6102-6111.
273. Criado, G., Simelyte, E., Inglis, J.J., Essex, D., and Williams, R.O. 2009. Indoleamine 2,3 dioxygenase-mediated tryptophan catabolism regulates accumulation of Th1/Th17 cells in the joint in collagen-induced arthritis. *Arthritis and rheumatism* 60:1342-1351.
274. Iannitti, R.G., Carvalho, A., Cunha, C., De Luca, A., Giovannini, G., Casagrande, A., Zelante, T., Vacca, C., Fallarino, F., Puccetti, P., et al. 2013. Th17/Treg imbalance in murine cystic fibrosis is linked to indoleamine 2,3-dioxygenase deficiency but corrected by kynurenines. *American journal of respiratory and critical care medicine* 187:609-620.
275. Hayden, M.S., and Ghosh, S. 2004. Signaling to NF-kappaB. *Genes & development* 18:2195-2224.
276. Yang, F., Tang, E., Guan, K., and Wang, C.Y. 2003. IKK beta plays an essential role in the phosphorylation of RelA/p65 on serine 536 induced by lipopolysaccharide. *Journal of immunology* 170:5630-5635.
277. Hacker, H., Tseng, P.H., and Karin, M. 2011. Expanding TRAF function: TRAF3 as a tri-faced immune regulator. *Nature reviews. Immunology* 11:457-468.
278. Solan, N.J., Miyoshi, H., Carmona, E.M., Bren, G.D., and Paya, C.V. 2002. RelB cellular regulation and transcriptional activity are regulated by p100. *The Journal of biological chemistry* 277:1405-1418.
279. Jacque, E., Tchenio, T., Piton, G., Romeo, P.H., and Baud, V. 2005. RelA repression of RelB activity induces selective gene activation downstream of TNF receptors. *Proceedings of the National Academy of Sciences of the United States of America* 102:14635-14640.
280. Saito, T., Sasaki, C.Y., Rezanka, L.J., Ghosh, P., and Longo, D.L. 2010. p52-Independent nuclear translocation of RelB promotes LPS-induced attachment. *Biochemical and biophysical research communications* 391:235-241.
281. Sasaki, C.Y., Ghosh, P., and Longo, D.L. 2011. Recruitment of RelB to the Csf2 promoter enhances RelA-mediated transcription of granulocyte-macrophage colony-stimulating factor. *The Journal of biological chemistry* 286:1093-1102.
282. Caparros, E., Munoz, P., Sierra-Filardi, E., Serrano-Gomez, D., Puig-Kroger, A., Rodriguez-Fernandez, J.L., Mellado, M., Sancho, J., Zubiaur, M., and Corbi, A.L. 2006. DC-SIGN ligation on dendritic cells results in ERK and PI3K activation and modulates cytokine production. *Blood* 107:3950-3958.
283. Hovius, J.W., de Jong, M.A., den Dunnen, J., Litjens, M., Fikrig, E., van der Poll, T., Gringhuis, S.I., and Geijtenbeek, T.B. 2008. Salp15 binding to DC-SIGN inhibits cytokine expression by impairing both nucleosome remodeling and mRNA stabilization. *PLoS pathogens* 4:e31.
284. Hodges, A., Sharrocks, K., Edelmann, M., Baban, D., Moris, A., Schwartz, O., Drakesmith, H., Davies, K., Kessler, B., McMichael, A., et al. 2007. Activation of the lectin DC-SIGN induces an immature dendritic cell phenotype triggering Rho-GTPase activity required for HIV-1 replication. *Nature immunology* 8:569-577.

285. Foster, S.L., and Medzhitov, R. 2009. Gene-specific control of the TLR-induced inflammatory response. *Clinical immunology* 130:7-15.
286. Medzhitov, R., and Horng, T. 2009. Transcriptional control of the inflammatory response. *Nature reviews. Immunology* 9:692-703.
287. Harden, J.L., and Egilmez, N.K. 2012. Indoleamine 2,3-dioxygenase and dendritic cell tolerogenicity. *Immunological investigations* 41:738-764.
288. Bessede, A., Gargaro, M., Pallotta, M.T., Matino, D., Servillo, G., Brunacci, C., Bicciato, S., Mazza, E.M., Macchiarulo, A., Vacca, C., et al. 2014. Aryl hydrocarbon receptor control of a disease tolerance defence pathway. *Nature* 511:184-190.
289. O'Neill, L.A., Golenbock, D., and Bowie, A.G. 2013. The history of Toll-like receptors - redefining innate immunity. *Nature reviews. Immunology* 13:453-460.
290. Biswas, S.K., and Lopez-Collazo, E. 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends in immunology* 30:475-487.
291. Pena, O.M., Pistolic, J., Raj, D., Fjell, C.D., and Hancock, R.E. 2011. Endotoxin tolerance represents a distinctive state of alternative polarization (M2) in human mononuclear cells. *Journal of immunology* 186:7243-7254.
292. O'Carroll, C., Fagan, A., Shanahan, F., and Carmody, R.J. 2014. Identification of a unique hybrid macrophage-polarization state following recovery from lipopolysaccharide tolerance. *Journal of immunology* 192:427-436.
293. Cavaillon, J.M., and Adib-Conquy, M. 2006. Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. *Critical care* 10:233.
294. Fallarino, F., Pallotta, M.T., Matino, D., Gargaro, M., Orabona, C., Vacca, C., Mondanelli, G., Allegrucci, M., Boon, L., Romani, R., et al. 2014. LPS-conditioned dendritic cells confer endotoxin tolerance contingent on tryptophan catabolism. *Immunobiology*.
295. Netea, M.G., Simon, A., van de Veerdonk, F., Kullberg, B.J., Van der Meer, J.W., and Joosten, L.A. 2010. IL-1beta processing in host defense: beyond the inflammasomes. *PLoS pathogens* 6:e1000661.
296. Patenaude, J., D'Elia, M., Cote-Maurais, G., and Bernier, J. 2011. LPS response and endotoxin tolerance in Flt-3L-induced bone marrow-derived dendritic cells. *Cellular immunology* 271:184-191.
297. Amor, S., Peferoen, L.A., Vogel, D.Y., Breur, M., van der Valk, P., Baker, D., and van Noort, J.M. 2014. Inflammation in neurodegenerative diseases--an update. *Immunology* 142:151-166.
298. Hotamisligil, G.S. 2006. Inflammation and metabolic disorders. *Nature* 444:860-867.
299. Grivennikov, S.I., Greten, F.R., and Karin, M. 2010. Immunity, inflammation, and cancer. *Cell* 140:883-899.
300. Marshall, J.C. 2014. Why have clinical trials in sepsis failed? *Trends in molecular medicine* 20:195-203.
301. Huttunen, R., Syrjanen, J., Aittoniemi, J., Oja, S.S., Raitala, A., Laine, J., Pertovaara, M., Vuento, R., Huhtala, H., and Hurme, M. 2010. High activity of indoleamine 2,3 dioxygenase enzyme predicts disease severity and case fatality in bacteremic patients. *Shock* 33:149-154.
302. Tattevin, P., Monnier, D., Tribut, O., Dulong, J., Bescher, N., Mourcin, F., Uhel, F., Le Tulzo, Y., and Tarte, K. 2010. Enhanced indoleamine 2,3-

dioxygenase activity in patients with severe sepsis and septic shock. *The Journal of infectious diseases* 201:956-966.

303. Darcy, C.J., Davis, J.S., Woodberry, T., McNeil, Y.R., Stephens, D.P., Yeo, T.W., and Anstey, N.M. 2011. An observational cohort study of the kynurenine to tryptophan ratio in sepsis: association with impaired immune and microvascular function. *PLoS One* 6:e21185.
304. Jung, I.D., Lee, M.G., Chang, J.H., Lee, J.S., Jeong, Y.I., Lee, C.M., Park, W.S., Han, J., Seo, S.K., Lee, S.Y., et al. 2009. Blockade of indoleamine 2,3-dioxygenase protects mice against lipopolysaccharide-induced endotoxin shock. *Journal of immunology* 182:3146-3154.
305. Serhan, C.N., Chiang, N., and Van Dyke, T.E. 2008. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nature reviews. Immunology* 8:349-361.
306. Wysocka, M., Robertson, S., Riemann, H., Caamano, J., Hunter, C., Mackiewicz, A., Montaner, L.J., Trinchieri, G., and Karp, C.L. 2001. IL-12 suppression during experimental endotoxin tolerance: dendritic cell loss and macrophage hyporesponsiveness. *Journal of immunology* 166:7504-7513.
307. Sharabi, A.B., Aldrich, M., Sosic, D., Olson, E.N., Friedman, A.D., Lee, S.H., and Chen, S.Y. 2008. Twist-2 controls myeloid lineage development and function. *PLoS biology* 6:e316.
308. Albrecht, V., Hofer, T.P., Foxwell, B., Frankenberger, M., and Ziegler-Heitbrock, L. 2008. Tolerance induced via TLR2 and TLR4 in human dendritic cells: role of IRAK-1. *BMC immunology* 9:69.
309. Lawrence, T., Gilroy, D.W., Colville-Nash, P.R., and Willoughby, D.A. 2001. Possible new role for NF-kappaB in the resolution of inflammation. *Nature medicine* 7:1291-1297.
310. Needham, B.D., Carroll, S.M., Giles, D.K., Georgiou, G., Whiteley, M., and Trent, M.S. 2013. Modulating the innate immune response by combinatorial engineering of endotoxin. *Proceedings of the National Academy of Sciences of the United States of America* 110:1464-1469.
311. Adib-Conquy, M., Adrie, C., Moine, P., Asehnoune, K., Fitting, C., Pinsky, M.R., Dhainaut, J.F., and Cavaillon, J.M. 2000. NF-kappaB expression in mononuclear cells of patients with sepsis resembles that observed in lipopolysaccharide tolerance. *American journal of respiratory and critical care medicine* 162:1877-1883.
312. Ziegler-Heitbrock, L. 2001. The p50-homodimer mechanism in tolerance to LPS. *Journal of endotoxin research* 7:219-222.
313. Bohuslav, J., Kravchenko, V.V., Parry, G.C., Erlich, J.H., Gerondakis, S., Mackman, N., and Ulevitch, R.J. 1998. Regulation of an essential innate immune response by the p50 subunit of NF-kappaB. *The Journal of clinical investigation* 102:1645-1652.
314. Chen, X., Yoza, B.K., El Gazzar, M., Hu, J.Y., Cousart, S.L., and McCall, C.E. 2009. RelB sustains IkappaBalpha expression during endotoxin tolerance. *Clinical and vaccine immunology : CVI* 16:104-110.
315. Yoza, B.K., Hu, J.Y., Cousart, S.L., Forrest, L.M., and McCall, C.E. 2006. Induction of RelB participates in endotoxin tolerance. *Journal of immunology* 177:4080-4085.
316. Kumagai, Y., Takeuchi, O., and Akira, S. 2008. TLR9 as a key receptor for the recognition of DNA. *Advanced drug delivery reviews* 60:795-804.

317. Gursel, M., Verthelyi, D., Gursel, I., Ishii, K.J., and Klinman, D.M. 2002. Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. *Journal of leukocyte biology* 71:813-820.
318. Verthelyi, D., Ishii, K.J., Gursel, M., Takeshita, F., and Klinman, D.M. 2001. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *Journal of immunology* 166:2372-2377.
319. Avalos, A.M., Latz, E., Mousseau, B., Christensen, S.R., Shlomchik, M.J., Lund, F., and Marshak-Rothstein, A. 2009. Differential cytokine production and bystander activation of autoreactive B cells in response to CpG-A and CpG-B oligonucleotides. *Journal of immunology* 183:6262-6268.
320. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., et al. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740-745.
321. Vollmer, J., Weeratna, R., Payette, P., Jurk, M., Schetter, C., Laucht, M., Wader, T., Tluk, S., Liu, M., Davis, H.L., et al. 2004. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *European journal of immunology* 34:251-262.
322. Gray, R.C., Kuchtey, J., and Harding, C.V. 2007. CpG-B ODNs potently induce low levels of IFN-alphabeta and induce IFN-alphabeta-dependent MHC-I cross-presentation in DCs as effectively as CpG-A and CpG-C ODNs. *Journal of leukocyte biology* 81:1075-1085.
323. Kerkmann, M., Costa, L.T., Richter, C., Rothenfusser, S., Battiany, J., Hornung, V., Johnson, J., Englert, S., Ketterer, T., Heckl, W., et al. 2005. Spontaneous formation of nucleic acid-based nanoparticles is responsible for high interferon-alpha induction by CpG-A in plasmacytoid dendritic cells. *The Journal of biological chemistry* 280:8086-8093.
324. Hartmann, G., Weiner, G.J., and Krieg, A.M. 1999. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America* 96:9305-9310.
325. Kuwajima, S., Sato, T., Ishida, K., Tada, H., Tezuka, H., and Ohteki, T. 2006. Interleukin 15-dependent crosstalk between conventional and plasmacytoid dendritic cells is essential for CpG-induced immune activation. *Nature immunology* 7:740-746.
326. Hoene, V., Peiser, M., and Wanner, R. 2006. Human monocyte-derived dendritic cells express TLR9 and react directly to the CpG-A oligonucleotide D19. *Journal of leukocyte biology* 80:1328-1336.
327. Hardenberg, G., Planelles, L., Schwarte, C.M., van Bostelen, L., Le Huong, T., Hahne, M., and Medema, J.P. 2007. Specific TLR ligands regulate APRIL secretion by dendritic cells in a PKR-dependent manner. *European journal of immunology* 37:2900-2911.
328. Chakarov, S., and Fazilleau, N. 2014. Monocyte-derived dendritic cells promote T follicular helper cell differentiation. *EMBO molecular medicine* 6:590-603.
329. Krug, A., Rothenfusser, S., Selinger, S., Bock, C., Kerkmann, M., Battiany, J., Sarris, A., Giese, T., Speiser, D., Endres, S., et al. 2003. CpG-A oligonucleotides induce a monocyte-derived dendritic cell-like phenotype that preferentially activates CD8 T cells. *Journal of immunology* 170:3468-3477.

330. Behboudi, S., Chao, D., Klenerman, P., and Austyn, J. 2000. The effects of DNA containing CpG motif on dendritic cells. *Immunology* 99:361-366.
331. Schattenberg, D., Schott, M., Reindl, G., Krueger, T., Tschoepe, D., Feldkamp, J., Scherbaum, W.A., and Seissler, J. 2000. Response of human monocyte-derived dendritic cells to immunostimulatory DNA. *European journal of immunology* 30:2824-2831.
332. Coccia, E.M., Severa, M., Giacomini, E., Monneron, D., Remoli, M.E., Julkunen, I., Cella, M., Lande, R., and Uze, G. 2004. Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. *European journal of immunology* 34:796-805.
333. Erhardt, M., Gorschluter, M., Sager, J., Ziske, C., Strehl, J., Lilienfeld-Toal, M.V., and Schmidt-Wolf, I.G. 2005. Transfection of human monocyte-derived dendritic cells with CpG oligonucleotides. *Immunology and cell biology* 83:278-285.
334. Hillyer, P., Mane, V.P., Schramm, L.M., Puig, M., Verthelyi, D., Chen, A., Zhao, Z., Navarro, M.B., Kirschman, K.D., Bykadi, S., et al. 2012. Expression profiles of human interferon-alpha and interferon-lambda subtypes are ligand- and cell-dependent. *Immunology and cell biology* 90:774-783.
335. Vollmer, J., and Krieg, A.M. 2009. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Advanced drug delivery reviews* 61:195-204.
336. Mellor, A.L., Baban, B., Chandler, P.R., Manlapat, A., Kahler, D.J., and Munn, D.H. 2005. Cutting edge: CpG oligonucleotides induce splenic CD19+ dendritic cells to acquire potent indoleamine 2,3-dioxygenase-dependent T cell regulatory functions via IFN Type 1 signaling. *Journal of immunology* 175:5601-5605.
337. Baban, B., Chandler, P.R., Johnson, B.A., 3rd, Huang, L., Li, M., Sharpe, M.L., Francisco, L.M., Sharpe, A.H., Blazar, B.R., Munn, D.H., et al. 2011. Physiologic control of IDO competence in splenic dendritic cells. *Journal of immunology* 187:2329-2335.
338. Volpi, C., Fallarino, F., Pallotta, M.T., Bianchi, R., Vacca, C., Belladonna, M.L., Orabona, C., De Luca, A., Boon, L., Romani, L., et al. 2013. High doses of CpG oligodeoxynucleotides stimulate a tolerogenic TLR9-TRIF pathway. *Nature communications* 4:1852.
339. Munn, D.H., and Mellor, A.L. 2013. Indoleamine 2,3 dioxygenase and metabolic control of immune responses. *Trends in immunology* 34:137-143.
340. Schmidt, S.V., and Schultze, J.L. 2014. New Insights into IDO Biology in Bacterial and Viral Infections. *Frontiers in immunology* 5:384.
341. Zelante, T., Fallarino, F., Bistoni, F., Puccetti, P., and Romani, L. 2009. Indoleamine 2,3-dioxygenase in infection: the paradox of an evasive strategy that benefits the host. *Microbes and infection / Institut Pasteur* 11:133-141.
342. Obojes, K., Andres, O., Kim, K.S., Daubener, W., and Schneider-Schaulies, J. 2005. Indoleamine 2,3-dioxygenase mediates cell type-specific anti-measles virus activity of gamma interferon. *Journal of virology* 79:7768-7776.
343. Pfefferkorn, E.R. 1984. Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan. *Proceedings of the National Academy of Sciences of the United States of America* 81:908-912.

344. Murray, H.W., Szuro-Sudol, A., Wellner, D., Oca, M.J., Granger, A.M., Libby, D.M., Rothermel, C.D., and Rubin, B.Y. 1989. Role of tryptophan degradation in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophages. *Infection and immunity* 57:845-849.
345. Loughman, J.A., and Hunstad, D.A. 2012. Induction of indoleamine 2,3-dioxygenase by uropathogenic bacteria attenuates innate responses to epithelial infection. *The Journal of infectious diseases* 205:1830-1839.
346. Boasso, A., Herbeuval, J.P., Hardy, A.W., Anderson, S.A., Dolan, M.J., Fuchs, D., and Shearer, G.M. 2007. HIV inhibits CD4+ T-cell proliferation by inducing indoleamine 2,3-dioxygenase in plasmacytoid dendritic cells. *Blood* 109:3351-3359.
347. Furset, G., Floisand, Y., and Sioud, M. 2008. Impaired expression of indoleamine 2, 3-dioxygenase in monocyte-derived dendritic cells in response to Toll-like receptor-7/8 ligands. *Immunology* 123:263-271.
348. Thomas, S.R., Mohr, D., and Stocker, R. 1994. Nitric oxide inhibits indoleamine 2,3-dioxygenase activity in interferon-gamma primed mononuclear phagocytes. *The Journal of biological chemistry* 269:14457-14464.
349. Fujigaki, H., Saito, K., Lin, F., Fujigaki, S., Takahashi, K., Martin, B.M., Chen, C.Y., Masuda, J., Kowalak, J., Takikawa, O., et al. 2006. Nitration and inactivation of IDO by peroxynitrite. *Journal of immunology* 176:372-379.
350. Thomas, S.R., Terentis, A.C., Cai, H., Takikawa, O., Levina, A., Lay, P.A., Freewan, M., and Stocker, R. 2007. Post-translational regulation of human indoleamine 2,3-dioxygenase activity by nitric oxide. *The Journal of biological chemistry* 282:23778-23787.
351. Thomas, S.R., Salahifar, H., Mashima, R., Hunt, N.H., Richardson, D.R., and Stocker, R. 2001. Antioxidants inhibit indoleamine 2,3-dioxygenase in IFN-gamma-activated human macrophages: posttranslational regulation by pyrrolidine dithiocarbamate. *Journal of immunology* 166:6332-6340.
352. Hucke, C., MacKenzie, C.R., Adjogble, K.D., Takikawa, O., and Daubener, W. 2004. Nitric oxide-mediated regulation of gamma interferon-induced bacteriostasis: inhibition and degradation of human indoleamine 2,3-dioxygenase. *Infection and immunity* 72:2723-2730.
353. Lee, S.H., Lee, J.G., Kim, J.R., and Baek, S.H. 2007. Toll-like receptor 9-mediated cytosolic phospholipase A2 activation regulates expression of inducible nitric oxide synthase. *Biochemical and biophysical research communications* 364:996-1001.
354. Ghosh, D.K., Misukonis, M.A., Reich, C., Pisetsky, D.S., and Weinberg, J.B. 2001. Host response to infection: the role of CpG DNA in induction of cyclooxygenase 2 and nitric oxide synthase 2 in murine macrophages. *Infection and immunity* 69:7703-7710.
355. Lavieri, R., Piccioli, P., Carta, S., Delfino, L., Castellani, P., and Rubartelli, A. 2014. TLR Costimulation Causes Oxidative Stress with Unbalance of Proinflammatory and Anti-Inflammatory Cytokine Production. *Journal of immunology* 192:5373-5381.
356. Trinchieri, G., and Sher, A. 2007. Cooperation of Toll-like receptor signals in innate immune defence. *Nature reviews. Immunology* 7:179-190.
357. Whitmore, M.M., DeVeer, M.J., Edling, A., Oates, R.K., Simons, B., Lindner, D., and Williams, B.R. 2004. Synergistic activation of innate immunity by

double-stranded RNA and CpG DNA promotes enhanced antitumor activity. *Cancer research* 64:5850-5860.

358. Bafica, A., Scanga, C.A., Feng, C.G., Leifer, C., Cheever, A., and Sher, A. 2005. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *The Journal of experimental medicine* 202:1715-1724.
359. Bafica, A., Santiago, H.C., Goldszmid, R., Ropert, C., Gazzinelli, R.T., and Sher, A. 2006. Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in Trypanosoma cruzi infection. *Journal of immunology* 177:3515-3519.
360. Byrnes, A.A., Ma, X., Cuomo, P., Park, K., Wahl, L., Wolf, S.F., Zhou, H., Trinchieri, G., and Karp, C.L. 2001. Type I interferons and IL-12: convergence and cross-regulation among mediators of cellular immunity. *European journal of immunology* 31:2026-2034.
361. De Nardo, D., De Nardo, C.M., Nguyen, T., Hamilton, J.A., and Scholz, G.M. 2009. Signaling crosstalk during sequential TLR4 and TLR9 activation amplifies the inflammatory response of mouse macrophages. *Journal of immunology* 183:8110-8118.
362. Grohmann, U., and Bronte, V. 2010. Control of immune response by amino acid metabolism. *Immunological reviews* 236:243-264.
363. Coste, A., Dubourdeau, M., Linas, M.D., Cassaing, S., Lepert, J.C., Balard, P., Chalmeton, S., Bernad, J., Orfila, C., Seguela, J.P., et al. 2003. PPARgamma promotes mannose receptor gene expression in murine macrophages and contributes to the induction of this receptor by IL-13. *Immunity* 19:329-339.
364. Egan, B.S., Abdolrasulnia, R., and Shepherd, V.L. 2004. IL-4 modulates transcriptional control of the mannose receptor in mouse FSDC dendritic cells. *Archives of biochemistry and biophysics* 428:119-130.
365. Linehan, S.A., Coulson, P.S., Wilson, R.A., Mountford, A.P., Brombacher, F., Martinez-Pomares, L., and Gordon, S. 2003. IL-4 receptor signaling is required for mannose receptor expression by macrophages recruited to granulomata but not resident cells in mice infected with Schistosoma mansoni. *Laboratory investigation; a journal of technical methods and pathology* 83:1223-1231.
366. Martinez-Pomares, L., Reid, D.M., Brown, G.D., Taylor, P.R., Stillion, R.J., Linehan, S.A., Zamze, S., Gordon, S., and Wong, S.Y. 2003. Analysis of mannose receptor regulation by IL-4, IL-10, and proteolytic processing using novel monoclonal antibodies. *Journal of leukocyte biology* 73:604-613.
367. Tachado, S.D., Zhang, J., Zhu, J., Patel, N., Cushion, M., and Koziel, H. 2007. Pneumocystis-mediated IL-8 release by macrophages requires coexpression of mannose receptors and TLR2. *J Leukoc Biol* 81:205-211.
368. Pathak, S.K., Skold, A.E., Mohanram, V., Persson, C., Johansson, U., and Spetz, A.L. 2012. Activated apoptotic cells induce dendritic cell maturation via engagement of Toll-like receptor 4 (TLR4), dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN), and beta2 integrins. *The Journal of biological chemistry* 287:13731-13742.
369. Parsons, M.W., Li, L., Wallace, A.M., Lee, M.J., Katz, H.R., Fernandez, J.M., Saijo, S., Iwakura, Y., Austen, K.F., Kanaoka, Y., et al. 2014. Dectin-2 Regulates the Effector Phase of House Dust Mite-Elicited Pulmonary Inflammation Independently from Its Role in Sensitization. *Journal of immunology*.

370. Kingeter, L.M., and Lin, X. 2012. C-type lectin receptor-induced NF-kappaB activation in innate immune and inflammatory responses. *Cellular & molecular immunology* 9:105-112.
371. Yonekawa, A., Saijo, S., Hoshino, Y., Miyake, Y., Ishikawa, E., Suzukawa, M., Inoue, H., Tanaka, M., Yoneyama, M., Oh-Hora, M., et al. 2014. Dectin-2 is a direct receptor for mannose-capped lipoarabinomannan of mycobacteria. *Immunity* 41:402-413.
372. Rooney, A.A., Luebke, R.W., Selgrade, M.K., and Germolec, D.R. 2012. Immunotoxicology and its application in risk assessment. *EXS* 101:251-287.
373. Zhang, J., Zhu, J., Bu, X., Cushion, M., Kinane, T.B., Avraham, H., and Koziel, H. 2005. Cdc42 and RhoB activation are required for mannose receptor-mediated phagocytosis by human alveolar macrophages. *Molecular biology of the cell* 16:824-834.
374. Nikolic, D.S., Lehmann, M., Felts, R., Garcia, E., Blanchet, F.P., Subramaniam, S., and Piguet, V. 2011. HIV-1 activates Cdc42 and induces membrane extensions in immature dendritic cells to facilitate cell-to-cell virus propagation. *Blood* 118:4841-4852.
375. Sweet, L., Singh, P.P., Azad, A.K., Rajaram, M.V., Schlesinger, L.S., and Schorey, J.S. 2010. Mannose receptor-dependent delay in phagosome maturation by Mycobacterium avium glycopeptidolipids. *Infection and immunity* 78:518-526.
376. Rajaram, M.V., Brooks, M.N., Morris, J.D., Torrelles, J.B., Azad, A.K., and Schlesinger, L.S. 2010. Mycobacterium tuberculosis activates human macrophage peroxisome proliferator-activated receptor gamma linking mannose receptor recognition to regulation of immune responses. *Journal of immunology* 185:929-942.
377. Garrido, V.V., Dulgerian, L.R., Stempin, C.C., and Cerban, F.M. 2011. The increase in mannose receptor recycling favors arginase induction and Trypanosoma cruzi survival in macrophages. *International journal of biological sciences* 7:1257-1272.
378. Lefevre, L., Lugo-Villarino, G., Meunier, E., Valentin, A., Olagnier, D., Authier, H., Duval, C., Dardenne, C., Bernad, J., Lemesre, J.L., et al. 2013. The C-type lectin receptors dectin-1, MR, and SIGNR3 contribute both positively and negatively to the macrophage response to Leishmania infantum. *Immunity* 38:1038-1049.
379. Astarie-Dequeker, C., N'Diaye, E.N., Le Cabec, V., Rittig, M.G., Prandi, J., and Maridonneau-Parini, I. 1999. The mannose receptor mediates uptake of pathogenic and nonpathogenic mycobacteria and bypasses bactericidal responses in human macrophages. *Infection and immunity* 67:469-477.
380. Kerur, N., Veettil, M.V., Sharma-Walia, N., Sadagopan, S., Bottero, V., Paul, A.G., and Chandran, B. 2010. Characterization of entry and infection of monocytic THP-1 cells by Kaposi's sarcoma associated herpesvirus (KSHV): role of heparan sulfate, DC-SIGN, integrins and signaling. *Virology* 406:103-116.
381. Szanto, A., Balint, B.L., Nagy, Z.S., Barta, E., Dezso, B., Pap, A., Szeles, L., Poliska, S., Oros, M., Evans, R.M., et al. 2010. STAT6 transcription factor is a facilitator of the nuclear receptor PPARgamma-regulated gene expression in macrophages and dendritic cells. *Immunity* 33:699-712.

382. Ahmadian, M., Suh, J.M., Hah, N., Liddle, C., Atkins, A.R., Downes, M., and Evans, R.M. 2013. PPARgamma signaling and metabolism: the good, the bad and the future. *Nature medicine* 19:557-566.
383. Varga, T., and Nagy, L. 2008. Nuclear receptors, transcription factors linking lipid metabolism and immunity: the case of peroxisome proliferator-activated receptor gamma. *European journal of clinical investigation* 38:695-707.
384. Nencioni, A., Grunebach, F., Zobywlaski, A., Denzlinger, C., Brugger, W., and Brossart, P. 2002. Dendritic cell immunogenicity is regulated by peroxisome proliferator-activated receptor gamma. *Journal of immunology* 169:1228-1235.
385. Faveeuw, C., Fougeray, S., Angeli, V., Fontaine, J., Chinetti, G., Gosset, P., Delerive, P., Maliszewski, C., Capron, M., Staels, B., et al. 2000. Peroxisome proliferator-activated receptor gamma activators inhibit interleukin-12 production in murine dendritic cells. *FEBS letters* 486:261-266.
386. Szatmari, I., Torocsik, D., Agostini, M., Nagy, T., Gurnell, M., Barta, E., Chatterjee, K., and Nagy, L. 2007. PPARgamma regulates the function of human dendritic cells primarily by altering lipid metabolism. *Blood* 110:3271-3280.
387. Szatmari, I., Rajnavolgyi, E., and Nagy, L. 2006. PPARgamma, a lipid-activated transcription factor as a regulator of dendritic cell function. *Annals of the New York Academy of Sciences* 1088:207-218.
388. Klotz, L., Dani, I., Edenhofer, F., Nolden, L., Evert, B., Paul, B., Kolanus, W., Klockgether, T., Knolle, P., and Diehl, L. 2007. Peroxisome proliferator-activated receptor gamma control of dendritic cell function contributes to development of CD4+ T cell anergy. *Journal of immunology* 178:2122-2131.
389. Nagy, L., Szanto, A., Szatmari, I., and Szeles, L. 2012. Nuclear hormone receptors enable macrophages and dendritic cells to sense their lipid environment and shape their immune response. *Physiological reviews* 92:739-789.
390. Agostini, M., Schoenmakers, E., Mitchell, C., Szatmari, I., Savage, D., Smith, A., Rajanayagam, O., Semple, R., Luan, J., Bath, L., et al. 2006. Non-DNA binding, dominant-negative, human PPARgamma mutations cause lipodystrophic insulin resistance. *Cell metabolism* 4:303-311.
391. Hammad, H., de Heer, H.J., Soullie, T., Angeli, V., Trottein, F., Hoogsteden, H.C., and Lambrecht, B.N. 2004. Activation of peroxisome proliferator-activated receptor-gamma in dendritic cells inhibits the development of eosinophilic airway inflammation in a mouse model of asthma. *The American journal of pathology* 164:263-271.
392. Angeli, V., Llodra, J., Rong, J.X., Satoh, K., Ishii, S., Shimizu, T., Fisher, E.A., and Randolph, G.J. 2004. Dyslipidemia associated with atherosclerotic disease systemically alters dendritic cell mobilization. *Immunity* 21:561-574.
393. Nakken, B., Varga, T., Szatmari, I., Szeles, L., Gyongyosi, A., Illarionov, P.A., Dezso, B., Gogolak, P., Rajnavolgyi, E., and Nagy, L. 2011. Peroxisome proliferator-activated receptor gamma-regulated cathepsin D is required for lipid antigen presentation by dendritic cells. *Journal of immunology* 187:240-247.
394. Angeli, V., Hammad, H., Staels, B., Capron, M., Lambrecht, B.N., and Trottein, F. 2003. Peroxisome proliferator-activated receptor gamma inhibits the migration of dendritic cells: consequences for the immune response. *Journal of immunology* 170:5295-5301.

395. Le Naour, F., Hohenkirk, L., Grolleau, A., Misek, D.E., Lescure, P., Geiger, J.D., Hanash, S., and Beretta, L. 2001. Profiling changes in gene expression during differentiation and maturation of monocyte-derived dendritic cells using both oligonucleotide microarrays and proteomics. *The Journal of biological chemistry* 276:17920-17931.
396. Gosset, P., Charbonnier, A.S., Delerive, P., Fontaine, J., Staels, B., Pestel, J., Tonnel, A.B., and Trottein, F. 2001. Peroxisome proliferator-activated receptor gamma activators affect the maturation of human monocyte-derived dendritic cells. *European journal of immunology* 31:2857-2865.
397. Szatmari, I., Gogolak, P., Im, J.S., Dezso, B., Rajnavolgyi, E., and Nagy, L. 2004. Activation of PPARgamma specifies a dendritic cell subtype capable of enhanced induction of iNKT cell expansion. *Immunity* 21:95-106.
398. Klotz, L., Hucke, S., Thimm, D., Classen, S., Gaarz, A., Schultze, J., Edenhofer, F., Kurts, C., Klockgether, T., Limmer, A., et al. 2009. Increased antigen cross-presentation but impaired cross-priming after activation of peroxisome proliferator-activated receptor gamma is mediated by up-regulation of B7H1. *Journal of immunology* 183:129-136.
399. Straus, D.S., Pascual, G., Li, M., Welch, J.S., Ricote, M., Hsiang, C.H., Sengchanthalangsy, L.L., Ghosh, G., and Glass, C.K. 2000. 15-deoxy-delta 12,14-prostaglandin J2 inhibits multiple steps in the NF-kappa B signaling pathway. *Proceedings of the National Academy of Sciences of the United States of America* 97:4844-4849.
400. Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., and Santoro, M.G. 2000. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. *Nature* 403:103-108.
401. Appel, S., Mirakaj, V., Bringmann, A., Weck, M.M., Grunebach, F., and Brossart, P. 2005. PPAR-gamma agonists inhibit toll-like receptor-mediated activation of dendritic cells via the MAP kinase and NF-kappaB pathways. *Blood* 106:3888-3894.
402. Ouaaz, F., Arron, J., Zheng, Y., Choi, Y., and Beg, A.A. 2002. Dendritic cell development and survival require distinct NF-kappaB subunits. *Immunity* 16:257-270.
403. Benezech, C., Mader, E., Desanti, G., Khan, M., Nakamura, K., White, A., Ware, C.F., Anderson, G., and Caamano, J.H. 2012. Lymphotoxin-beta receptor signaling through NF-kappaB2-RelB pathway reprograms adipocyte precursors as lymph node stromal cells. *Immunity* 37:721-734.
404. Lovas, A., Radke, D., Albrecht, D., Yilmaz, Z.B., Moller, U., Habenicht, A.J., and Weih, F. 2008. Differential RelA- and RelB-dependent gene transcription in LTbetaR-stimulated mouse embryonic fibroblasts. *BMC genomics* 9:606.
405. Alexander, D.L., Ganem, L.G., Fernandez-Salguero, P., Gonzalez, F., and Jefcoate, C.R. 1998. Aryl-hydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis. *Journal of cell science* 111 (Pt 22):3311-3322.
406. Shimba, S., Wada, T., and Tezuka, M. 2001. Arylhydrocarbon receptor (AhR) is involved in negative regulation of adipose differentiation in 3T3-L1 cells: AhR inhibits adipose differentiation independently of dioxin. *Journal of cell science* 114:2809-2817.
407. Hanlon, P.R., Cimafranca, M.A., Liu, X., Cho, Y.C., and Jefcoate, C.R. 2005. Microarray analysis of early adipogenesis in C3H10T1/2 cells: cooperative

inhibitory effects of growth factors and 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology and applied pharmacology* 207:39-58.

408. Mesman, A.W., Zijlstra-Willems, E.M., Kaptein, T.M., de Swart, R.L., Davis, M.E., Ludlow, M., Duprex, W.P., Gack, M.U., Gringhuis, S.I., and Geijtenbeek, T.B. 2014. Measles virus suppresses RIG-I-like receptor activation in dendritic cells via DC-SIGN-mediated inhibition of PP1 phosphatases. *Cell host & microbe* 16:31-42.
409. Kimura, A., Naka, T., Nakahama, T., Chinen, I., Masuda, K., Nohara, K., Fujii-Kuriyama, Y., and Kishimoto, T. 2009. Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. *The Journal of experimental medicine* 206:2027-2035.