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The Role of mir-511-3p in Modulating Human Dendritic Cell Function

Submitted by

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Abstract

MicroRNAs (miRNA) are functional, non-coding RNA molecules that negatively regulate gene expression by repressing target mRNAs. Recently, microRNA-511-3p (miR-511-3p) has emerged as a key player in regulating the function of human DCs and in controlling TLR4-mediated signalling. Previously, our group showed that c-type lectin receptors on DCs such as the mannose receptor (MR) is involved in uptake of allergens and downstream events leading to Th2 allergic responses. Interestingly, miR-511-3p is embedded within the *MRC1* gene that encodes MR. Therefore, in this study, it was hypothesised that miR-511-3p maybe a key player in regulating MR expression on DCs and downstream events affecting Th polarisation. Additionally, miR-511-3p is highlighted to putatively target PPARγ, a potent suppressor of immune responses; however the link between miR-511-3p and PPARγ and its influence on DC function within the context of LPS-induced inflammatory responses is unknown.

Using a selection of miR-511-3p inhibitors and mimics, this study has shown for the first time that up or downregulation of miR-511-3p has opposing effects on mRNA and protein levels of MR and another CLR (DC-SIGN) on human DCs. In addition, knockdown of miR-511-3p induced 1) an increase in IDO enzyme activity (after treatment with mannan and LPS); 2) upregulation of the PDL-1 surface marker and 3) an increase in IL-10 production, thereby promoting an anti-inflammatory DC phenotype and generation of T cells with increased IL-4 and decreased IL-17/IFN-γ production. This was in contrast to observations with miR-511-3p mimics, which promoted a pro-inflammatory DC phenotype. Furthermore, LPS stimulation of DCs, following knockdown of miR-511-3p was also able to upregulate RelB and A20 protein levels, which are key repressors of NF-κB activation, compared to their overexpressed counterparts, further highlighting the impact of miR-511-3p expression on human DCs.

Lastly, this study has demonstrated that changes in miR-511-3p expression inversely correlate with PPARγ expression and transcriptional activity following PPARγ activation with rosiglitazone (RSG), in the presence or absence of LPS. Interestingly, inhibition of miR-511-3p was also able to promote an anti-inflammatory DC characterised by increased IL-10 production following stimulation with RSG and LPS, whereas overexpression of miR-511-3p promoted IL-6 pro-inflammatory cytokine production. This suggests miR-511-3p targets PPARγ to inhibit its suppressive role.

Taken together, these observations highlight the complexity of miR-511-3p induced regulation of human DC phenotype and function and could ultimately pave way for rational design of therapies against a range of inflammatory disorders.

Publications and Conferences

Publications from this work

1- Awuah D, Latif A, Salazar F, Emes R.D. and Ghaemmaghami A.M. The cross-talk between miR-511-3p and c-type lectin receptors on dendritic cells modulates dendritic cell function. (Manuscript submitted for publication).

Other publications

2- Singh S, Awuah D, Rostam H, Emes R, Kandola N, Onion D, Htwe S, Rajchagool B, Cha B, Kim D, Tighe PJ, Vrana NE, Khademhosseini A, and Ghaemmaghami A (2017). Unbiased analysis of the impact of micropatterned biomaterials on macrophage behaviour provides insights beyond predefined polarization states. *ACS Biomaterials Science & Engineering* 3 (6), 969-978

3- Salazar F, Awuah D, Negm O, Shakib F and Ghaemmaghami A (2017). The role of indoleamine 2,3-dioxygenase-aryl hydrocarbon receptor pathway in the TLR4-induced tolerogenic phenotype in human DCs. *Scientific Reports* volume 7: 43337 doi:10.1038/ srep43337

4- Salazar F, Hall L, Negm O, Awuah D, Tighe PJ, Shakib F, Ghaemmaghami AM (2016). 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. *J Allergy Clin Immunol*; 137(6):1841-1851.e2

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Abbreviations

15d-PGJ_2	15-Deoxy-Delta-12,14-prostaglandin J2
AAM	Alternatively activated macrophages
AFU	Arbitrary fluorescence unit
AGO	Argonaute
AhR	Aryl hydrocarbon receptor
APC	Antigen presenting cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CLR	C-type lectin receptor
CRD	Carbohydrate recognition domain
CTLD	C-type lectin-like domain
CR	Cysteine rich
CT	Transfected controls
DAMP	Damage associated molecular patterns
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific (ICAM)-3 grabbing non-integrin
DGCR8	DiGeorge syndrome critical region 8
FABP4	Fatty acid binding protein 4
FACS	Fluorescence activated cell sorting
FcεR	Fragment of crystallization epsilon receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN2	General control non-derepressible 2
GM-CSF	Granulocyte-macrophage colony stimulating factor
HDM	House dust mite

HLA	Human leukocyte antigen
ICAM	Intracellular adhesion molecule
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB Kinase
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
IκB	Inhibitor of kappa B
KYN	Kynurenine
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MD2	Myeloid differentiation protein 2
MFI	Median fluorescence intensity
miRNA	MicroRNA
Mo-DC	Monocyte-derived dendritic cell
MR	Mannose receptor
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response protein 88
NF-κB	Nuclear factor –κB
NIK	NF-κB inducing kinase
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction

PDL	Programmed death ligand
PFA	Paraformaldehyde
PGE_2	Prostaglandin E2
PI	Propodium iodide
PPARγ	Peroxisome proliferator activated receptor gamma
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real time PCR
RISC	RNA induced silencing complex
RNU6-2	RNA, U6 Small Nuclear 2
RSG	Rosiglitazone
siRNA	Small interfering RNA
TGF-β	Transforming growth factor beta
Th	T helper cell
TLR	Toll-like Receptor
TNF-α	Tumour necrosis factor alpha
TNFAIP3	TNF-α-induced protein 3
TRAF	TNF receptor-associated factor
Treg	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRP	Tryptophan
TZD	Thiazolidinediones
UTR	Untranslated region

Chapter 1: General Introduction

Chapter 1: General Introduction

1.1 The immune system

The immune system has evolved to efficiently recognize and eliminate specific disease-causing microorganisms such as bacteria and viruses, as well as larger multicellular parasites, while maintaining tolerance to endogenous (self) antigens. It is made up of specialized cells and tissues that employ diverse effector mechanisms to protect the host from harm. The inability of the immune system to mount effective and appropriate responses could result in an immunodeficiency, an attack against healthy tissues (i.e. autoimmunity) or harmless substances (allergy). In general, the immune system is divided into two categories, innate and adaptive immunity.

Initial responses against pathogens are generated by innate immunity, which elicits first line, non-specific defence through physical and chemical barriers such as the skin and mucosae of the airways and gut (1). Furthermore, innate immunity relies on germline-encoded components to detect structurally conserved molecular patterns on invading pathogens; however, this does not guarantee successful elimination of the threat. Following pathogen recognition, innate immunity activates the adaptive immune response, which although slower, exhibits a high degree of specificity and memory, resulting from previous encounter with the same pathogen (2). Cells comprising innate and adaptive immunity originate from hematopoietic stem cells (HSCs), which are sub-divided into two lineages; myeloid and lymphoid progenitors. The myeloid lineage gives rise to monocytes, granulocytes and megakaryocytes, whereas the lymphoid lineage develops into B and T cells as well as NK cells respectively (Figure 1.1) (3). Nonetheless, the effectiveness of an immune response depends on the interplay between the innate and adaptive immune responses, both of which are highly regulated by elaborate mechanisms to ensure beneficial outcomes in response to foreign invaders.

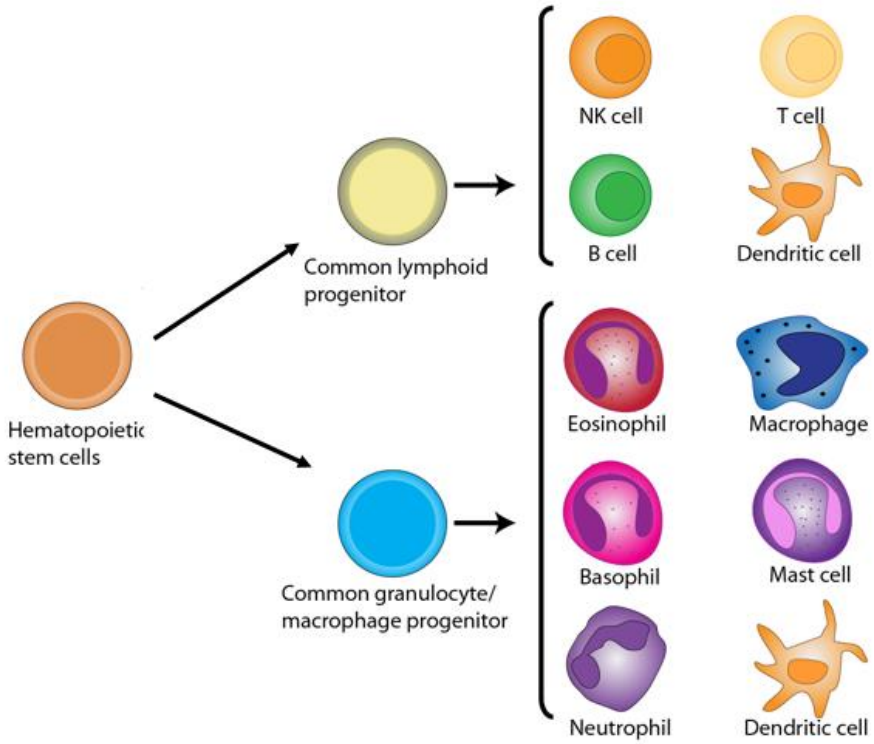


Figure 1.1 Cells of the immune system.

1.2 Innate immune recognition

Cells of the innate immune system, such as macrophages, neutrophils and dendritic cells, utilize a unique set of germline-encoded pattern recognition receptors (PRRs) to recognise diverse conserved structures on microbial species, popularly referred to as PAMPs (4). Evidence indicates that PRRs also recognise molecular signatures released from damaged endogenous cells (i.e. DAMPs), which are often associated with stress or inflammation (5). Four classes of PPR families have been identified so far namely; Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), which are typically transmembrane proteins (although some exist as cytoplasmic proteins); as well as intracellular receptors such as the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and the nucleotide oligomerization domain (NOD)-like receptors (NLRs) (5,6). Sensing of PAMPs or DAMPs by PRRs leads to the transcription of genes that regulate

immune responses, however, the expression of genes depends on the nature of antigen recognised and PRRs that are activated.

1.2.1 Toll-like Receptors

TLRs are type I transmembrane proteins defined by the presence of an extracellular N-terminal recognition domain consisting of multiple leucine-rich repeats and a C-terminal cytoplasmic Toll/Interleukin-1 receptor (TIR) domain (7,8). It is one of the best-studied PRR families responsible for detecting invading pathogens. More than 10 TLRs have been identified in both humans and mice, each of which is activated by a limited repertoire of PAMPs as well as self-antigens. Engagement of TLRs trigger signaling pathways through distinct pathways such as MyD88 and TRIF (8). Currently, five TIR domain-containing intracellular signaling proteins have been identified, however signaling through the MyD88 is the most common pathway for several TLRs, although TRIF and TRAM (Trif-related adaptor molecule) can activate a MyD88-independent pathway downstream of TLR3 or TLR4 (9). TLRs are primarily expressed on monocytes, DCs and macrophages, but can also be expressed on non-haematopoietic cells. Additionally, TLRs that recognise nucleotide and/or viral structures (i.e. TLRs 3, 7-9), are expressed in cytoplasmic compartments mainly endosomes or endoplasmic reticulum (9,10). TLR signaling activates NF-kB and MAPK pathways that influence not only inflammatory gene expression but also cell maturation and survival.

1.2.1.1 Toll-like receptor 4

TLR4 recognizes lipopolysaccharide (LPS), a component derived from the extracellular membrane (cell wall) of Gram-negative bacteria. LPS can initiate a strong immune response and serves as an early signal of bacterial infection or sepsis. Recognition of

LPS by DCs and other immune cells requires two other molecules namely myeloid differentiation factor 2 (MD2) on the cell surface and CD14. LPS initially binds to the LPS-binding protein (LBP) in serum and transfers the LPS to CD14, which exists as a soluble molecule or a glycosylphosphatidylinositol-anchored transmembrane molecule. CD14 subsequently presents bound LPS to the TLR4-MD2 complex, leading to activation of signaling components and production of inflammatory cytokines (Figure 1.2) (11,12). Several studies have highlighted the importance of TLR4 pathway in inflammation. For instance, CD14-deficient mice injected with high dose LPS produce little or no cytokines (TNF and IL-1) and showed no signs of endotoxin shock, similar to MD2 and TLR4-deficient mice (13). Furthermore, Roger et al, showed that mice lacking TLR4 and MyD88 ($TLR4^{-/-}$ and $MyD88^{-/-)}$ were resistant to *E. coli*–induced septic shock, whereas $TLR2^{-/-}$ and wild-type mice rapidly died of sepsis (14).

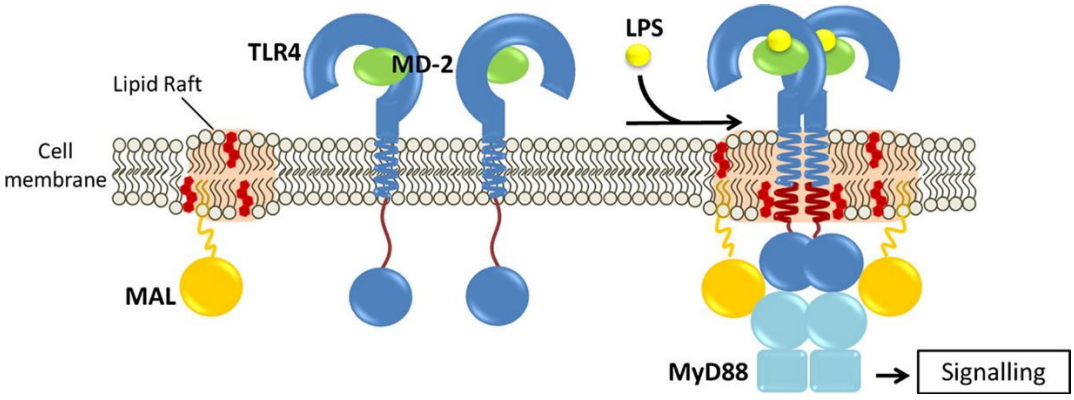


Figure 1.2. TLR4 activation in immune cells. Inactive TLR4s exists as monomers in cell membranes before stimulation with LPS. Following stimulation, monomers move into close proximity and TIR domains interact with each other to initiate downstream signaling cascades e.g. via MyD88 (15).

1.2.2 C-type lectin receptors

CLRs comprise a large family of protein receptors that are involved in the recognition and uptake of carbohydrate antigens (16). They are originally named for their ability to bind carbohydrates in a Ca^{2+}-dependent manner through conserved c-type lectin like domains (CTLDs), (Figure 1.3) and are primarily expressed on leukocytes such as DCs and macrophages, where they function as PRRs in sensing ligands from PAMPs or DAMPs (17,18). The CTLDs / CRDs of many C-type lectins can recognise a broader repertoire of ligands including lipids and proteins, due to lack of components such as the CA^{2+} binding site motif, needed for Ca^{2+}-dependent carbohydrate recognition (18,19). In mammals, C-type lectins can be secreted as soluble molecules, where they function as opsonins or growth factors; or as transmembrane proteins with functions in signal transduction and modulation of cellular and immune responses (19,20). Currently, CLRs are grouped into 17 categories on the basis of molecular structure and phylogeny (21). In terms of receptor signaling, some CLRs possess Syk-coupled, immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails, whereas others possess inhibitory motifs (ITIMs). In addition, there are CLRs without distinct ITAMs or ITIMs, such as the mannose receptor (MR) and dendritic cell–specific intracellular adhesion molecule 3–grabbing non-integrin (DC-SIGN). DCs express a variety of CLRs that play a key role in the immune response to endogenous and foreign antigens. Different studies performed by our group and others have shown the key role of CLRs, particularly MR and DC-SIGN in uptake of different antigens as well as clinically relevant allergens (20,22-26).

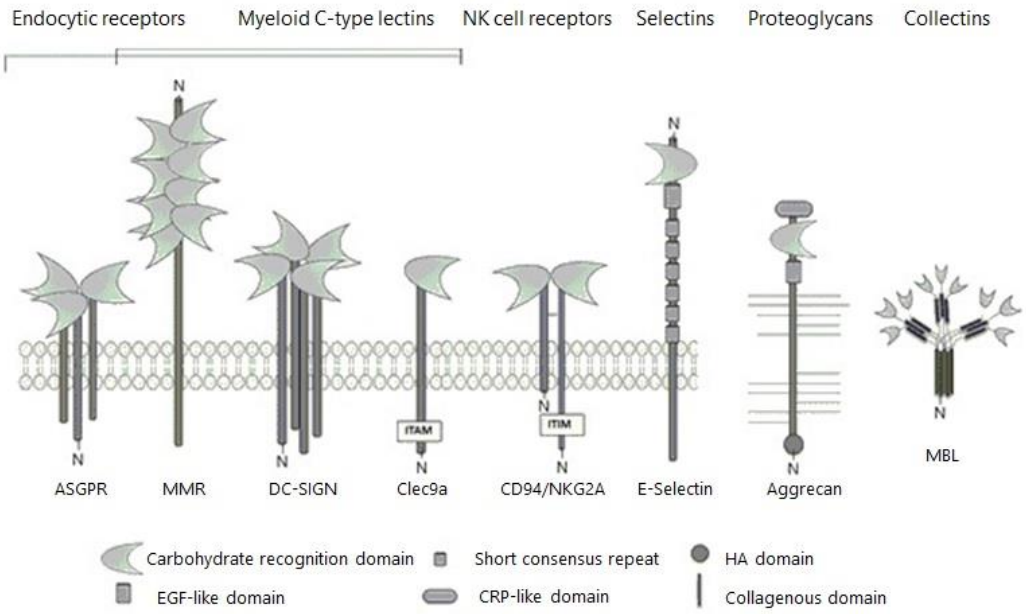


Figure 1.3. C-type lectins: Soluble or membrane-bound C-type lectins possess at least one CRD and are grouped based on structure and phylogeny. Within the cytoplasmic domains of the CLRs are conserved motifs that are important in antigen internalisation or signaling (i.e. ITIM, ITAM and Proline-rich regions) (27)

1.2.2.1 Mannose Receptor

The mannose receptor (MR) is a 175-kDa group V transmembrane CLR present on human and mouse macrophages as well as DCs (26). It consists of an extracellular, N-terminal cysteine-rich domain, a fibronectin type II-repeat domain, eight CTLDs and a short cytoplasmic tail. Soluble MR is also shed into the serum through proteolytic cleavage of transmembrane receptor. MR binds ligands with sugars terminating in mannose, fucose or GlcNAc mostly found on bacterial and fungal cell walls (24,28,29). Studies show that MR mediates ligand uptake via receptor-mediated endocytosis and phagocytosis (30), which targets ligands through the endocytic pathway for MHC II loading (31). Moreover, little is known regarding signaling cascades induced by MR, since this has been associated with the lack of signaling motifs within its cytoplasmic domain (32,33). Nonetheless MR along with CLRs such as Dectin-1 and TLR2 have

been shown to induce downstream signaling and modulate Th17 responses during anti-fungal immunity to *Candida albicans* leading to the production of IL-17 (34,35).

1.2.2.2 DC-SIGN

DC-SIGN is a 44-kDa type II transmembrane CLR expressed exclusively on DCs and some macrophage subsets and capable of binding high mannose or fucose residues in a Ca^{2+} dependent manner (36). It was discovered based on the finding that DCs bind ICAM-3 with very high affinity (37). Structurally, DC-SIGN consists of a c-terminal CRD, a hinge region with seven tandem-repeats and a short transmembrane region connected to a cytoplasmic tail. The cytoplasmic tail houses a number of internalisation and/or activation motifs. In addition to its role in antigen capture, DC-SIGN functions to regulate cell adhesion and trafficking as well as formation of a T cell synapse (38). Unlike the MR, DC-SIGN has been shown to activate intracellular signaling pathways involving Raf-kinase, ERK1 and ERK2; modulate TLR signaling and induce IL-10 production (39). This pathway of DC-SIGN signaling has been shown to be targeted by several pathogens to suppress immune responses as shown for HIV and Mycobacteria through ManLAM (40,41). Additionally, pathogens that bind DC-SIGN disrupt downstream antigen presentation, which in turn modifies the T cell response (39,42).

1.3 Adaptive immunity

The human adaptive immune system consists primarily of specialised lymphocytes that have evolved to mediate antigen-specific immunity to a great diversity of pathogens, following non-specific innate immune response. Adaptive immunity is divided into the humoral response, which is mediated by antibody-producing B cells; as well as cell-mediated responses, generated by T cells (43,44). T cells are further divided into $CD8^+$

cytotoxic T lymphocytes (CTLs) and $CD4^+$ T helper (Th) cells, which play different functional roles in the immune system (45). Both B and T cells recognise antigen using specialised receptors (BCR and TCR respectively) with hypervariable regions, capable of generating approximately 10^{13} unique antigen receptors. The highly diverse receptor repertoire is due to a process of clonal selection, where a limited number of somatic genes undergo VDJ rearrangement (46,47). Moreover, somatic hyper-mutations contributes an additional mechanism in immunoglobulin (Ig) genes to further enhance the diversity of BCR repertoire to accommodate the increasing diversity of microbial pathogens (48). The randomness of the process means that some cells are generated with specificity for self-antigens resulting in range of autoimmune and inflammatory diseases (49). B cells can recognise native antigen, which induces B cell maturation into plasma (antibody-producing) cells. T cells on the other hand, recognise antigenic peptides only within the context of MHC molecules present on antigen presenting cells.

1.4 Dendritic cells

The initiation and control of primary immune responses depends largely on dendritic cells (DCs), a class of specialised antigen presenting cells (APCs) derived from myeloid and lymphoid progenitors in the bone marrow (50). They serve as a critical link between the innate and adaptive immune systems through their ability to shape acute inflammatory responses by recognising and responding to pathogen- and/or damage-associated signals. Their characteristic ability to shape the adaptive immune response arises from their ability to process and present extracellular and endogenous antigen to naïve T cells within the context of MHC (HLA) and co-stimulatory molecules (51). In addition to T cell activation, DCs are key players in the development of immune

tolerance, the ability to discriminate between self and non-self-proteins. In the steady state, DCs are immature where they patrol peripheral tissues and maintain homeostasis.

1.4.1 Human DC subsets

Currently, comparative gene profiling studies classify human blood DCs on the basis of lineage into plasmacytoid DCs (pDCs) and two types of conventional/ myeloid DCs (mDC1 and mDC2) (52,53). Both populations are highly migratory cells that move out of blood into tissue and are present in lymphoid organs such as tonsils, spleen and lymph nodes. Plasmacytoid DCs are distinguished as $CD11c^{-}/CD123^{high}$ DCs and express an array of surface markers such as CD303 (BDCA-2) and FcέRI, known to regulate their main function, the production of type-1 interferons (IFNs). pDCs are primary innate sensors of viral infection through TLR7 and 9 (sensing ssRNA and dsDNA respectively) and produce large quantities of IFN-α/β that promote rapid induction of $CD8^{+}$ cytotoxic T lymphocytes (54).

Myeloid DC1 are present in blood and tissues at a ratio of 1:10 to mDC2 cells. They were originally characterised as $CD141^{high}$ (BDCA-3), but also express CD13/CD33 in common with mDC2 cells (55). Studies show that mDC1 have an intrinsic capacity for cross presentation using highly expressed MHC-I molecules. Additionally, they have been shown to promote Th1 cells through secretion of low quantities of IL-12. However, mDC1 cells are best known for their role in viral recognition through TLR3, TLR9 and TLR10 and production of type-III interferons (IFN-λ) (56).

The major constituents of myeloid DCs in human blood are mDC2 cells, which are distinguished based on expression of CD1c and CD11c antigens (50,57). In humans, $CD1c^{+}$ mDC2 have also been found in skin, liver, lung and intestine where they are

'resident cells' migrating directly from the blood. Myeloid DC2 cells express a variety of PRRs such as CLRs and TLRs for detection of a diverse array of microbial and glycolipid antigens. Additionally, they are potent producers of inflammatory cytokines IL-12, TNF-α and IL-10 although they produce relatively low levels of type-I and III IFNs (58,59).

Other subsets of human DCs include Langerhans cells, which are resident in the basal epidermis of the skin and express high levels of the invariant MHC-I molecule CD1a (60). They function primarily in tolerance to commensal flora in the epidermis, while maintaining the capacity for inflammatory responses to pathogens (61). Recently, single cell RNA sequencing identified subsets of DCs that clustered near pDCs and showed expression of AXL (TAM-receptor tyrosine kinase) and SIGLEC6 (sialic acid binding) proteins; however, under certain conditions, these cells can acquire phenotypic and functional properties that resemble mDCs (62).

1.4.2 DC maturation and T cell activation

Circulating DCs in peripheral blood make up approximately 1-2% of PBMCs and are difficult to isolate for *in vitro* studies. Therefore most work is carried out with DCs generated from monocytes under inflammatory conditions (i.e. treatment with cytokines such as IL-4 and GM-CSF). Although cytokine supplementation used for generation of DCs from monocytes *in vitro* have contributed to the vast understanding of DC biology, a number of limitations are associated with the use of this model system compared to conventional DCs, which are difficult to isolate in large numbers for *in vitro* culture. Firstly, cytokine concentrations used for *in vitro* studies are typically higher than expected *in vivo* and could interfere with development of physiologically normal pathways. For instance, the use of GM-CSF for *in vitro* DC differentiation is

not an essential requirement for normal DC differentiation *in vivo*. Secondly, DCs derived from monocytes, bone marrow or cord blood may differ in functional capacities thereby affecting their study or use as immunotherapeutic tools, and lastly, cytokine cultured DCs survive for only a short period of time, as these cultures do not support proliferation of progenitors so dying cells are not replaced by newly differentiating DCs (63). Regardless, cytokine generated DCs exhibit several characteristics of primary DCs, making them useful tools for study of *in vitro* immune responses.

Monocyte-derived DCs are efficient antigen presenting cells that exhibit several characteristics of primary DCs. Under steady state, circulating DCs are immature and express moderate levels of MHC-I and II as well as co-stimulatory molecules such as CD80 and CD86. They patrol the microenvironment and capture antigen via endocytosis or phagocytosis following PRR-mediated recognition and activation. Depending on the nature of the PAMP or DAMP (e.g. lipopolysaccharide), DC activation via specific PRR induces downstream recruitment of adapter proteins, signaling cascades and activation of key transcription factors, in particular NF-kB, that promote changes in gene expression. Simultaneously, DCs initiate a maturation program characterised by decreased affinity for antigen capture and up-regulation of MHC and co-stimulatory markers on the surface, enabling increased expression of processed antigen. During maturation, DCs migrate to draining lymph nodes via homing receptors such as the chemokine receptor CCR7, where they interact with naïve T cells in T cell areas.

The role of DCs in the functional characterisation of $CD4^+$ Th cells has been of great interest, particularly the mechanisms through which innate activation of DCs modulate adaptive responses. Studies indicate that optimal activation of T cells can only be achieved through the formation of an immunological synapse consisting of clusters of

DC and T cell receptor molecules. Fully activated DCs expressing MHC-peptide complexes interact with T cells through the TCR, which serves as the initial signal for T cell activation. Following MHC-TCR recognition, a second or co-stimulatory signal is subsequently provided by CD80 and C86, which binds CD28 on activated T cells. In addition to changes that occur in receptor expression, innate activation of DCs induces cytokine production, which has been shown to provide a differentiating signal to drive Th cell polarisation towards effector subsets. Th cell effectors play different roles in the immune system and are characterised by key transcription factors and cytokine signatures (Figure 1.4). For instance, IL-12 secretion by DCs is key in Th1 polarisation in both mice and humans, as Th1 development has been shown to be severely impaired in humans lacking expression of functional IL-12 or the IL-12 receptor, due to mutations in *IL12B* (64). More recently, Th9 and Th22 cells have also been identified, so-named for their predominant expression of IL-9 and IL-22, respectively (65,66). Interestingly, some studies have demonstrated that Th cells can exert a degree of plasticity following subset differentiation, whereby they remain responsive to stimuli from DCs enabling them to change their polarisation status. However, the physiological mechanisms through which these occur *in vivo* are still unknown (67,68).

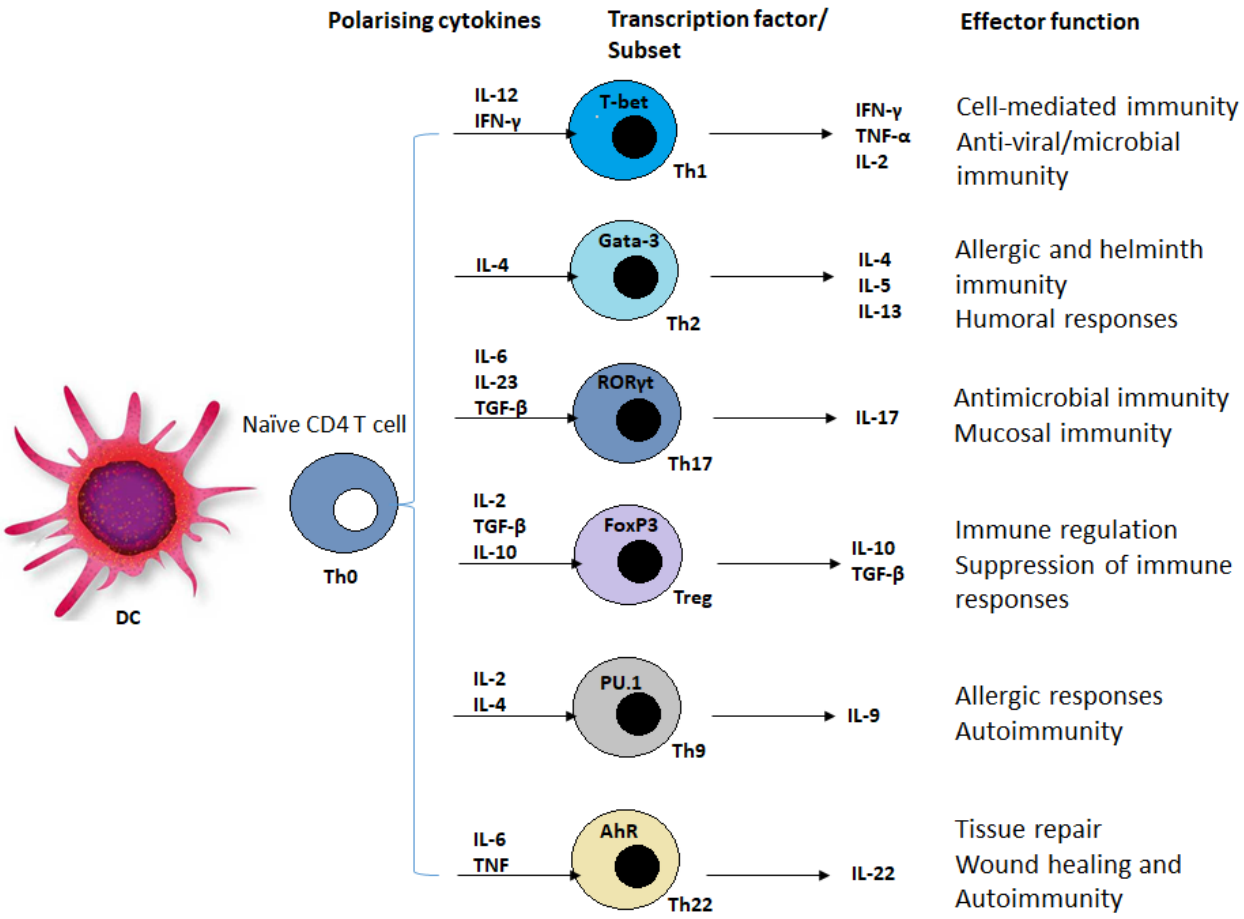


Figure 1.4. T cell polarisation by dendritic cells: Antigen specific naïve T cells expand following interaction with DCs and leads to development of distinct T cell subsets and production of signature cytokines. Adapted from (69).

1.5 Dendritic cells in allergy and immune regulation

Type I hypersensitivity or allergy is an exacerbated immune response to innocuous antigen known as allergen. Allergy currently affects millions of people worldwide and represents a huge burden in terms of morbidity and expenditure (70). In many allergic reactions, the synthesis of IgE antibodies and its effector functions play a key role in the pathophysiology of disease (71). In most of the population, exposure to common environmental allergens in air or food is often harmless, but as many as 40% of

population in certain countries could show an exaggerated immune response upon allergen encounter, a feature known as atopy (72). Such a response leads to the development of allergic sensitization and is a major risk factor for developing allergic disease (73,74). Allergic diseases typically include allergic asthma, allergic rhinitis, atopic eczema or dermatitis and food allergy, all of which are characterised as eliciting type 2 immune responses (involving Th2 cells, B cells, basophils, mast cells etc.). This involves a complex network of cytokines secreted from both the innate and adaptive immune response. Moreover, it is now clear that type 2 inflammatory responses during allergy are distinguished by high cellular plasticity that promotes adaptation to specific inflammatory milieu (75).

Initial interaction with allergens in the periphery (skin, airways or gut) is mediated by DCs, which have been shown to play a key role in the early response to allergen sensitization (20,76). Following uptake, DCs migrate to regional lymph nodes, where they present processed peptides to naïve $CD4^+$ T-cells within the context of MHC-II molecules (77). Depending on the context of antigen exposure (e.g. presence of different PAMPs) and the nature of cytokine produced, T-cells become polarised into various subsets; ; (e.g. Th1, Th2, or Th17) (78). In susceptible or atopic individuals, activated T-cells become polarised into IL-4 secreting Th2 cells (Figure 1.5) .(79)

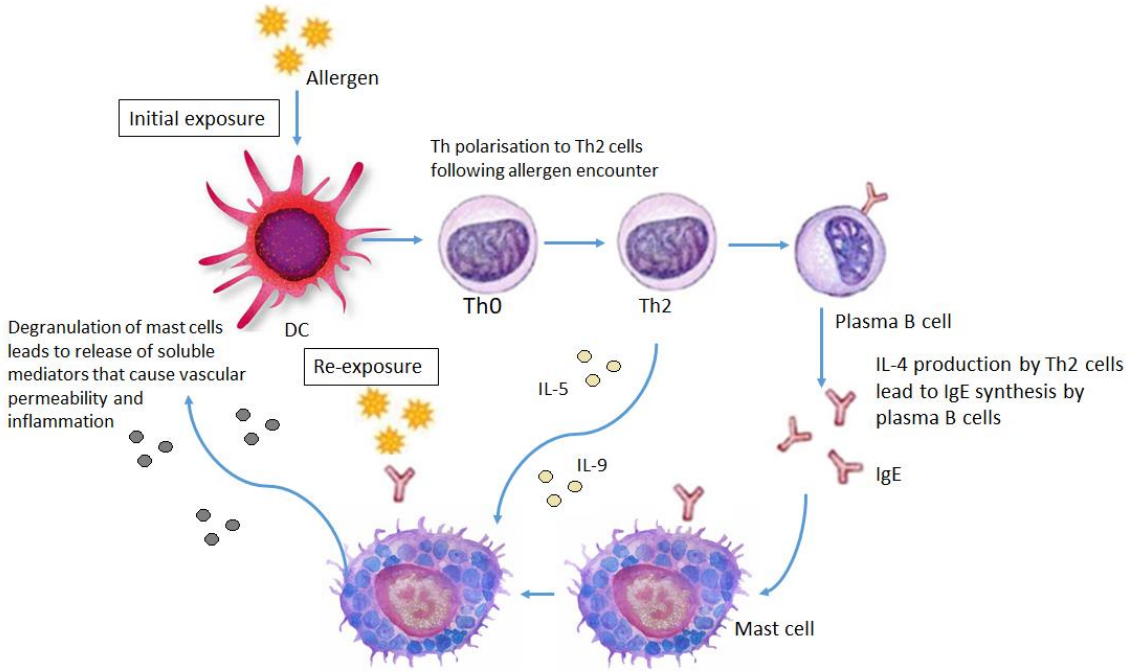


Figure 1.5: Allergic cascade. Initial exposure to innocuous allergen results in allergic sensitization and production of IgE antibodies by plasma B cells, which bind high affinity FcεRI on mast cells. Re-exposure to allergen at a later time leads to mast cell degranulation and release of soluble mediators of inflammation. Adapted from (77)

Following differentiation, Th2 cells migrate to B-cell areas within the lymph nodes, where in the presence of IL-4 and appropriate co-stimulatory signals; (CD40/CD40L and B7/CD28), B cells undergo immunoglobulin class-switching to produce allergen-specific IgE antibodies. IgE is then released into circulation where it binds high-affinity Fc receptors such as FcεRI on mast cells (77), thereby sensitizing these cells. During the effector phase, re-exposure to the allergen at a later time leads to development of the clinical features associated with allergy (vascular permeability, mucus production and release of inflammatory mediators such as prostaglandins). The importance of DCs in initiating and maintaining allergic diseases have been demonstrated. For instance, van Rijt et al, showed that depletion of lung $CD11c^+$ DCs during allergen challenge eliminates the characteristic features of asthma (80). Additionally, DCs were shown to regulate the generation of pulmonary Th2 and Th17 cells in response to intranasal

injection of protease allergens (81). Interestingly CD11b+ cDCs were identified as predominant subset of lung DCs inducing Th2-mediated immunity to HDM-specific T cells *in vitro* and *in vivo* (82). Other cells that contribute towards the development of allergic sensitization include epithelial cells (ECs), which form a tight junction barrier against allergen entry, and allergic patients have been shown to exhibit altered barrier function leading to development of allergic sensitization. Additionally, ECs induce tolerance and produce cytokines such as IL-25, IL-33 and TSLP that stimulate DCs and ILC2s, contributing to development of allergic sensitization (75,83,84).

1.5.1 Role of CLR and TLR crosstalk

Several studies have highlighted the role of CLRs on DCs, particularly MR and DC-SIGN in the recognition and uptake of allergens, which are usually (glyco-) protein in nature, and subsequent modulation of DC function. For instance, Chieppa *et al* (85) reported production of IL-10 and IL-1Ra in DCs and up-regulation of co-stimulatory molecules that led to Th2 polarisation when MR was stimulated with specific ligands *in vitro*. Similarly, Deslee et al (86) demonstrated that MR may be involved in the uptake of allergen in Der p 1-pulsed DCs from allergic individuals, and showed that pulsed DCs expressed higher levels of CD86 and secreted a cytokine profile that favoured the development of Th2 circuits. In line with these observations, previous work from our lab highlighted MR's involvement in uptake of allergens from sources including peanut (Ara h 1), dog (Can F 1), house dust mite (Der p 1, Der p 2) and cockroach (Bla g 2). More importantly, allergen recognition by MR in these studies resulted in Th2 polarisation and IgE production (24). MR was also implicated in the uptake of the major cat allergen, Fel d 1, with allergen uptake mediated by its cysteine-rich domain (22).

Uptake of allergens by DC-SIGN from airborne (Der p 1 and BG-60) as well as food sources such as peanut (Ara h 1) has been reported (25,86). Interestingly, studies by our group showed that knockdown of DC-SIGN expression favoured the development of Th2 circuits in DC-T co-cultures when DCs were stimulated with Der p 1. This was in contrast to observations with MR knockdown studies, which favoured differentiation of Th1 cells in DC-T co-cultures (24). It is worth noting that previous work from our group has demonstrated that Der p 1, which possesses a cysteine protease activity, is able to cleave DC-SIGN but not MR (73,87). This is in line with the observation that MR expression on DCs is higher in atopic individuals, whereas DC-SIGN expression is decreased in Der p 1-sensitised patients (88). It is therefore reasonable to suggest that the level of the level of DC-SIGN and MR expression on DCs play a part in determining the outcome of T cell responses to allergen exposure in different individuals. Other c-type lectins such as Dectin 1 and 2, mincle, MBL and DCIR have also been implicated in the development of inflammatory responses to allergens (74). For instance, Dectin 1 has been recently shown to recognise fungal antigens and initiate Th17 responses that promote hypersensitivity pneumonitis (89).

The crosstalk between PRRs particularly CLRs and TLRs has been demonstrated to be essential in maintaining the balance between immune tolerance and activation (40,90,91). For example, the recognition of self-antigens by CLRs alone in a state of immune inactivation induces homeostatic control, whereas the presence of danger signals (from pathogens as well as self-antigens) triggers both CLRs and TLRs and favours immune activation (91,92). On the other hand, some pathogens exploit the intracellular routing of CLRs to interfere with the crosstalk between CLRs and TLRs (90,91) as shown for MR and DC-SIGN (40,93). Induction of allergic responses by TLRs (94-96) has been reported to occur either via direct recognition of allergen or

through pathways involving crosstalk with other receptors (8). For instance, Der p 2, a major HDM allergen has been shown to induce inflammatory responses in a TLR2/MyD88-dependent manner, thereby activating airway smooth muscles (97). Moreover, allergens such as Der p 1 and Der p 2 have been shown to be glycosylated and their uptake is mediated by MR, suggesting a link between glycosylation and allergen recognition (24,98). The TLR4 ligand, LPS is a major microbial contaminant in many allergen extracts including HDM allergens, and the development of allergic sensitization has been linked to the levels of LPS contaminant in HDM.(76,94,95,99). Eisenbarth et al, (100) using mouse models of allergic sensitization, reported that low doses of LPS exposure, via a TLR4/MyD88–dependent signaling was sufficient for the induction of Th2 responses to inhaled aeroallergens, whereas high LPS doses resulted in down-regulation of Th2 responses and prevention of airway hyper-reactivity and inflammation. Collectively, these data provide evidence for synergistic interactions between CLRs and TLRs, which most likely work in concert to bias immune responses toward a Th2 phenotype after allergen exposure (Figure 1.6).

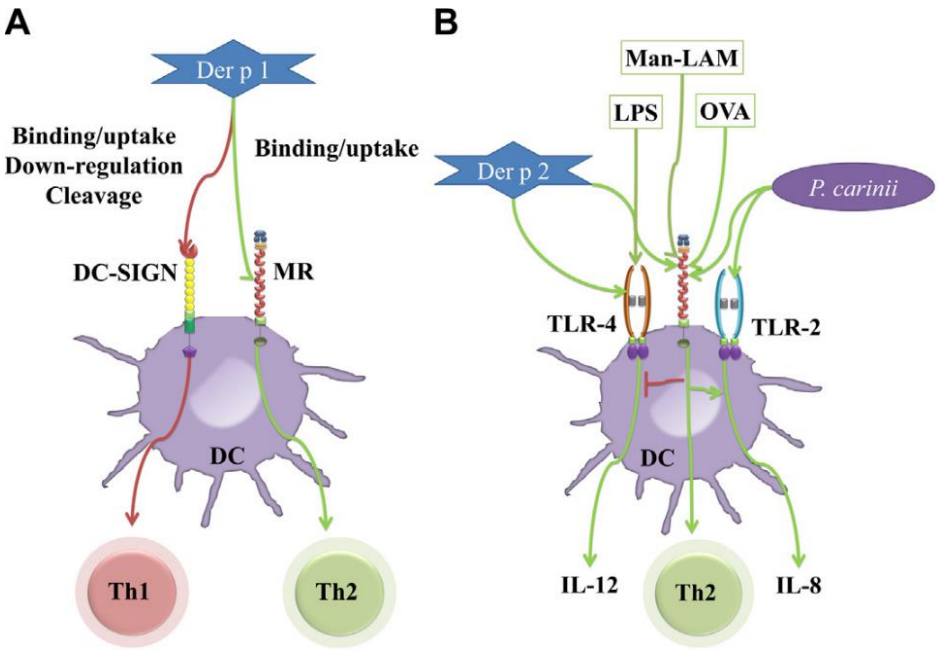


Figure 1.6: Interaction between CLRs and TLRs. A) Both MR and DC-SIGN are able to recognise Der p 1 but differ in downstream responses leading to Th polarisation. B). Interaction of MR and TLR2/4 in promoting Th2 responses following uptake of diverse allergens in the presence of LPS. Adapted from (74).

1.5.2 Role of indoleamine 2,3 dioxygenase (IDO)

A delicate balance exists between immunity and tolerance that serves to protect the host from invading pathogens while limiting damage to local tissues (101). One endogenous mechanism that contributes to this immune regulation is indoleamine 2,3-dioxygenase (IDO). IDO is a rate-limiting enzyme that mediates tryptophan (TRP) metabolism in a number of biological systems including mammalian reproduction, immune and nervous system regulation. IDO exists as two structurally similar isozymes; IDO1 (or IDO), is the most extensively studied and is ubiquitously expressed in mammalian cells, whereas IDO2 is restricted to a subset of cells and tissues such as the liver, kidneys and some APCs mainly DCs (102). The immunosuppressive effect of IDO was first demonstrated by Mellor and Munn et al, who showed that placental cells express IDO, which prevented foetal destruction by maternal T cells during pregnancy (103,104). In macrophages and DCs, IDO has been shown to influence peripheral tolerance and control regulatory processes (e.g. inflammation, anti-tumour and autoimmunity) by inhibiting effector T cells (105,106). The biological effect of IDO on T cells can be mediated by direct depletion of TRP or through immune modulatory TRP metabolites, known as kynurenines (KYN) (107). KYN has been shown to activate other regulatory molecules such as the AhR, leading to the generation of regulatory T cells (108). In T cells, TRP depletion by IDO in the microenvironment has been shown to signal activation of the stress kinase GCN2, which responds to elevations in uncharged tRNA, resulting from amino acid deficiency thereby driving Treg development.

DCs are a major source of IDO, and induce IDO production in response to a variety of signals. For instance, CTLA-4, an inhibitory marker expressed by T cells and other cell types, binds B7 on DCs to induce IFN-γ production, which acts in an autocrine or paracrine manner via Jak-STAT signaling to activate IDO expression (109,110). Local changes in TRP availability and production of KYN following IDO induction result in decreased clonal expansion and deletion of T cells. Other studies have demonstrated induction of IDO, which is independent of IFN-γ. For instance, ISS-ODN, which binds TLR9 on pDCs, has been shown to induce pulmonary IDO activity to inhibit Th2-driven experimental asthma (111). In addition, LPS and cytokines such as IL-1 and TNF have been demonstrated to act synergistically with IFN-γ to promote IDO expression *in vitro*; however, *in vivo*, additional pathways and cytokines may contribute towards IDO modulation by specific cell types, highlighting the complex nature of IDO regulation and control (112,113).

Several studies have reported the role of IDO in allergic responses. However, initial reports were contradictory as to whether IDO was a proinflammatory or tolerogenic enzyme in allergy. In addition, most studies were carried out with experimental mouse models (i.e. knockouts), which may behave differently from wild-type mice. For instance, Xu et al showed that lack of IDO did not impair development of immune tolerance, but impaired Th2 responses in the lung induced by repeated allergen exposure (114). In addition, they showed that antigen-induced airway hyper-responsiveness (AHR), cytokine responses and airway eosinophilia were also significantly lower in the $IDO^{-/-}$ mice in the Th2 model. However, it is now clear that induction of the TRP degradation pathway by IDO reduces allergic inflammation. Allergens may also subvert the inhibitory role of IDO on T cell responses through modulation of DC function. For instance, exposure to Der p 1 allergen suppressed IDO

activity in DCs from HDM-sensitive asthmatic patients and resulted in induction of Th2 responses (115). Similar studies by our group have highlighted the MR involvement in modulating IDO activity in DCs subsequent to allergen exposure (24). In these studies, up-regulation of IDO activity was observed in Der p 1 treated MR-deficient DCs, which resulted in a reversal of Th2 immune responses towards Th1. This was seen in the presence of small quantities of LPS. More recently, our group showed the molecular basis of IDO regulation through MR, and highlighted involvement of molecules such as AhR and RelB (116). Data from these studies suggest that MR engagement with allergen may be crucial in regulating immune responses via IDO within the context of allergic inflammation; however, further work is needed to determine signaling pathways involved in the induction of IDO activity via MR and whether these pathways involve crosstalk with other CLRs or TLRs.

1.5.3 Role of PPARγ

The peroxisome proliferator activated receptor gamma (PPARγ) belongs to the nuclear hormone receptor (NR) superfamily of ligand-inducible transcription factors (which includes PPARα and PPARβ/δ), with a variety of key roles in glucose metabolism, fatty acid synthesis, adipogenesis and immune regulation (117,118). It is expressed in a variety of immune cells including monocytes and macrophages, lymphocytes, platelets and DCs (119). Following ligand stimulation, PPARγ heterodimerizes with retinoid X receptors (RXRs) and specific co-activators to bind PPARγ response elements (PPRE) located in promoters of target genes, initiating the transcription of genes such as those involved in inflammation, metabolism and adipocyte differentiation (118-120) (Figure 1.7).

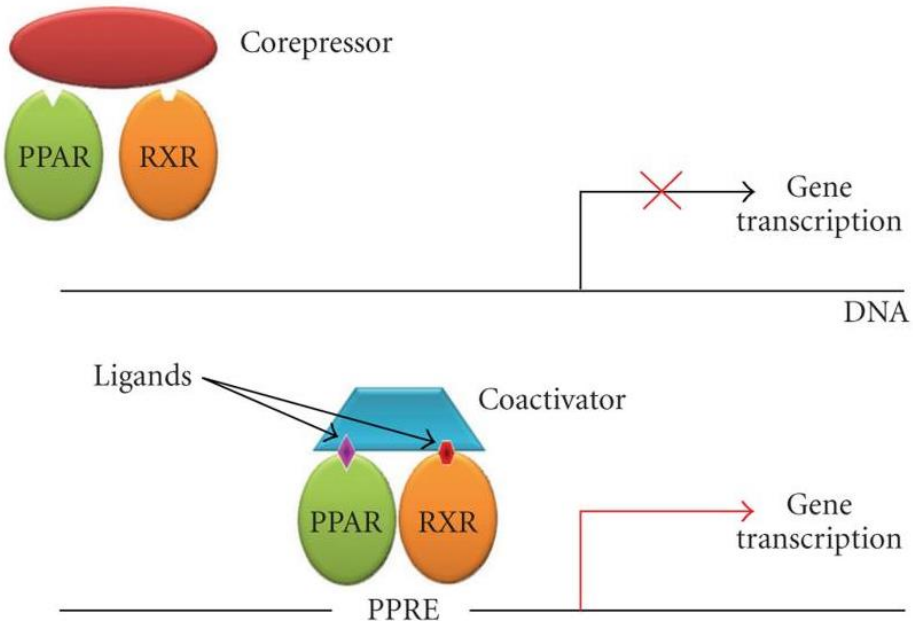


Figure 1.7: Mechanism of PPARγ activation. Ligand binding to the PPAR/RXR complex induces a conformational change that enables release of a corepressor and binding of a coactivator resulting in an increased affinity for the specific PPAR response element (PPRE), which modulates gene transcription. Source (120).

Several natural and synthetic ligands for PPARγ have been identified including prostaglandins (such as PGD_2 and 15d-PGJ_2), eicosanoids (e.g. arachidonic acid) and thiazolidinediones (e.g. rosiglitazone and pioglitazone) (121). In immune cells, PPARγ activation has been shown to exert potent anti-inflammatory effects by negatively interfering with pro-inflammatory signaling particularly NF-κB (119,122). Although PPARγ modulates metabolism and immune functions of monocytes/ macrophages (123), it is not essential for their differentiation. In DCs, PPAR affects several aspects of DC biology including differentiation, maturation and cytokine secretion among others (124-127). For instance, PPARγ activation in DC suppresses production of IL-12 needed for Th1 polarisation (128) and reduces CCL5 and CXCL10 chemokine expression required for Th1 recruitment (125). Additionally PPARγ activation was

shown to lead to downregulation of CCR7 on DCs, which further decreased the ability of DCs to migrate to lymph nodes in response to CCL19 and CCL21 chemo attractants (127,129,130). Positive regulation of PPARγ activation has also been demonstrated, particularly in promoting mannose receptor-mediated endocytosis of ovalbumin (OVA) antigen in mouse DCs in response to PPARγ agonists (131). Additionally, PPARγ was associated with increased expression of the scavenger receptor CD36, which promotes the uptake of apoptotic cells (123,125). Due to their role in lipid metabolism, PPARγ regulates the expression of CD1 proteins (CD1a, b, c and e), which are MHC-I-like molecules required for lipid antigen presentation to T cells (132,133). The anti-inflammatory role of PPARγ has also been reported in allergic airway inflammation. For instance, Khare et al demonstrated that CD11c-specific PPARγ deficient mice showed impaired induction of tolerance in the airways in response to inhaled antigen. Additionally, they showed that the PPARγ deficient mice were impaired in their ability to promote expression of Foxp3 in $CD4^+$ T cells (134). Likewise, Nobs et al recently showed that expression of PPARγ in DC and T cells controls the development of type-2 immunity in mice sensitized with HDM as model for allergic inflammation (135). Taken together, these studies highlight the regulatory role of PPARγ on DC function and downstream immune responses.

1.6 MicroRNAs

Protein coding genes comprise approximately 2% of the entire human genome, accounting for about 25,000 genes (136). The rest (~98.5%) consists of non-protein coding DNA sequences (previously referred to as 'junk' DNA), most of which are now considered to be transcribed into RNA at low levels (137). The human genome also encodes several thousands of long non-protein coding RNAs (lncRNAs) of more than 250 nucleotides in length, expressed at lower abundance compared to messenger RNAs (mRNAs) and functionally distinct from small RNA species (138). Over the years, multiple classes of small RNAs have been discovered in eukaryotes, which have primarily evolved to suppress expression of unwanted genetic material. These short non-coding RNAs (ncRNA) are defined based on length (ranging from 18-25 nucleotides) as well as association with the Argonaute family of proteins (AGO). These typically include microRNA (miRNA), piwi-interacting RNA (piRNA), short-interfering RNA (siRNA) and small nucleolar RNA (snoRNA), (138-142). Others such as the transfer RNA (tRNA) and ribosomal RNA (rRNA) are small ncRNA molecules that have key roles in protein synthesis. MicroRNAs were first discovered by Lee et al in 1993, working in the labs of Victor Ambros and Gary Ruvkun, (143).They showed that the *lin-4* gene in the nematode parasite *Caenorhabditis elegans,* did not encode protein, but rather transcribed two small RNAs complementary to the 3' UTR of lin-14 mRNA that controls developmental events in the parasite. Currently, hundreds of miRNAs have been identified in animals, plants and viruses, all of which are classified in an online database, miRBase (http://www.mirbase.org/) (142). MicroRNAs do not normally require perfect complementarity with their targets and each is thus predicted to bind several hundreds of targets, making microRNAs complex regulators of gene expression. Additionally, different cells express different microRNA signatures

therefore, different combinations of microRNAs may be important within different contexts.

1.6.1 MicroRNA biogenesis

In mammals, nearly 40% of all miRNAs are processed from introns of protein-coding genes (also known as 'intragenic/intronic miRNA'). The remaining (~50%) are located in intergenic spaces (previously unannotated regions of the human genome). Intergenic microRNAs are independently expressed and possess their own enhancer and promoter elements. Intronic miRNAs in contrast, can share a common transcription machinery with the host gene and may thus show concordant expression with the host gene (144). However, there have been several reports where expression of intronic miRNA and their respective host gene are not correlative hence the likelihood intronic miRNAs may be transcribed from distinct transcription units having their own promoters independent of their host genes. For instance, intronic miR-128-2, embedded in the *ARPP21* gene, was shown to be significantly overexpressed in patients with acute lymphoblastic leukaemia (ALL), but not in acute myeloid leukaemia (145). Furthermore, they showed that increased levels of miR-128-2 was not due to increased gene copy number (*ARPP21)* or promoter hypomethylation, indicating the presence of an independent miR-128-2 promoter (145). Mechanisms that could influence discordant expression between miRNA and host gene include post-transcriptional miRNA regulation, unequal transcript stabilities or independent expression of intronic miRNAs.

The canonical pathway of miRNA biogenesis is a multi-step process, beginning in the nucleus and then terminating in the cytoplasm (Figure 1.8). In general, majority of miRNAs are transcribed by RNA polymerase II (although a small subset are transcribed by RNA polymerase III), which generates a long primary miRNA (pri-miRNA)

transcript consisting of a number of hairpin structures, each possessing a stem and terminal loop (140,141,146). Pri-miRNAs are typically capped, spliced and polyadenylated and in most cases, give rise to more than one functional miRNA (147). The pri-miRNA is then cleaved by a microprocessor unit composed of Drosha, a ~160 kDa RNA pol III enzyme and its essential cofactor the DiGeorge syndrome critical region gene 8 (DGCR8) to generate a ~60-70 nucleotide hairpin precursor termed pre-miRNA (148-151). The microprocessor unit is also made up of other auxiliary cofactors, which are believed to promote the specificity of Drosha cleavage (152). The precursor hairpin is subsequently shuttled out of the nucleus into the cytoplasm by Exportin-5, in a GTP-dependent manner. Exportin-5 a member of the Ran-dependent nuclear transport receptor family (153-155). Once in the cytoplasm, another RNA pol III endonuclease, Dicer, processes the precursor into ~24 nucleotide, double-stranded RNA (dsRNA) duplex. Dicer is found in nearly all eukaryotes and is a highly conserved protein, which in humans, interacts with two other closely-related proteins namely TRBP (TAR RNA-binding protein) and PACT to enhance silencing activity. PACT and TRBP are double stranded RNA binding proteins that form a complex with Dicer to help facilitate its cleavage of the pre-miRNA strand (156,157). Dicer produces a duplex RNA of approximately 22 nucleotides, with a two nucleotide overhang at its 3' end (158,159). One strand of the duplex is selected as the mature miRNA strand, whereas the complementary strand is rapidly degraded (159-161).

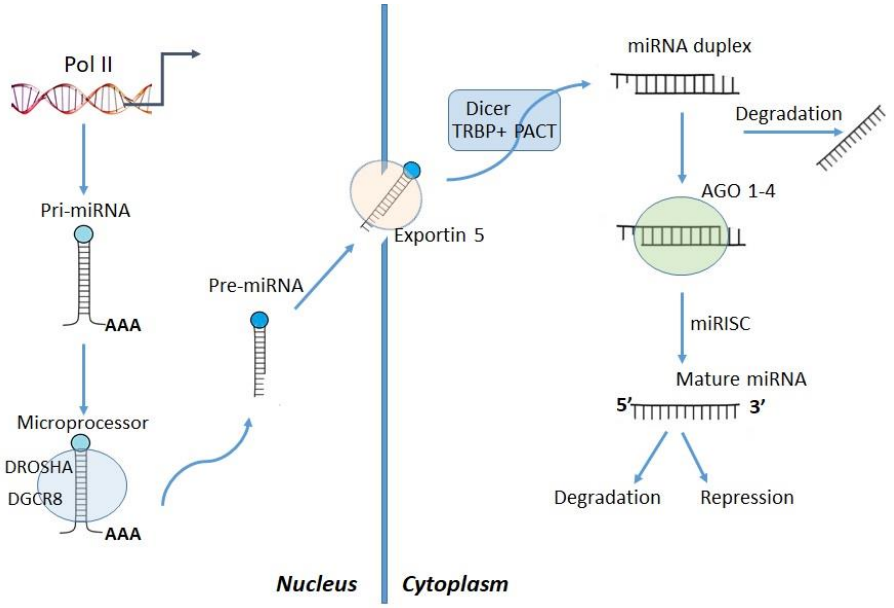


Figure 1.8: Schematic representation of miRNA biogenesis pathway: RNA pol II transcribes pri-miRNA hairpin structure, which is cleaved by Drosha/DGCR8 to generate the precursor miRNA. Pre-miRNA is then exported into the cytoplasm where further enzymatic cleavage results in generation of a functional strand. The mature miRNA is loaded with Argonaute proteins into the silencing complex (miRISC), where it guides RISC to repress mRNA through cleavage or degradation. Adapted from (147).

Strand selection has been shown to be dependent on the relative thermodynamic stability between the two ends of the duplex; i.e. the strand with less stable nucleotides terminating at the 5' end is selected as mature miRNAs (162,163). Mature miRNAs originating from the 5′ and 3′ strands of pre-miRNA are subsequently referred to as the 5p and 3p miRNAs respectively. In a process that is not clearly understood, Dicer promotes incorporation of the mature miRNA sequence into Argonaute protein complexes to form the miRNA-induced silencing complex (miRISC) (164-167). Four closely related Argonaute proteins have been identified in humans (AGO1-4) and each interacts with deadenylation factors and translational machinery to induce mRNA degradation and translational repression. The mature miRNA consequently confers

mRNA target specificity to the silencing complex. Interestingly, there have been reports of a dicer-independent mechanism of miRNA biogenesis that requires AGO2-dependent cleavage to form the mature miRNA strand. This has been shown for miR-451, which is not cleaved by dicer due to its short length, but rather incorporated directly into AGO2 (168-171). The non-canonical miRNA biogenesis pathway is a recently-discovered pathway for a subset of miRNAs known as mirtrons, which resemble canonical mRNAs functionally and structurally but follow unique maturation pathways that excludes steps of the canonical pathway.

1.6.2 Mechanism of miRNA-mediated silencing

Historically, miRNA-mediated mRNA silencing was thought to result in reduced transcription, without changes to the levels of mRNA (172). For instance, *lin-4*, one of the best studied miRNA in *C. elegans*, only inhibits translation of the LIN-14 protein but does not alter the transcription, polyadenylation or abundance of *lin-14* mRNA (166,173). Accumulating evidence however, suggests that miRNAs can mediate silencing through two distinct mechanisms; mRNA repression (slicer-independent) or degradation (slicer-dependent); however, mechanisms that pre-determine whether mRNA silencing occurs through either of these two mechanisms are still unclear. Slicer activity refers to the endonuclease cleavage of target genes by AGO proteins (such as AGO2), which requires extensive complementarity between mRNA and miRNA. This interaction is a crucial step in determining the specificity of miRNA targeting (174-177). To initiate silencing, miRNAs must initially recognise and bind complementary base-pairs in the 3'untranslated regions (UTRs) of their target mRNAs (178,179). Although binding to the 3'UTR of the target mRNA is the primary mechanism of action for most microRNAs, interactions between microRNAs and the 5' UTR of mRNAs

have also been reported. The 'seed sequence', which comprise nucleotides 2-7 at the 5' end of the miRNA, is important in determining target recognition and silencing. The more extensive the base pairing is at the seed region, the stronger the target mRNA response to miRNA silencing (178-180). It has been suggested that partial complementarity (i.e. binding within the seed sequence) is sufficient to induce miRISC activity, leading to a reduction in protein synthesis i.e. mRNA repression. Additionally, mismatches within seed region have been shown to occur during target recognition, but these can be compensated for by base pairing between the 3' end of the miRNA and 3' UTR of target (Figure 1.9).

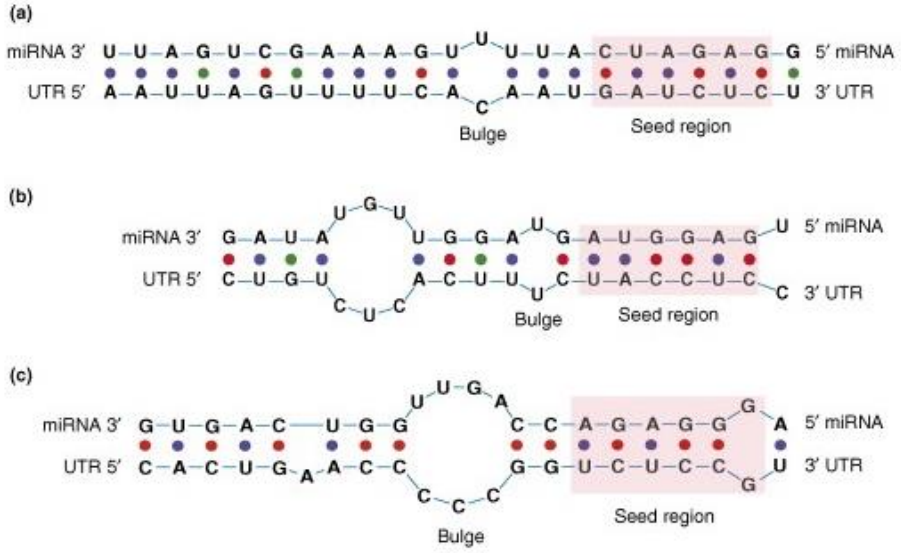


Figure 1.9. Types of target duplexes. (a) Sites with perfect complementarity at both 5′ and 3′ ends of the miRNA. **(b)** Seed regions have partial 5′ complementarity to miRNA but poor 3′ complementarity. **(c)** Compensatory sites have a mismatch in 5′ seed region but compensate through excellent complementarity at the 3′ end. Adapted from (181).

Conversely, miRNAs that possess imperfect complementarity to the 3'UTR of target mRNAs have been shown to regulate gene expression by targeting mRNA through decay pathways, where target genes are deadenylated (removal of poly-A tails),

decapped and ultimately degraded (177,182,183). Deadenylation occurs through a CAF1-CCR4-NOT deadenylase complex, which is recruited by GW182, a protein required for miRNA repression. GW182 associates with AGO proteins and the complex interacts with PABPC, resulting in mRNA deadenylation. The target mRNA is decapped by enzymes such as DCP2, which associates with specific activators for complete removal of the 5' cap of the mRNA. Subsequently, 5'-3' exonuclease enzymes degrade the deadenylated and decapped mRNA (177). Therefore, depending on the specific miRNA and the degree of complementarity to target sequences, miRISC can employ a variety of mechanisms to regulate target gene expression.

1.6.3 MicroRNA target prediction

Several methods relying on bioinformatics approaches and websites have been recently developed to enable quick identification of putative miRNA targets (Table 1). In most cases, these algorithms identify mRNA targets based on complementarity between 3' UTR of target gene and miRNA (184,185). One class of such target sites have perfect complementarity with mRNA at both ends (5'- 3') of the miRNA, whereas a second class possesses imperfect complementarity to the 'seed region' of the miRNA as previously stated (Figure 1.9). Furthermore, considering a single transcript can have several target sites for multiple miRNAs; this adds a level of complexity to regulatory mechanisms employed by miRNAs and makes it challenging to identify targets.

Table 1.1: Algorithms for miRNA target prediction. Adapted from (186)

Algorithm	Regions	Mechanism of prediction	Web server/URL
miRanda	3′ UTR	sequence complementarity, evolutionary conservation, free energy	http://www.microrna.org
mirSVR	No restriction	Ranks target sites with a supervised vector regression (SVR) model for secondary structure, accessibility and conservation.	http://www.microrna.org
PicTar	3′ UTR	Sequence complementarity, pairing stability	http://pictar.mdc-berlin.de/
TargetScan	3′ UTR, open reading frames	Sequence complementarity, evolutionary conservation	http://www.targetscan.org/
RNA22	No restriction	Uses patterns discovered from known mature miRNAs for predicting candidate miRNA-target sites in a sequence.	http://cm.jefferson.edu/rna22v1.0/
PITA	3′ UTR	Predicts targets using model that computes difference between free energy gained from formation of miRNA-target duplex and energetic cost of un-pairing the target to make it accessible to the miRNA.	http://genie.weizmann.ac.il/pubs/mir07/
DIANA-microT	3′ UTR and CDS	Sequence complementarity, evolutionary conservation, free energy, site accessibility, target-site abundance.	http://diana.cslab.ece.ntua.gr/microT/

The criteria used by these algorithms have a significant influence on outputs and binding patterns of miRNA for predicted targets; however, variations in criteria used may result in discrepancies and generation of false-negatives and positives as have been reported (187,188). In addition, algorithms that require strict pairing within seed regions to 3' UTRs, (demonstrated by majority of softwares) may miss genuine miRNA targets (186,189). For instance, Alexiou et al, compared the effectiveness of eight

commonly used prediction programs for mouse and human genomes using experimentally validated targets, and showed that selected algorithms exhibited approximately 50% precision and 12% sensitivity to validated targets (190). Despite these limitations, prediction softwares are widely accepted for identifying miRNA targets in a variety of species. Experimental validation of miRNA predicted targets can subsequently be achieved through the construction of luciferase reporter assays. This approach involves cloning the 3' UTR of the potential mRNA target into a luciferase reporter, which is then transfected into cells expressing the miRNA of interest. Luciferase activity is then measured, where decreased activity signifies mRNA target repression (191). High throughput methods for identification of miRNA targets include the use of microarrays, proteomics and biochemical methods such as CLIP-Seq. Microarrays (via transfection experiments) are able to detect mRNA degradation or dysregulation in disease and have demonstrated that several mRNAs are downregulated by overexpressing miRNA (192,193). Microarrays can be used in conjunction with bioinformatics algorithms to identify mRNAs with complementary pairing to miRNA (184). Staple isotope labelling with amino acids (SILAC) are high-throughput proteomic approaches used to assess changes in protein expression in response to miRNA levels following manipulation with inhibitors or mimics (194). CLIP-Seq (crosslinking immunoprecipitation) followed by high-throughput sequencing (first described by Chi et al.) is a method through which mRNA undergoing miRNA regulation by RISC components (i.e. Argonaute proteins) are immunoprecipitated. The target mRNA in the immunoprecipitated lysate is then subjected to high-throughput sequencing (195). Further modifications have been added to this method, whereby crosslinking of RNA to RNA binding proteins prior to IP, allows deep sequencing of transcripts bound in real time.

1.7 MicroRNAs and immunity

Over the past decade, several studies have demonstrated the pivotal role miRNAs play in regulating both the innate and adaptive immune responses, particularly in influencing development and differentiation of functional immune cell phenotypes, maintenance of homeostasis, regulating the outcome of allergic and other inflammatory diseases as well as cancer. More recently, advancements in profiling and/or sequencing methods have enabled identification and contribution of specific miRNAs to the regulation of distinct immune cells and their responses.

1.7.1 Regulation of innate and adaptive immunity

Studies show that knocking out Dicer, which is essential for biogenesis of miRNAs is lethal to the development of mice, as dicer knockout embryos are depleted entirely of HSCs (196). Additionally, HSC homeostasis and lineage commitment is modulated by miRNAs including miR-196 and miR-10 located in HOX loci that encodes genes vital for HSC homeostasis (197,198). The significance of miRNAs in the development and function of innate immune cells is well documented (Figure 1.10). For instance, *in vitro* studies have demonstrated a role for miR-223 in promoting the development of granulocytes. The transcription factor, C/EBPα was shown to drive expression of miR-233 and mice lacking miR-233 developed inflammatory lung lesions following endotoxin challenge as a result of hyerfunctional neutrophils (199,200). Moreover, neutrophils lacking miR-223 as opposed to wild type cells, were shown to undergo increased oxidative burst to efficiently kill *Candida albicans* (199). MicroRNAs such as the miR-17~92 cluster, miR-20a and miR-106a are highly expressed in early myeloid progenitors, but downregulated during monocyte/macrophage differentiation from human $CD34^+$ cells. All three miRNAs target Runx1, which promotes transcription of

macrophage colony stimulating factor (M-CSF), and their downregulation enables increased expression of Runx1 (201,202). In natural killer cells, forced expression of miR-181 inhibits Notch signaling by targeting the Nemo-like kinase (NLK), which drives early NK cell development. In addition, the let-7 miRNA family has been demonstrated to regulate iNKT cell function, particularly secretion of IFN-γ and IL-17 by modulating expression of the promyelocytic leukaemia zinc finger (PLZF) transcription factor (203). MiRNAs that influence development of other innate immune cells such as basophils, eosinophils and mast cells are not well known, although miR-221/222 have been shown to be upregulated in mast cells and control proliferation of these cells (204).

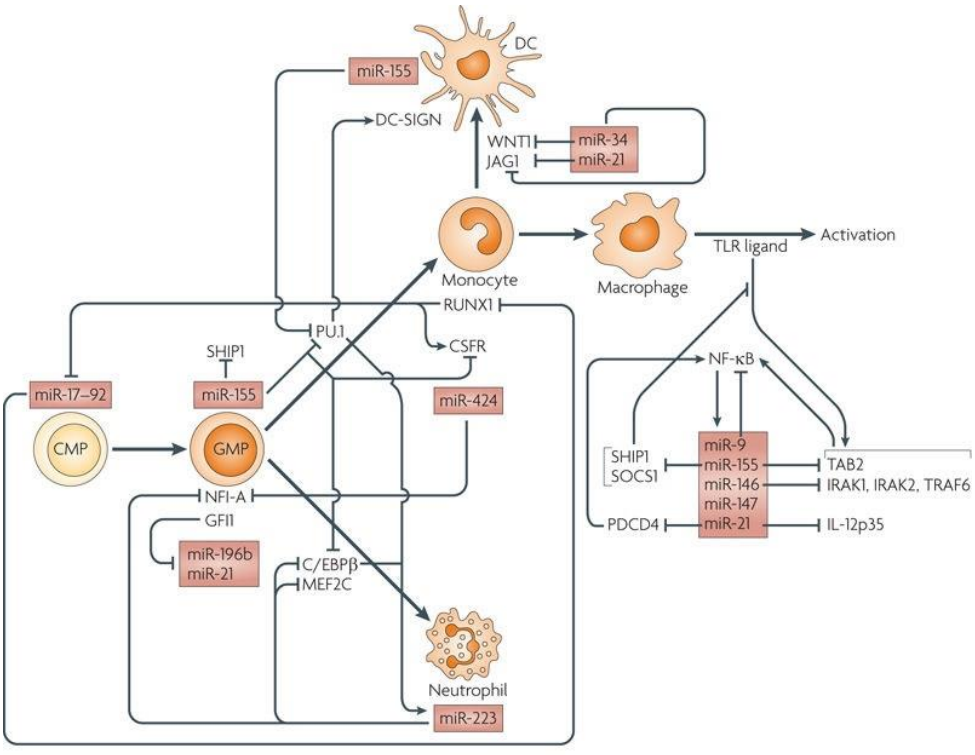


Figure 1.10 MicroRNA-mediated regulation of myeloid cell development and function. The generation of different myeloid cell types is regulated by the interactions between miRNAs and relevant transcription factors (205).

The adaptive immune system mainly comprises B and T lymphocytes, and studies show that miRNAs can affect various aspects of their development and function. For instance, miR-181a is shown to be highly expressed in thymocytes and plays a crucial role in T cell differentiation by promoting sensitivity of TCR signaling through inhibition of several protein tyrosine phosphatases, such as SHP-2, PTPN22m and DUSP6, involved in signal transduction events (206). Interestingly, miR-181a is also a positive regulator of B lymphocyte differentiation, demonstrated by the fact that ectopic expression of miR-181a in mouse HSC resulted in an increase in B cell population. Another miRNA, miR-150 has been implicated in B and T cell maturation; however, its expression is downregulated during activation of $CD4^+$ and $CD8^+$ T cells by ectopic expression of Foxp3, a characteristic transcription factor for regulatory T cell (Treg) differentiation. In studies by Zhou et al, overexpression of miR-150 in haematopoietic progenitors resulted in a significantly reduced level of mature B cells, although no changes were observed in the levels of circulating T cells. Further studies demonstrated that miR-150 exerted its effect by targeting the transcription factor c-Myb, required for early B cell transitioning from pro-B to pre-B cells. MicroRNAs have also been demonstrated to promote the function of B and T cells. For example, mice lacking miR-155 failed to develop protective immune responses to bacteria following immunization. Also, lack of miR-155 has been associated with impaired Th1 and Th17 subset differentiation, but promote Th2 responses and cytokine production (207,208). Changes following knockout of miR-155 in these mice were attributed to the effect of miR-155 on several target genes, including T cell-specific transcription factors activated during development such as c-Maf. In contrast to these, miR-182 was shown to be is upregulated in Th1, Th2 and Th17 cells following stimulation of TCR with IL-2; however, this upregulation was shown to be inhibited by STAT5, which suggests that

IL-2 may be important for late phase clonal expansion of T cell subsets (209). In B cells, mice lacking miR-155 were shown to have impaired antibody production and class switching due to defect in germinal centre formation as compared to mice ectopically expressing miR-155

1.7.2 Regulation of DC function

MicroRNAs fine-tune several aspects of DC biology including development and function. For instance, Notch/Wnt signaling is crucial in the development of several immune cell types including DCs and previous studies indicate that miR-34a and miR-21 bind the 3'UTR of *WNT1* and *JAG1* (encodes the Notch ligand jagged-1) to impair Notch signaling and disrupt monocyte-derived DC (MoDC) differentiation (210). Additionally, Lu et al demonstrated that miR-221, which is highly induced in immature MoDCs, targets $p27^{kip}$ (inhibitor of cell cycle), to enable progression through cell cycle, as miR-221 inhibition was shown to induce apoptosis in MoDCs (211). In DCs as well as monocytes/macrophages, miRNAs have been shown to play a key role in maturation and function of these cells primarily through the regulation of TLR signaling. Signaling through TLRs starts with the recruitment of adaptor molecules, followed by a series of cascade events that lead to activation of transcription factors such as AP1 and NF-kB, thereby inducing gene expression (10). Recently, miR-146a, miR-155 and miR-21, three well-studied miRNAs, were shown to be highly expressed in immune cells such as monocytes (212), DCs and macrophages (213,214), after LPS treatment and/or NF-kB activation, and function to regulate inflammation. For instance, miR-146a was shown to target IRAK-1 and TRAF6 adaptor proteins and also downregulate IL-1 and IL-8 production by suppressing NF-kB activation (215). In DCs differentiated from monocytes *in vitro*, upregulation of miR-155 distinctly characterises DC maturation

and function. For example, miR-155 was shown to be highly induced in human and mouse DCs following treatment with various TLR ligands and DCs lacking miR-155 were unable to efficiently activate antigen-specific T-cells due to impaired antigen presentation, reduced co-stimulation (CD40 and CD86) and cytokine production (IL-12p40, IL-12p35 and TNF-α) (207,216). Interestingly, miR-155 also magnifies LPS-induced signaling in a feed-forward loop by downregulating the SH2-containing inositol phosphatase (SHIP1) (217) and downregulation of miR-155 in DCs also down-regulates suppressor of cytokine signaling 1 (SOCS1) (211). SHIP is a negative regulator of immune responses (218) and macrophages lacking SHIP for instance, were shown to increase cytokine production in response to TLR4 stimulation (219,220). McCoy et al, however, showed that IL-10 signaling is able to downregulate miR-155 through STAT3 to promote SHIP1 and SOCS1 expression (221). In DCs, miR-142 was shown to bind the 3'UTR of IL-6, and repression of miR-142-3p in bone-marrow derived DCs resulted in enhanced production of IL-6 in immature and LPS activated DCs (222). Like miR-146a, miR-21 is up regulated in monocytes following LPS stimulation but negatively regulates TLR signaling (223,224). In addition to its role in DC differentiation, miR-21 also contributes to DC tolerance induction by inhibiting pro-inflammatory cytokine production. Several other miRNAs have been identified to regulate the development and function of DCs including the miR-148 family, let-7i and miR-221

1.8 Role of miR-511 in immunity

In the human genome, *MRC1*, which encodes the mannose receptor (MR) and houses miR-511 in its fifth intron, undergoes duplication to enable expression of two hsa-miR-511 coding units within chromosome 10, while in mice, a single mmu-miR-511 coding

unit is present on chromosome 2.2 (225). In both species, the *MRC1/miR-511* loci exhibits a high degree of conservation regarding intron-exon organisation. Also, bioinformatics analysis demonstrated the existence of conserved binding sites for transcription factors such as PU.1, a master regulator of myeloid development previously shown to bind the *Mrc1* promoter in mice and rats (226).

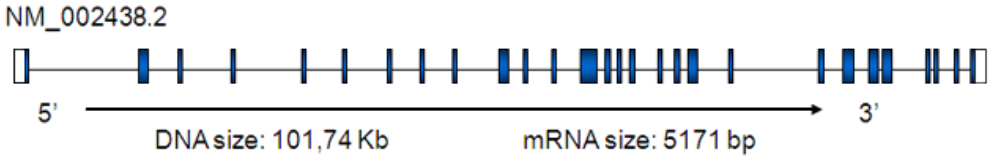


Figure 1.11. Exon-intron structure of human MRC1 gene. Blue boxes represent exons, while white boxes correspond to introns. MRC1 is a functional gene of 101.74 kb comprising 30 exons and 29 introns. Source: http://AtlasGeneticsOncology.org

The role of miR-511 in immunity has been demonstrated particularly in regulating human cancers (227-230). For instance, miR-511 was shown to inhibit growth and metastasis of human hepatocellular cancer cells by targeting PIK3R3 (231). Recently, Curtale et al demonstrated that glucocorticoids such as dexamethasone (Dex) are able to downregulate TLR4 induced inflammation by targeting miR-511-5p. Furthermore they showed that miR-511-5p is the active strand of miR-511 in human monocytes, and mediates anti-inflammatory responses induced by Dex and other stimuli such as TGF-β (225). This is in contrast to that previously reported for murine macrophages, where miR-511-3p is the active strand (232). Glucocorticoids were also reported to induce miR-511 expression in mice and inhibits endotoxemia and experimental hepatitis by downregulating TNFR1 (233).

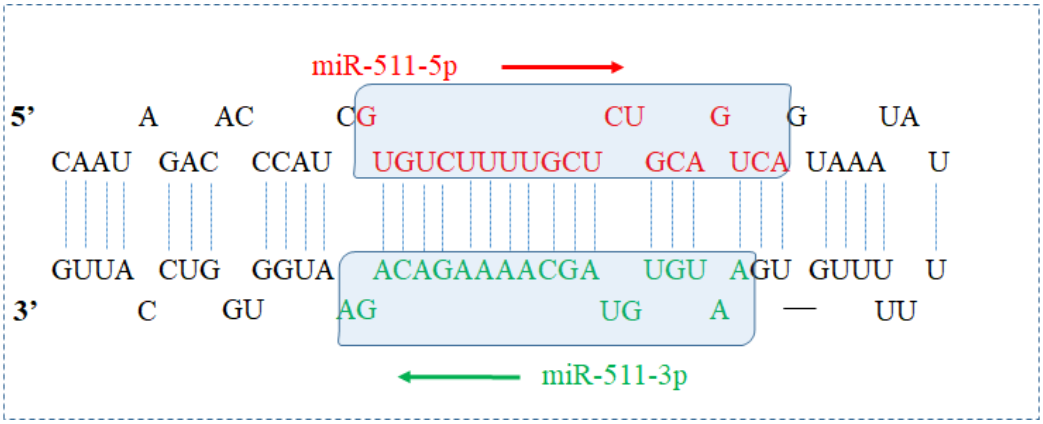


Figure 1.12: Stem loop structure of miR-511 in humans. Adapted from miRBase: http://www.mirbase.org

Previously, Squadrito et al showed bioactivity of miR-511-3p in *Mrc1*$^{+}$ alternatively activated and tumour associated macrophages (AAM and TAM) from mice; however, miR-511-3p overexpression was shown to downregulate expression of AAM genes, but did not influence proinflammatory gene signature of CD11c^{+} macrophages (232). Moreover, they also showed that miR-511-3p targeted Rock2, which phosphorylates the IRF4 transcription factor essential for AAM activation. Enforced expression of miR-511-3p in TAMs inhibited blood vessel formation and tumour growth by attenuating its pro-tumoural genetic signature suggesting that miR-511-3p is able to reprogram TAMs to an anti-tumour phenotype (232).

In a mouse model of allergic airway disease, miR-511-3p expression was shown to be elevated by the Th2 cytokines IL-4 and IL-13 *in vitro* and *in vivo* (234). Transcriptome analysis following miR-511-3p overexpression suggested the ability of this miRNA to modulate cell proliferation, metabolism and immune responses in AAM. More recently, allergen uptake through MR has been shown to modulate macrophage polarisation and allergic inflammation through miR-511-3p. In these studies, Zhou et al demonstrated that cockroach allergen uptake was significantly reduced in *Mrc1*$^{-/-}$ lung macrophages

compared to WT mice, and exhibited increased lung inflammation mediated by allergen specific Th2/Th17 cytokines (235). Interestingly, *Mrc1*$^{-/-}$ macrophages also showed significant reduction in miR-511-3p expression and displayed characteristic M1 macrophage phenotype. Following miR-511-3p overexpression *Mrc1*$^{-/-}$ macrophages exhibited an M2 phenotype, highlighting a protective role of miR-511-3p against allergen-induced lung inflammation (235). In line with this, miR-511-3p was found to be significantly lower in plasma from asthmatic patients compared with non-asthmatic subjects (235).

1.9 Hypothesis and aims

In a number of previous publications, miR-511 has been shown to be a key player in regulating several human diseases, particularly cancers and also the development and differentiation of immune cells. Considering miR-511-3p (located at the 3' end of the mature miRNA strand) is embedded within the human *MRC1* gene that encodes MR, it was hypothesized that it may play a role in regulating MR expression on human DC, which would in turn modulate other downstream events such as IDO and PPARγ activity as well as cytokine production, leading to Th polarisation. The overall aim of this study was therefore to determine the immune regulatory role of miR-511-3p on human DC phenotype and function.

Objectives

1. To examine the expression profile of miR-511-3p in human monocyte-derived dendritic cells (DCs) under steady state and after treatment with LPS and CLR ligands and their effect on surface marker expression.
2. To evaluate the effect of miR-511-3p up or downregulation on DC surface phenotype and function within the context of LPS-induced cytokine production and IDO enzyme activity and its effect on T cell polarisation.
3. To evaluate the link between miR-511-3p and PPARγ in modulating LPS-induced proinflammatory phenotype in DCs.
4. To elucidate downstream intracellular pathways in DCs involved in the crosstalk between miR-511-3p and PRRs.

Chapter 2: Materials and Methods

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2.1 Antibodies and reagents

Mouse monoclonal antibodies against human CD14 (clone TÜK4), CD86 (clone FM95), CD11c (clone MJ4-27G12), HLA-DR (clone AC122) and DC-SIGN (clone DCN47.5) were purchased from Miltenyi Biotec, UK. Antibodies against human CD83 (clone HB15e) and CD80 (clone 2D10.4) were purchased from eBioscience. Anti-PDL1 (clone MIH1), PDL2 (clone MIH18) and ICOSL (clone 2D3/B7-H2) antibodies were purchased from BD Biosciences (San Jose, California). The TLR4 antibody, anti-CD284 (clone HTA125) was obtained from AbD Serotec and the anti-CD206 (MR) antibody (clone 15-2) was purchased from Biolegend. The TLR4 agonist lipopolysaccharide (LPS) from *E. coli* 0111:B4 and mannan from *Saccharomyces cerevisiae* were purchased from Sigma, UK. The PPARγ agonist (rosiglitazone) and antagonist (GW9662) were purchased from Cayman chemicals, UK.

2.2 Generation of monocyte-derived DCs

Human DCs were generated from monocytes as previously described (22,24,236,237). Briefly, buffy coats were obtained from healthy donors following ethics committee approval (National Blood Service, Sheffield, UK) and PBMCs were separated by density gradient centrifugation on Histopaque (Sigma-Aldrich, St Louis, Mo). Monocytes were purified from PBMCs by positive selection using a magnetic cell separation system (MACS) with mouse anti-human CD14 antibodies conjugated to magnetic beads (Miltenyi Biotec, UK). The purity of $CD14^+$ cells was always above 90% as determined by flow cytometry (FC 500; Beckman Coulter, USA). Following isolation, monocytes ($1x10^6$ cells/ ml) were re-suspended in RPMI medium 1640 (Sigma), supplemented with 10% heat inactivated FBS, 100U/ml penicillin, 100U/ml

streptomycin and 2mM L-glutamine. Cells were incubated at 37 °C with 5% CO_2 in a humidified incubator. DC differentiation was carried out over 6 days in the presence of 250 U/ml IL- 4 and 50ng/ml GM-CSF (Miltenyi Biotec).

2.3 RNA interference

Pre-designed miR-511-3p inhibitors and mimics were purchased from Qiagen, UK and transfection was carried out using the HiPerFect Transfection Reagent (Qiagen) according to manufacturer's protocol. The miRNA targeted sequence was 5'-AAUGUGUAGCAAAAG-ACAGA-3'. Briefly, $CD14^+$ monocytes (1 x 10^6 cells/ ml) were seeded in Opti-MEM® reduced serum media in 24 well plates and incubated at 37 °C (with 5% CO_2). Prior to transfection, miR-511-3p inhibitor or mimic was diluted in 50µl serum-free media (with 3µl transfection reagent) in separate tubes for 10 minutes at room temperature, before adding drop wise unto cells. The miScript Negative Control Inhibitor and the AllStars Negative Control siRNA were used as controls for inhibitor and mimic respectively (Qiagen, UK). Transfection of small interfering RNAs (siRNA) was carried out as previously described (24,116). MR siRNA was the SMARTpool ON-TARGET plus siRNA from GE Healthcare, Sweden. The ON-TARGET plus, non-targeting control siRNA was used as negative control. All transfections were carried out at a final concentration of 50nM as determined during optimizations. Monocytes were differentiated into DCs after 6 hours of incubation, with fresh Opti-MEM® supplemented with 250 U/ml IL-4 and 50ng/ml GM-CSF. Transfection efficiency of different transfection reagents (DharmaFECT 2, 4 and HiPerFect) used during optimizations were determined using the fluorescently labelled siGLO RISC-free control siRNA (GE Healthcare). Expression of miRNA and/or mRNA was assessed on day 6.

2.4 Flow cytometry analysis

Control and transfected cells were collected and washed twice in cold PBA (PBS buffer containing 0.5% BSA and 0.1% sodium azide (Sigma Aldrich)). Staining with labelled antibodies was then carried out in the dark at 4 °C for 20 minutes according to manufacturer's instructions. Unless otherwise stated, specific antibodies were conjugated to Fluorescein Isothiocyanate (FITC), Phycoerythrin (PE) or Phycoerythrin Cyanine 5.1 (PE/Cy5). Following staining, samples were washed twice with PBA buffer and fixed in 0.5% formaldehyde solution before analysis. Non-reactive, isotype-matched antibodies were used as controls. Flow cytometry was carried out on the FC500 Flow Cytometer (Beckman Coulter, UK) and data was analysed using Weasel Software for Windows.

2.5 Viability assay

Dendritic cell samples following treatment with inhibitors or mimics and transfection reagents were washed with ice-cold PBS, and staining was carried out using the Annexin-A5-FITC Apoptosis detection kit (Beckman coulter, UK) according manufacturer's instructions. Briefly, cells were suspended in binding buffer (1x) and stained with 5µl of Propodium Iodide (PI) and 1µl of annexin-A5 FITC. Samples were subsequently incubated on ice in the dark for 15 minutes. Analysis was then carried out within 30 minutes using the FC 500 Flow Cytometer (Beckman Coulter). Cells treated with 4% formaldehyde solution were used as positive control.

2.6 Quantification of IDO activity

IDO activity was determined as described before (24,236,238). DCs ($2.5x10^5$cells/ml) were seeded in a 24 well plate with complete RPMI media 1640, supplemented with

100µM L-tryptophan (TRP) (Sigma Aldrich, UK), in separate wells. Depending on the experimental setup, cells were treated with mannan and LPS or with PPARγ agonist RSG in the presence or absence of antagonist GW9662. IDO activity was determined after 24 hours of stimulation by measuring the levels of L-kynurenine (KYN) produced in culture supernatant. This was done using a colorimetric assay. Briefly, 150µl of 30% trichloroacetic acid (TCA) (Fisher Scientific) was added to 300µl of culture supernatant. The mixture was vortexed and centrifuged at 13,000 rpm for 5 minutes. Subsequently, 350µl of the soluble phase was added to 350µl of Ehrlich`s reagent (100mg of 4-dimethylamino benzaldehyde (Sigma) in 5ml of glacial acetic acid (Fisher Scientific)). Finally, 200µl was transferred to a 96-well micro-titre plate (Greiner bio-one) and the optical density (OD) was read at 492nm using a plate reader (Omega). The concentration of L-KYN was calculated from a standard curve of defined concentrations from 0 to 200µM.

2.7 Live/Dead assay

Cell viability following treatment with RSG and GW9662 was determined using the LIVE/DEAD assay kit according to manufacturer's guidelines (Thermo Fisher, UK). Cells were imaged using the Etaluma LS720 Microscope (Carlsbad, CA). Data was analysed using image J software.

2.8 Total RNA isolation and cDNA synthesis

Monocytes and DC samples were washed twice with cold PBS and stored at - 80 °C or in RNA later until RNA isolation. miR-511-3p transfected cells as well as controls were stimulated with different ligands (mannan/LPS or RSG) on day 6 for 24 hours before harvesting. Total RNA, including small RNAs was isolated with Trizol Reagent using

the miRNeasy Mini kit (Qiagen). The concentration of purified RNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific). First-strand cDNA was generated with the miScript Reverse Transcription (RT) II kit (Qiagen). Briefly, 0.6µg total RNA was reverse transcribed in a final volume of 20µl using 2µl Reverse Transcription mix, 4µl 5X miScript HiFlex buffer and appropriate volumes of RNase-free water. The reaction was incubated for 60 minutes at 37°C and at 95 °C for 5 minutes to inactivate the reverse transcriptase enzyme. Reverse transcription was done with the T100 Thermal Cycler (Bio-Rad) and the 5X miScript HiFlex buffer was used for parallel real-time PCR quantification of mature miRNA and mRNA.

2.9 Real time PCR (qRT-PCR)

Comparative real-time PCR for miR-511-3p expression was carried out on the MxPro 3005P qRT-PCR system (Stratagene, CA, USA), using the miScript SYBR Green PCR kit (Qiagen), according to manufacturer's instructions. Briefly, 12.5µl 2X QuantiTect SYBR-Green Master mix, 2.5µl 10X Universal Primer and 2.5µl Primer Assay (forward primer) was mixed with 3ng reverse transcription product in RNase-free water. qRT-PCR cycling was initiated at 95 °C for 15 minutes, followed by 40 cycles of 94 °C for 15 seconds, 55 °C for 30 seconds and 70 °C for 30 seconds. Mature miR-511-3p-specific primers (MIR511-1) were obtained from Qiagen. All experimental procedures were done in triplicate and relative expression was normalised U6 (RNU6-2) small nuclear RNAs (Qiagen). qRT-PCR for mRNA expression was performed with the Brilliant III Ultra-Fast SYBR Green PCR Master Mix (Agilent Technologies, USA) as previously described (116). The two-step cycling reaction was initiated at 95 °C for 3 minutes followed by 40 cycles of 95 °C for 20 seconds and 60 °C for 20 seconds. Gene expression levels were normalised to that of GAPDH and calculated using the

comparative *C*t method, which determines the difference in *C*t values between the gene of interest and housekeeping gene. Forward and reverse primers for specific mRNAs were purchased from Eurofins Scientific, UK.

Table 2.1: Primers for real-time PCR

Genes	**Primer**	**Sequence (5'-3')**
GAPDH	Forward	GAGTCAACGGATTTGGTCGT
	Reverse	GACAAGCTTCCCGTTCTCAG
MR	Forward	CGTTTACCAAATGGCTTCGT
	Reverse	CCTTGGCTTCGTGATTTCAT
DC-SIGN	Forward	CCAAAGGAGGAGACAAGCAG
	Reverse	GGACGACAGCTTCAGTGTGA
PPARG	Forward	TGTGAAGCCCATTGAAGACA
	Reverse	ACTCAGGGTGGTTCAGCTTC
FABP4	Forward	GGATGGAAAATCAACCACCA
	Reverse	GGAAGTGACGCCTTTCATGA

2.10 Cytokine ELISA

Cell-free culture supernatants were collected and stored at -20°C before analysis. The levels of cytokines IL-10, IL-12p70, IL-1β, IL-6 and TNF-α were measured by sandwich ELISA using the Duo Set ELISA kit (R&D Systems, UK) according to manufacturer's instructions.

2.11 Reverse phase protein microarray

DCs were harvested after treatment, washed twice with ice-cold PBS and lysed in 50 µL of RIPA buffer containing protease and phosphatase inhibitors (all from Thermo

Fisher Scientific). Protein quantification was done using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). For the reverse phase protein microarray, the procedure described by Negm et al (239) was followed. 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) and 0.1% Tween-20 in PBS (Sigma-Aldrich)) at 48 °C. After washing, slides were incubated with primary antibodies overnight at 48 °C. β-Actin antibody was included as a loading control. All antibodies were purchased from Cell Signaling Technologies (CST, UK). After washing, slides were incubated with infrared Licor secondary antibodies for 30 minutes 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, California). Protein signals were finally determined by using background subtraction and normalization to the internal housekeeping targets. Signal values represented on the colour scale for the heat map are log2 transformed from arbitrary fluorescence units and normalized by using the SD. Heat maps were generated with ClustVis software.

2.12 DC-T cell co-culture

This was done as previously described (23,116). Briefly, human DCs treated with miR-511-3p inhibitors or mimics were co-cultured in the presence of 0.1μg/ml LPS in 96-well round-bottom plates (Corning Life Sciences) with $CD3^+$ autologous T cells, at a ratio of 1:10. T cells were purified by negative selection using a cocktail of immunomagnetic beads (Miltenyi Biotec). Following isolation, cells were re-suspended

in RPMI media supplemented with 5% human AB serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (Sigma Aldrich). The co-culture media was refreshed every 3 days with media containing 5 ng/ml IL-2 (Miltenyi Biotec). After 6 days, T cells were harvested and re-stimulated with 2 µg/ml anti-CD3 (clone OKT3) and anti-CD28 (clone 28.2) antibodies (Sigma Aldrich, UK) for 18 hours, following which culture supernatants were collected and stored.

2.13 Human T cell cytokine array

T cell cytokine expression was measured in co-culture medium using the RayBio® human T cell response array (AAH-TCR-1-2) according to manufacturer's instructions. Briefly, antibody spotted membranes were blocked for 30 minutes and incubated with 1ml culture supernatants overnight at 4°C. Membranes were subsequently washed 5 times (5x) with wash buffers I (3x) and II (2x) for 5 minutes and then incubated with the biotinylated antibody cocktail for 2 hours at room temperature. Membranes were washed as before, incubated with the HRP-conjugated streptavidin secondary antibody for 2 hours and developed by incubation with detection buffers for 2 minutes. Chemiluminescent signals were captured by means of the Chemi-XRQ G: Box (Syngene) and exposure time was varied between 5 and 15 minutes to optimize signal to noise ratio of the images. Image analysis was done with the Odyssey Image Studio software (version 3.1.4) for windows.

2.14 Library construction and RNA-Seq

Total RNA was isolated with Trizol® reagent using the PureLink RNA mini kit, (Ambion, Thermo Fisher Scientific) according to manufacturer's instruction. The quality of purified RNA was assessed using the RNA Nano 6000 assay kit on the

Agilent 2100 Bioanalyzer (Agilent Technologies, USA). All samples had RNA Integrity Number (RIN) score ≥9. mRNA was then purified from total RNA using poly-T oligo-attached magnetic beads and fragmented. Strand-specific libraries were subsequently constructed using the NEB Next Ultra Directional RNA Library Prep Kit (New England Biolabs) and RNA sequencing was done using the Illumina HiSeq PE150 platform. Library preparation and sequencing was done by Novogene Bioinformatics Technology Co. Ltd (Hong Kong, China) (Figure 2.1).

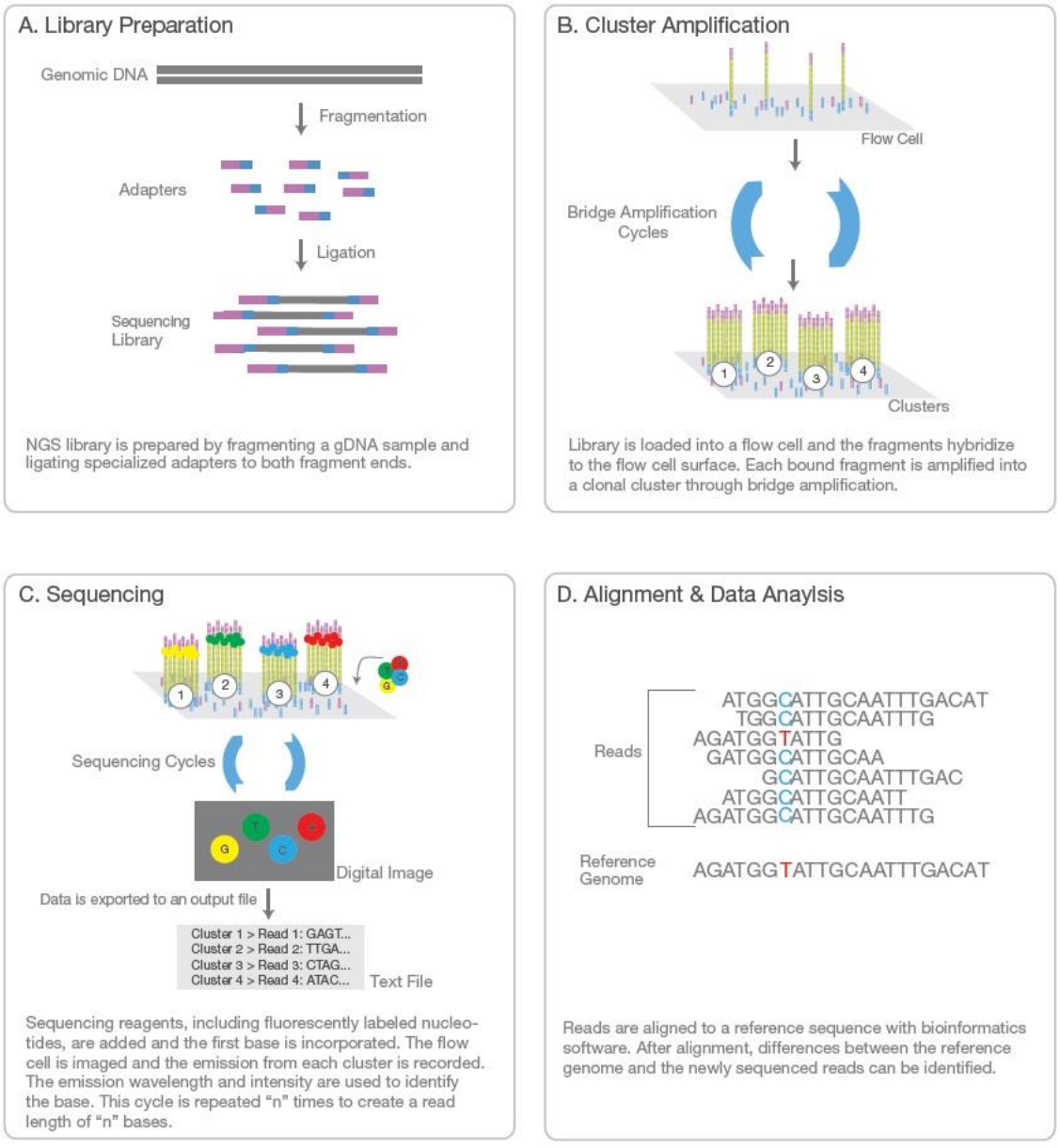


Figure 2.1: Overview of RNA sequencing workflow

2.15 RNA-Seq data analysis

Downstream RNA-Seq analysis was performed using a combination of programs including Bowtie2, Tophat2, HTseq, and Cufflink. Alignments were parsed using Tophat program and differential expressions were determined through DESeq2/ DEGseq. Gene ontology (GO) and KEGG enrichment were implemented by the GOseq R package and KOBAS respectively.

2.16 Statistical analysis

Data were analysed using GraphPad Prism v7.02 for Windows (GraphPad Software, CA) and values expressed as mean ± Standard deviation (SD) from independent experiments unless otherwise stated. Data was assumed to follow a normal distribution. Unpaired student t-test was used to compare two groups or ANOVA (to compare three of more groups) with Bonferroni post-hoc testing. A p value <0.05 was considered statistically significant.

Chapter 3: Expression Profile of miR-511-3p in Human Dendritic Cells

Chapter 3: Expression profile of miR-511-3p in human dendritic cells

3.1 Introduction

Dendritic cells (DCs) are a heterogeneous group of bone-marrow derived cells that are crucial in initiating and regulating antigen-specific immune responses (240). They are the most efficient antigen presenting cells (APCs) with a unique capacity to internalize and present antigens to naïve T cells. Additionally, DCs coordinate interactions with other immune cells such as B cells and natural killer (NK) cells to tailor the innate as well as adaptive immune response to disease conditions such as inflammation, infection, allergy and autoimmunity (241-243). Under certain conditions, DCs can also be derived from circulating blood monocytes, where they are distinguished from myeloid (bone-marrow) precursors on the basis of phenotypic expression, local microenvironment and functional activity (cytokine profile) (244-246).

DCs express a variety of unique pattern recognition receptors (PRRs) that play different roles in the immune system (247,248). Under steady-state conditions, tissue resident DCs are immature (iDC) and express high levels of receptors for antigen capture such as the mannose receptor (MR) (30) and DC-specific intracellular adhesion molecule (ICAM) 3 non-integrin (DC-SIGN) (40,249), which are typical C-type lectin receptors, as well as the toll-like receptors, such as TLR4 (250,251). However, the presence of ‘danger signals’ or tissue damage resulting from pathogenic infection, initiates a complex maturation program in DCs characterised by an upregulation of co-stimulatory molecules CD80, CD86, MHC-II (HLA-DR) and CD40 as well as homing receptors such as CCR7 for migration to lymph nodes, enhancing their ability to activate T cells (252-254). In addition to their potent stimulatory capacity, DCs have been shown to play a crucial role in tolerance induction and regulation of specific T cell responses. Despite their significant roles in the immune system, the mechanisms that regulate DC

development and function are not fully understood. The relatively recent discovery of microRNAs (miRNAs) and their ability to regulate various aspects of cell development and function will pave the way for better understanding of mechanisms that regulate DC function involving miRNAs.

miRNAs are small, evolutionarily conserved, noncoding RNA molecules that regulate gene expression by degrading target mRNAs or repressing translation (255). Hundreds of miRNAs have been identified in humans and each is predicted to regulate multiple genes simultaneously. Emerging evidence suggests that miRNAs are key regulators of immune function including antibody production, development and differentiation of immune cells, cytokine release and regulation of innate and adaptive immune responses (256). In DCs, miRNAs are crucial regulators of lineage commitment, maturation, antigen presentation and cytokine production. For example, miR-155 has been shown to be rapidly induced in human and mouse DCs following TLR-induced maturation and is important for DC function, as mice deficient in miR-155 showed impaired responses to pathogens and a reduced expression of CD86 and CD40 molecules (207,216). Also, up-regulation of let-7i miRNA in bone marrow-derived DCs (BMDC) after TLR4 activation has been demonstrated to be important for normal DC maturation and function, evidenced by the decreased expression of CD80 and CD86 co-stimulatory molecules and pro-inflammatory cytokine secretion, when let-7i was inhibited (240). Conversely, some miRNAs have been shown to exert a negative influence on DC function following TLR activation. For instance, increased expression of miR-146a after TLR7/9 ligation in plasmacytoid DCs (pDCs) inhibited upregulation of activation markers (CD86, CD80, CD40, HLA-DR and CCR7) as well as cytokine production, impairing their ability to activate allogenic T cell responses (257). This has also been demonstrated for miR-142 in bone marrow derived DCs (BMDCs). Although mice

deficient in miR-142 showed increased expression of maturation and activation markers, they failed to induce CD4 T cell responses compared to normal DCs, due to miR-142 targeting IL-6 mRNA. In addition to changes in DC maturation, several miRNAs have been shown to affect DC survival (e.g. miR-29b, miR-126, miR-146 and miR-155), antigen processing and presentation (e.g. miR-150 and miR-223) (258,259) and tolerance induction (e.g. miR-10, miR-99b and miR-148/152) (260-262).

Recently, studies have highlighted miR-511 as a multifunctional miRNA with roles in innate and adaptive immune responses and in regulation of human diseases. For instance, miR-511 was shown to suppress the proliferative capacity of human lung adenocarcinomas by targeting the *TRIB2* oncogene involved in cell division (228). Furthermore, dysregulated expression of miR-511-3p, the active strand of miR-511 (in mice), interfered with the genetic signature of tumour associated macrophages (TAMs) thereby affecting tumour growth in mouse models of cancer (232). Interestingly, miR-511-3p is embedded within the *MRC1* gene that encodes the mannose receptor (MR), and has been shown to contribute to the development and differentiation of macrophages and DCs (232,263). Although studies regarding the role of miR-511-3p in human diseases are slowly emerging, its role in the pathogenesis of inflammation is mostly uncharacterised. The aim of this chapter is to characterise the expression profile of miR-511-3p in human monocyte-derived DCs (Mo-DCs) and examine how miR-511-3p is modulated in these cells in the presence or absence of TLR4 and CLR stimulation with known ligands. Furthermore, optimization of transfection efficiencies (for induction or suppression of miR-511-3p) was performed in this chapter for studies in later chapters on investigating the role of miR-511-3p on human DC phenotype and function.

3.2 Results

3.2.1 miR-511-3p expression is induced in human DCs compared to monocytes

To determine whether miR-511-3p may play a role in the differentiation of human DCs, its expression levels was examined by qRT-PCR on different days of DC differentiation and compared to $CD14^+$ monocytes. DCs were generated from blood monocytes (Mo-DC) by culturing in the presence of IL-4 and GM-CSF for 6 days. The purity of cell preparations was further examined by flow cytometry for confirmation of CD14 and CD11c-specific markers for monocytes and DC respectively (Figure 3.1).

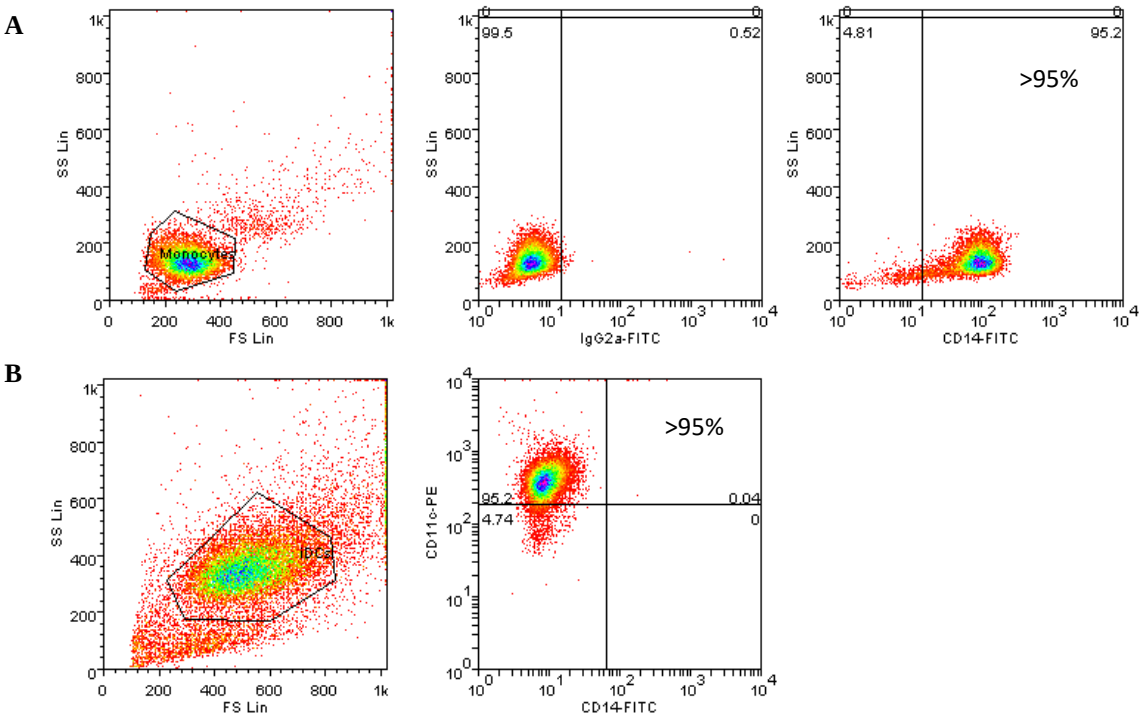
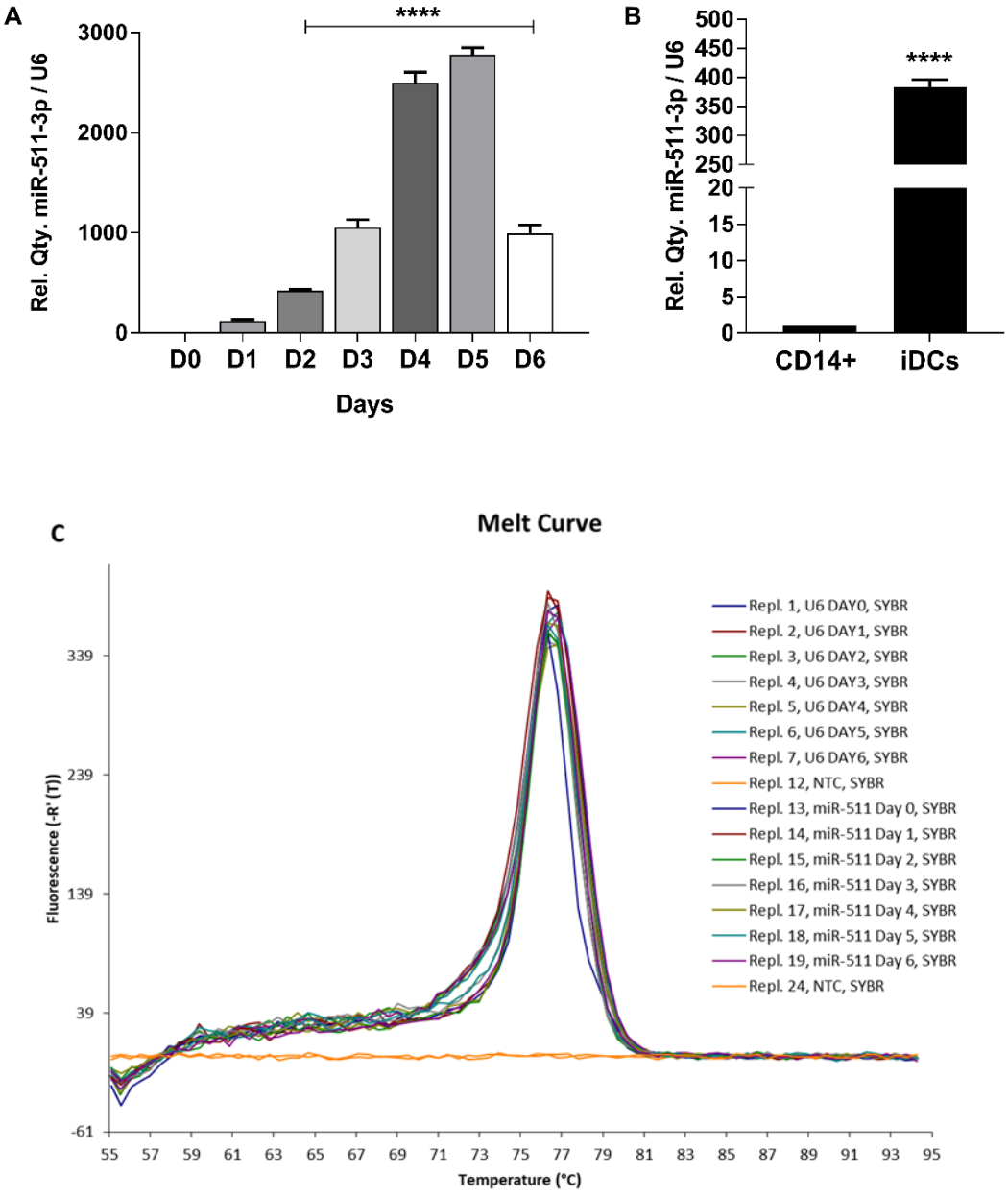


Figure 3.1: Purity of monocyte and DC preparations. Dot plots for specific antibody staining. From left to right A) FS/SS scatter for monocytes, isotype control and $CD14^+$ cells. B) Dot plots for characteristic DC markers showing FS/SS scatter and double staining for CD11c (>95%) and CD14 (<5%). Data in A and B is representative of one independent experiment with one donor out of a total of five donors.

As shown below, monocytes expressed relatively low levels of miR-511-3p in comparison with differentiating DCs, which conversely showed a steady increase in miR-511-3p expression from day 1 to day 5 and decreased on day 6. Furthermore, immature DCs (iDCs) (day 6) also showed a significant increase in miR-511-3p expression compared to cultured monocytes (Figure 3.2).



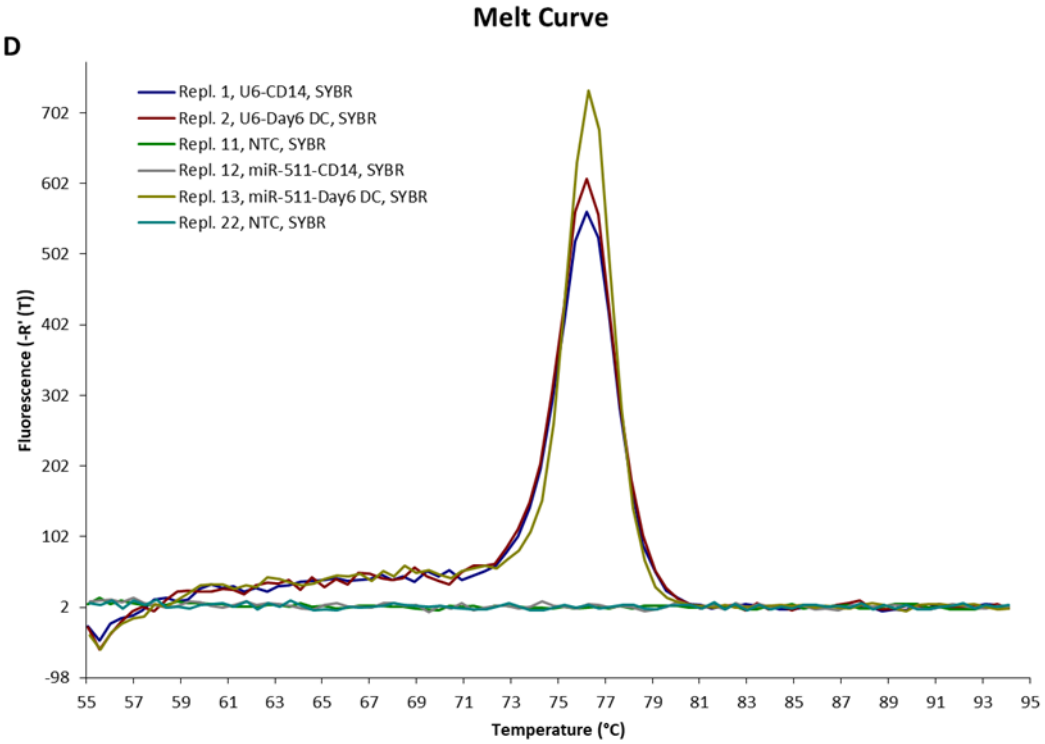


Figure 3.2: Expression of miR-511-3p is induced in human DCs compared to monocytes A) Relative expression of miR-511-3p in monocytes and differentiating DCs (day 1-6). Data is shown as mean ± SD of one independent experiment with two donors. ****p < 0.0001. B) Relative miR-511-3p expression between $CD14^+$ monocytes and day 6 immature DCs. Data is shown as mean± SD of three independent experiments from three donors. ****p < 0.0001 Data is from three independent experiments each with 3 replicates. Representative qRT-PCR melt curves shown for miR-511-3p expression in C) differentiating DCs and D) $CD14^+$ vs. immature DCs.

3.2.2 TLR4 activation downregulates miR-511-3p expression in human DCs

To determine whether DC maturation influenced the level of miR-511-3p expression, iDCs on day six were exposed to increasing concentrations (0.001, 0.01, 0.1 and 1µg/ml) of the TLR4 agonist, LPS for 24 hours (Figure 3.3). In line with a previous report (234), the data obtained showed a bell-shaped, dose-response curve of miR-511-3p expression in mature DCs (mDCs), all of which were significantly downregulated compared to unstimulated DCs (-).

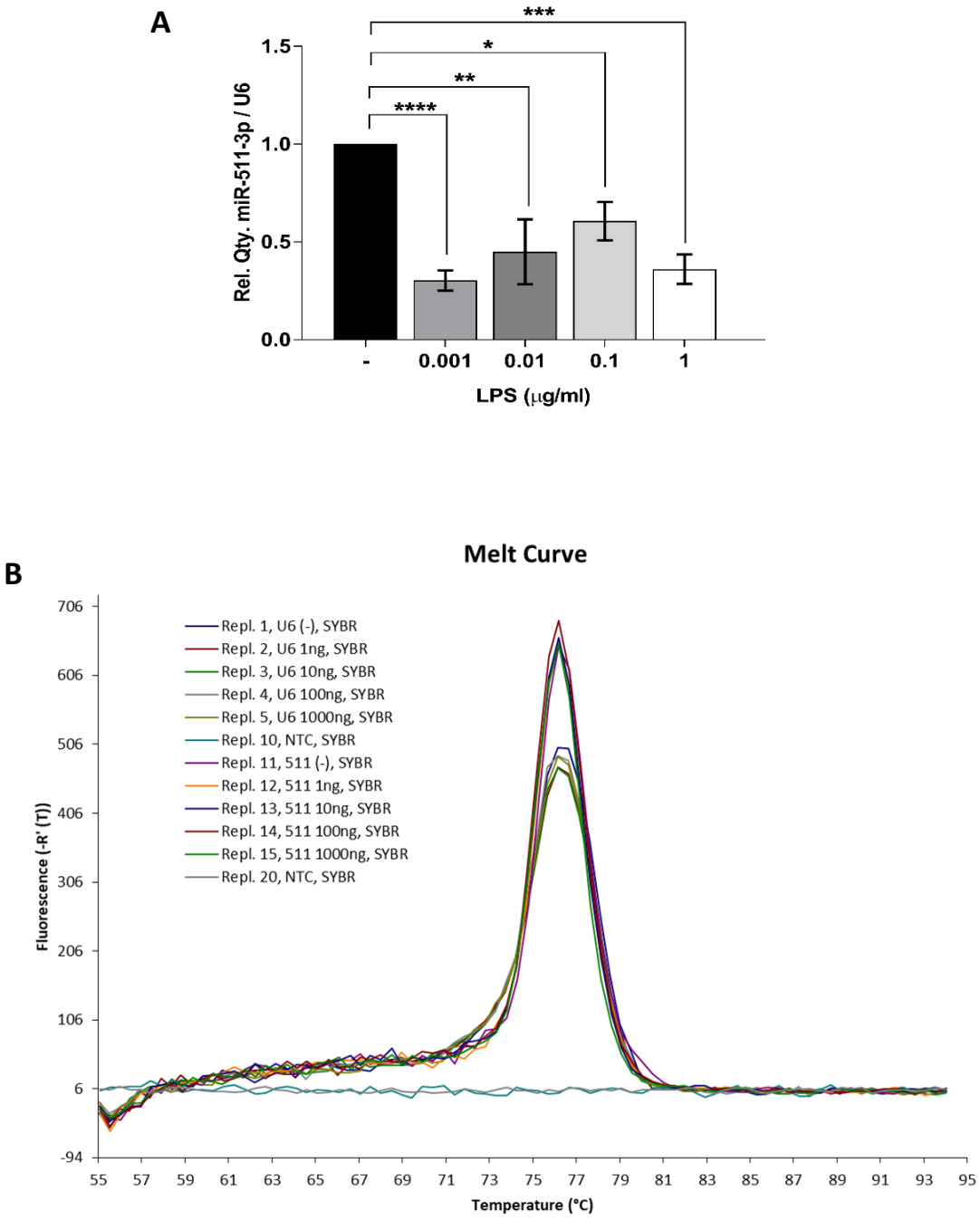
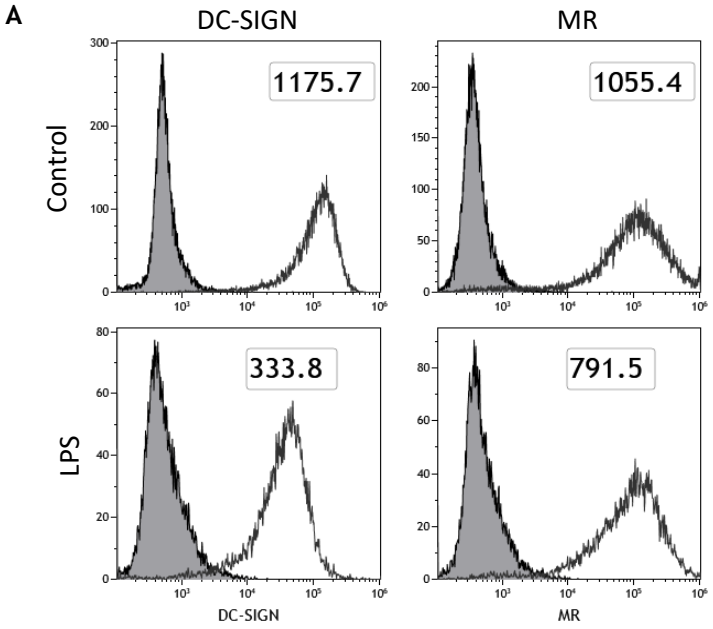


Figure 3.3. Expression of miR-511-3p is downregulated by LPS treatment. A) miR-511-3p expression in human DCs treated with increasing dose LPS from *E. coli* for 24hours. Data is represented as mean ± SD of two independent experiments with two biological replicates (donors) performed in triplicates *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (one-way ANOVA with Bonferroni post-hoc test). DC differentiation was carried out over 6 days and miR-511-3p expression was assessed by qRT-PCR. Data is normalised to RNU6. B) Representative qRT-PCR melt curve analysis of miR-511-3p expression.

Additionally, the phenotype of iDCs versus mDCs was examined for acquisition of maturation status following LPS treatment. Flow cytometry analysis revealed a typical phenotype for iDCs (control); as expected, these cells expressed high levels of MR and DC-SIGN, which are receptors for antigen capture (264,265) (Figure 3.4A). On the other hand, mDCs showed increased expression of the classical activation markers CD83, CD80 (B7-1), CD86 (B7-2) and HLA-DR (MHC-II), which were previously low in the immature cells. Conversely, expression of the above-mentioned CLRs, MR and DC-SIGN was downregulated in the mature cells. TLR4 expression however, remained unchanged between iDCs and mDCs (Figure 3.4).



B

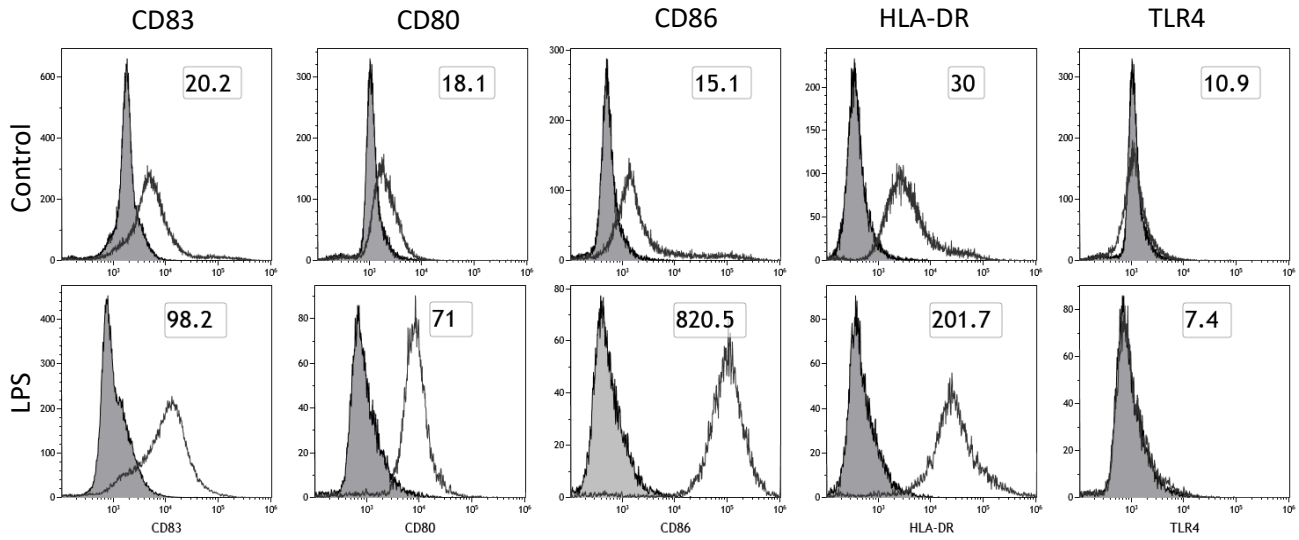


Figure 3.4: Flow cytometry analysis of human DC markers. A) Representative histograms of MR and DC-SIGN expression on immature (control) and mature DC (LPS). B) Representative histograms of DC maturation and activation markers between immature (control) and mature DC (LPS). Immature cells were treated with 0.1 μg/ml LPS for 24 hours before staining with labelled antibodies. Filled histograms (dark grey) represent unstained cells and black lines represent specific marker expression. Data in A and B is representative of one independent experiment with one donor out of a total of five donors. Median fluorescence intensity (MFI) values are shown.

3.2.3 Carbohydrate ligands promote miR-511-3p expression in human DCs

Next, the effect of CLR engagement on the pattern of miR-511-3p expression in DCs was also examined. Immature DCs were harvested on day 6 and treated with mannan for 24 hours. Mannan is a complex polysaccharide derived from *S. cerevisiae* and known to bind MR, DC-SIGN and Dectin receptors particularly Dectin-2 (266-269). In contrast to LPS treatment, increasing concentrations of mannan (1, 10 and 100μg/ml) were found to upregulate miR-511-3p expression in human Mo-DCs (Figure 3.5). Subsequent analysis of DC phenotype showed no significant changes to DC activation

markers CD83, CD86 and HLA-DR as well as DC-SIGN; however, there was a decrease in MR expression (MFI) following treatment with mannan (Figure 3.6).

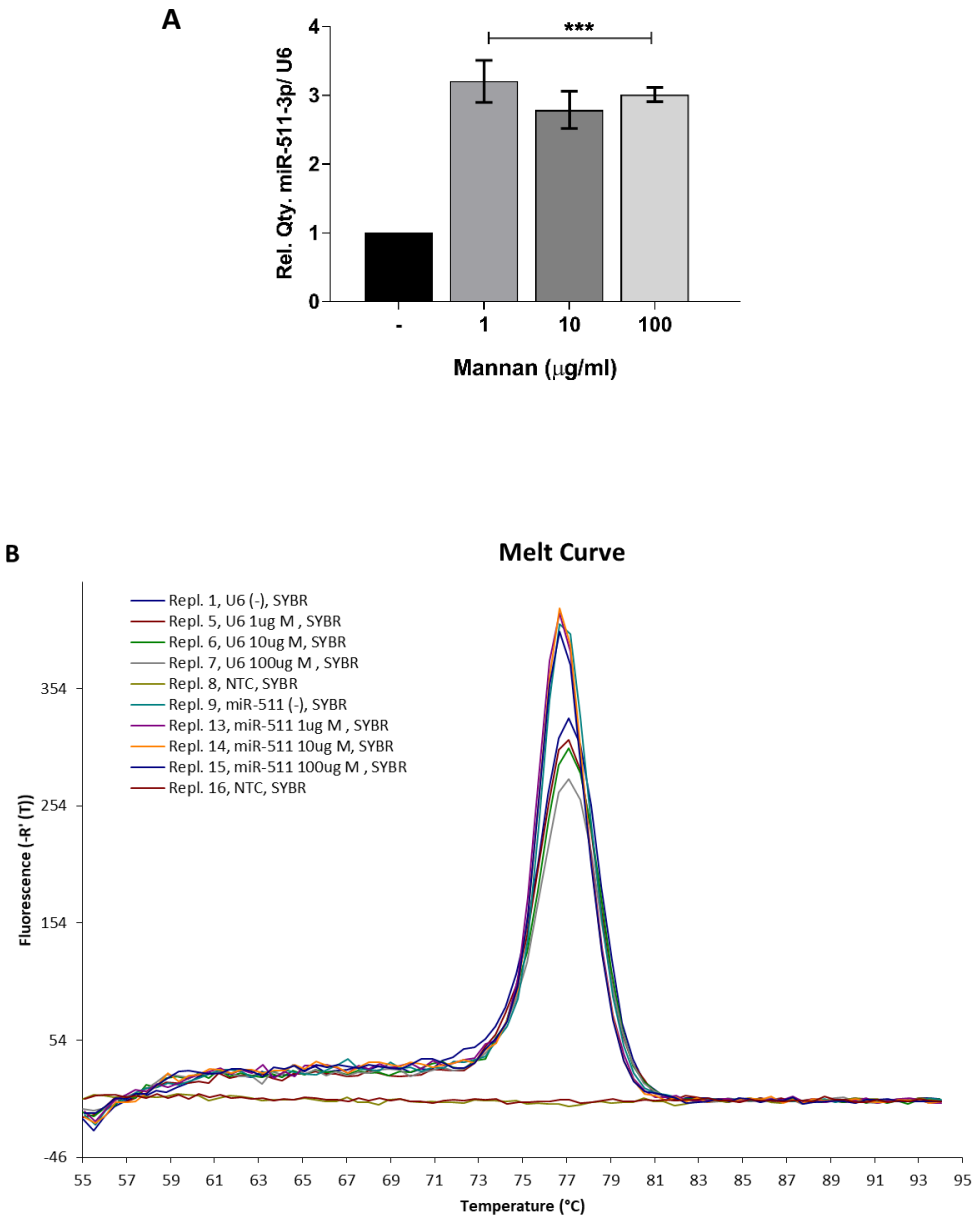


Figure 3.5. Relative expression of miR-511-3p in DCs. A) Expression of miR-511-3p in immature DCs treated with increasing dose mannan from for 24hours. Relative expression of miR-511-3p is normalized to RNU6. Data is represented as mean ± SD of one independent experiment with two biological replicates (donors) performed in triplicates. ***p < 0.001 (one-way ANOVA with Bonferroni post-hoc test). B) Representative qRT-PCR melt curve analysis of miR-511-3p expression.

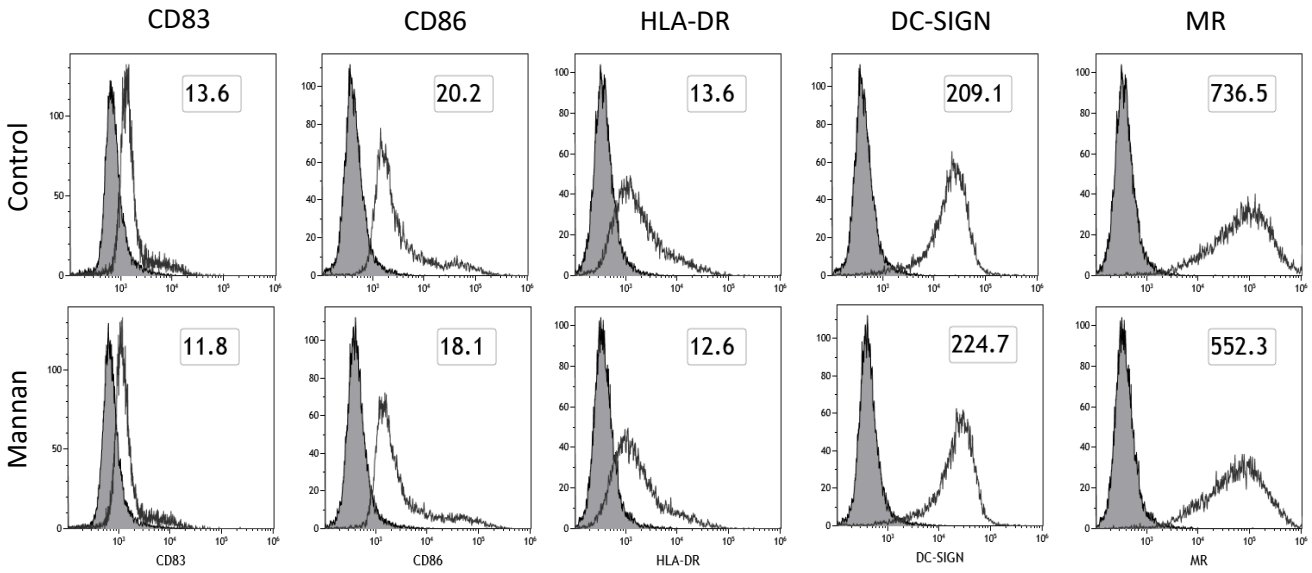


Figure 3.6: Analysis of human DC markers following treatment with mannan. Representative histograms of DC maturation and activation markers between immature DCs (control) and DCs treated with mannan. Immature cells were treated with 10 μg/ml mannan for 24 hours before staining with labelled antibodies. Filled histograms (dark grey) represent unstained cells and black lines represent specific marker expression. Data is representative of one independent experiment with one donor out of a total of two donors. Median fluorescence intensity (MFI) values are shown.

Considering miR-511-3p is expressed within the *MRC1* gene, the effect of MR-specific ligand on the miRNA expression was also examined. To this end, iDCs were also treated with increasing concentrations (1, 10 and 100μg/ml) of sulphated galactose (SO_4-3-Gal-PAA), (a ligand that binds the cysteine rich (CR) domain of the mannose receptor) for 24 hours. In contrast to mannan, treatment with 1 and 100 μg/ml sulphated galactose downregulated miR-511-3p expression in human DCs, whereas no change was seen following 10μg/ml treatment (Figure 3.7). In addition, no significant changes to activation markers were observed. Like mannan treatment, MR expression was downregulated following treatment with SO_4-3-Gal-PAA (Figure 3.8).

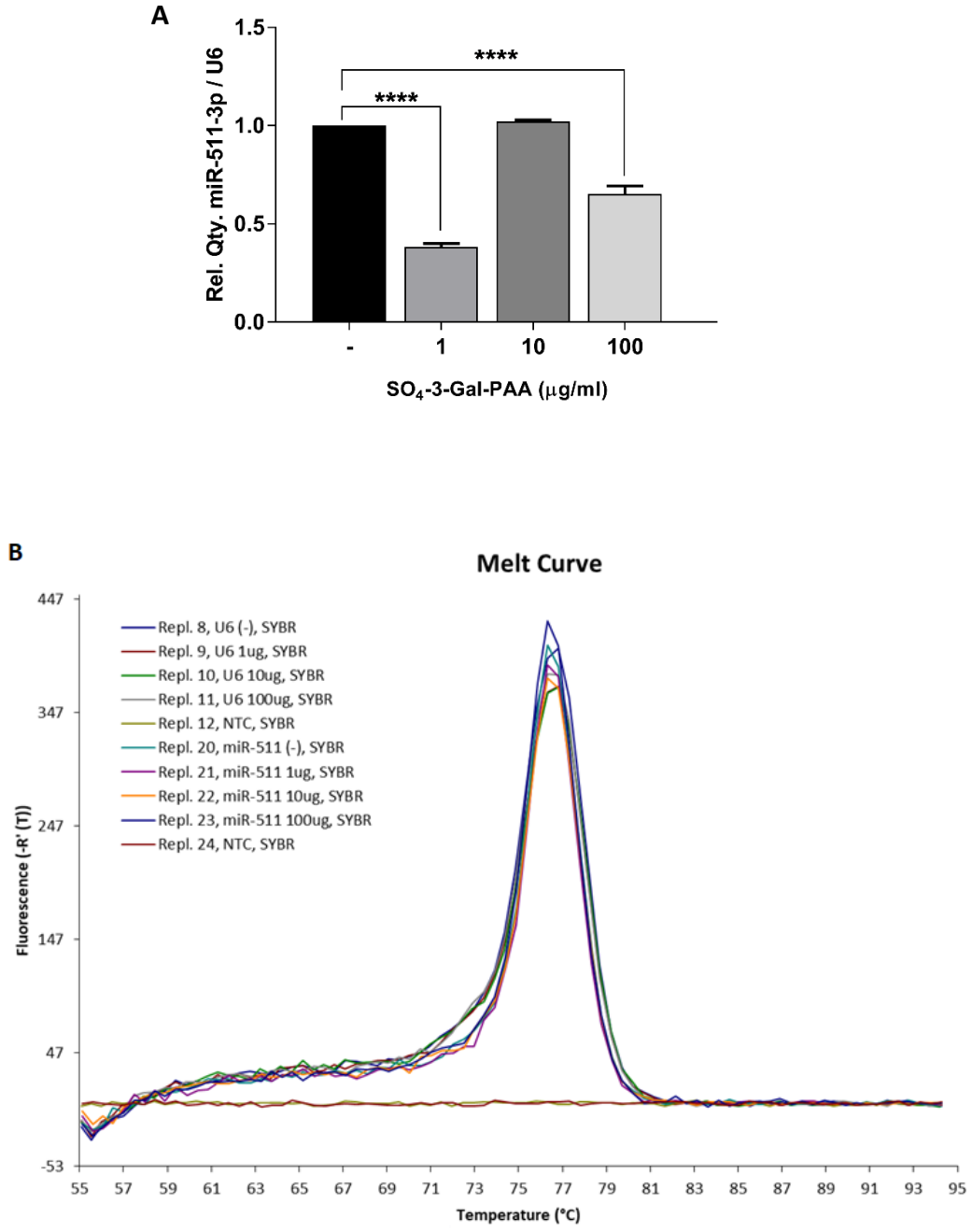


Figure 3.7. Relative expression of miR-511-3p in DCs. A) Expression of miR-511-3p in immature DCs treated with increasing concentration of SO_4-3-Gal-PAA from for 24hours. Relative expression of miR-511-3p is normalized to RNU6. Data is represented as mean ± SD of one independent experiment with two biological replicates (donors) performed in triplicates. ****$p < 0.0001$ (one-way ANOVA with Bonferroni post-hoc test). B) Representative qRT-PCR melt curve analysis of miR-511-3p expression.

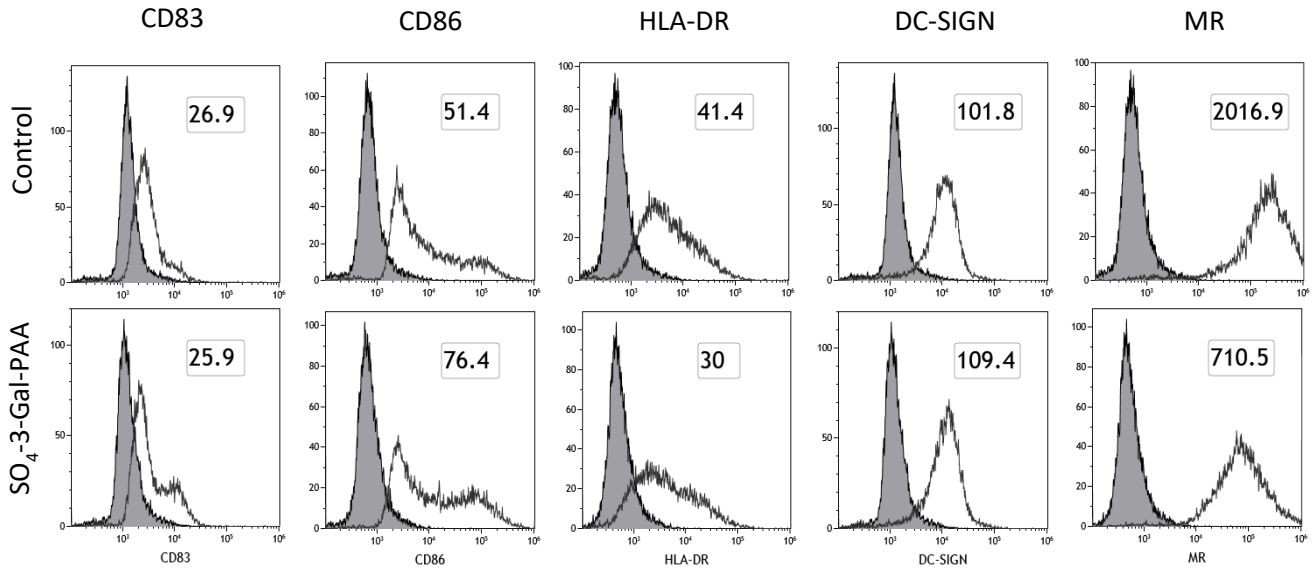


Figure 3.8: Analysis of human DC markers following treatment with SO4-3-Gal-PA. Representative histograms of DC maturation and activation markers between immature DCs (control) and DCs treated with sulphated galactose. Immature cells were treated with 10 μg/ml SO4-3-Gal-PA for 24 hours before staining with labelled antibodies. Filled histograms (dark grey) represent unstained cells and black lines represent specific marker expression. Data is representative of one independent experiment with one donor out of a total of two donors. Median fluorescence intensity (MFI) values are shown.

3.2.4 *In vitro* screening of transfection reagents.

The suitability of commercially available transfection reagents to promote uptake of siRNA by human Mo-DCs was examined for use in miR-511-3p silencing experiments. Day 0 monocytes were transfected with varying concentrations (25, 50 and 100nM) of the PE-labelled, siGLO RISC-free, non-targeting control siRNA (Dharmacon), using three liposome-based transfection reagents; DharmaFECT (DF) 2, DF4 (GE Healthcare) and HiPerFect (Qiagen). Cells were then differentiated into DCs after 6

hours of incubation. Transfection reagents were tested within range suggested by the manufacturers (3ul for HiPerFect and 250X dilution for DF reagents) and efficiency of uptake was examined by flow cytometry on day 6 (Figure 3.9).

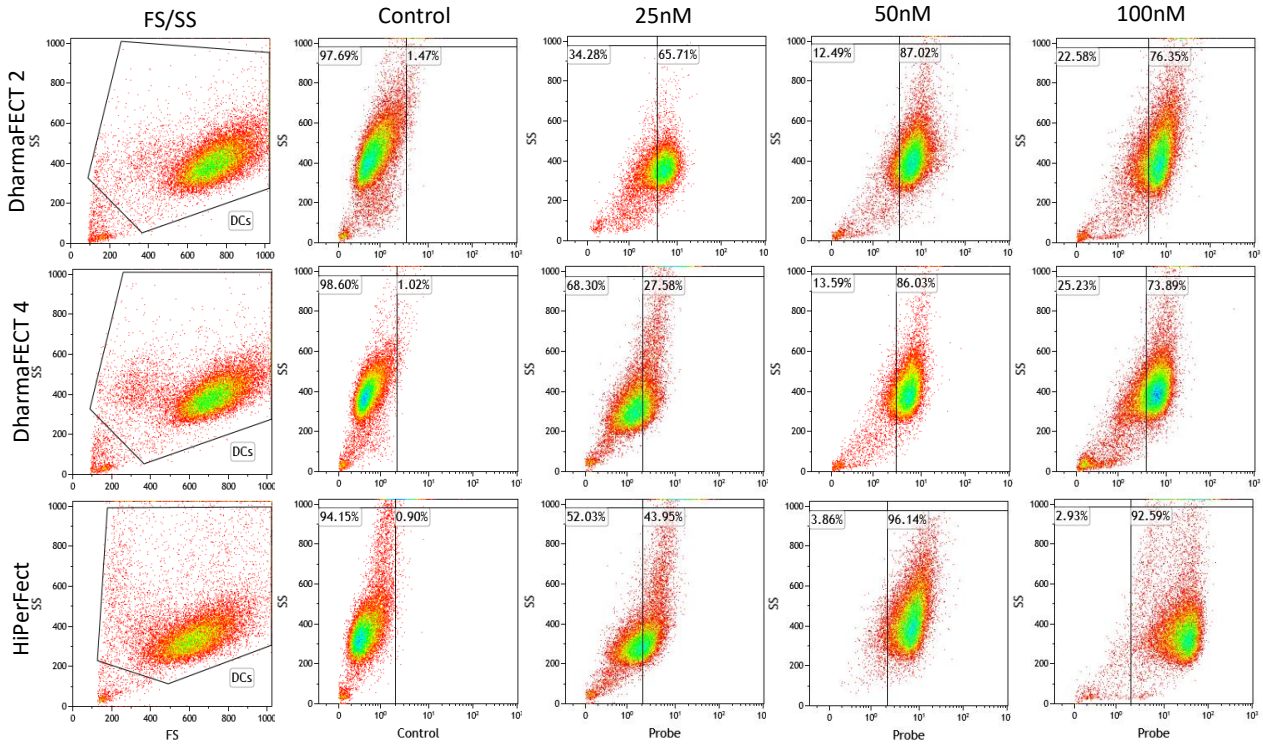


Figure 3.9: Transfection efficiency of human DCs using transfection system: Representative dot plots of monocyte-derived DCs transfected with increasing concentration (25nM, 50nM and 100nM) of siGLO RISC-free Green transfection indicator using DF2, DF4 and HiPerFect transfection reagents. Monocytes were transfected on day 0 and differentiated into DCs after 6 hours of incubation. Transfection efficiency was assessed on day 6 by flow cytometry. Data is representative of one independent experiment with one donor out of a total of two donors.

As shown, all transfection reagents promoted uptake of labelled siRNA, particularly more than 70% with higher concentrations (50nM and 100nM) compared to 25nM. Additionally, the highest efficiencies recorded in each case (i.e. 87% with DF2, 86% with DF4 and 96% with HiPerFect) was with 50nM siRNA concentration. Although

the use of DF2 and DF4 promoted high siRNA uptake in each case, both also resulted in considerable cell toxicity (as determined by Trypan blue staining) particularly when used with the highest (100nM) siRNA concentration. Conversely, the use of HiPerFect promoted high uptake without significant loss of cell viability (Figure 3.10).

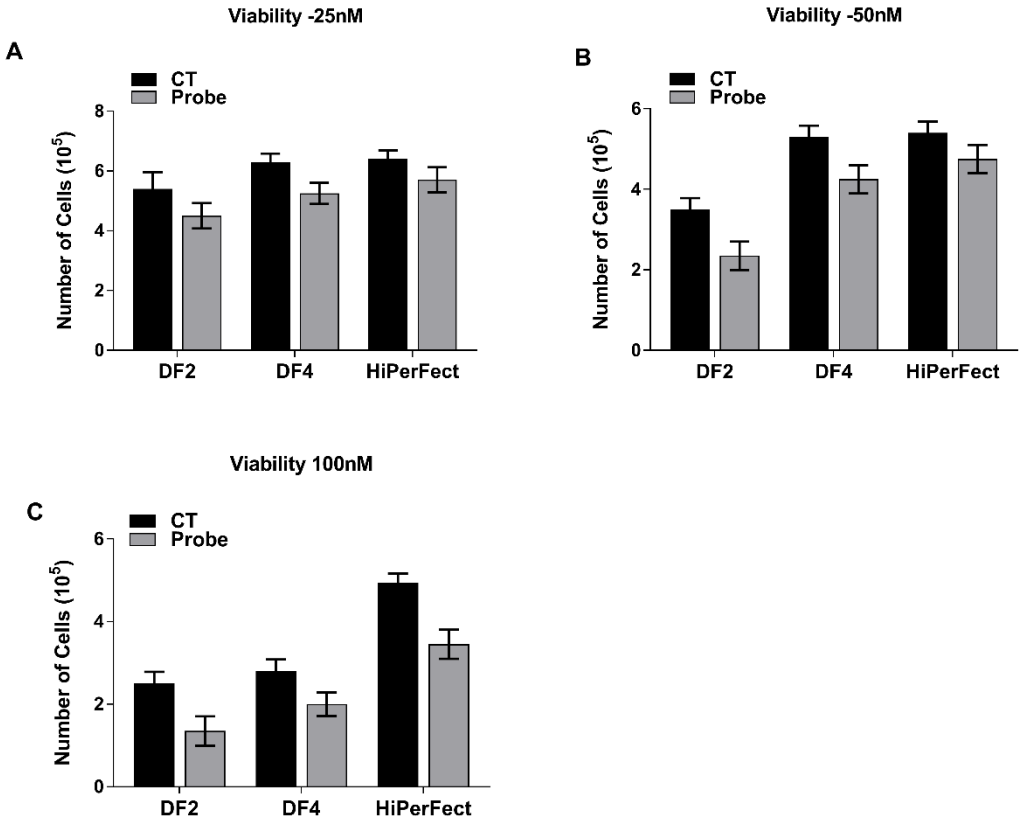


Figure 3.10: Monocyte-derived DC viability following siRNA transfection: Cells (10^6) were seeded in 24 well plates and transfected with increasing concentrations of siGLO RISC-free green transfection indicator using DF2, DF4 and HiPerFect transfection reagents respectively. Data is shown as mean ± SD of three independent experiments with two donors. Transfection efficiency was assessed on day 6 and cell viability was determined using Trypan blue staining. Monocytes were transfected on day 0 and differentiated into DCs after 6 hours of incubation.

3.3 Discussion

MicroRNAs represent a class of newly discovered gene regulators that bind the 3'UTR of target genes and cause translational inhibition or mRNA degradation (144,255). Although the functions of miRNAs in regulating the outcome of human diseases have been clearly demonstrated, their role in the development and function of antigen presenting cells particularly DCs is still being investigated. Here, the expression profile of miR-511-3p in human monocyte-derived DCs was examined and how its expression is modulated after treatment with LPS (TLR4 agonist) and CLR ligands (mannan and sulphated galactose). Tissue-resident DCs typically originate from bone marrow-derived progenitors including $CD14^+$ monocytes, which migrate to peripheral tissues and further develop into DCs (270,271). Factors that regulate DC differentiation and acquisition of immune stimulatory capacity are still subjects of intense investigation. Inflammatory cytokines such as GM-CSF and IL-4 have been demonstrated to drive DC differentiation from monocytes (272). However, several other signals may also play an important role in the overall process.

Initial examination of miRNA expression showed that miR-511-3p is highly expressed in monocyte derived DCs compared to their monocyte precursors. Interleukin (IL)-4 and IL-13, cytokines that play key roles in Th2 mediated allergic responses, have been demonstrated to induce miR-511 expression in M2 type macrophages in mice, as opposed to M1 macrophages (234). Moreover, IL-4 in particular drives the *Mrc1* gene expression in mice, which houses miR-511 in its fifth intron (232,273). In the present study, DCs were differentiated in the presence of IL-4 and GM-CSF over 6 days, which could account for the observed upregulation in miR-511-3p expression in DCs. Some studies have also indicated that miR-511 is significantly induced in $CD14^+$ monocytes

from rheumatoid arthritis (RA) patients, but expressed at low copy numbers in healthy monocytes (http://theses.gla.ac.uk/6418/; unpublished data).

The data obtained in this study indicates that miR-511-3p expression in human Mo-DCs is significantly downregulated upon treatment with LPS. Similar results relating to suppression of miR-511 expression has been demonstrated in mice following treatment of macrophages with IFN-γ and heat-inactivated *E.coli* (234). Several miRNAs are differentially expressed in immune cells following LPS treatment, most likely to protect against TLR4-mediated inflammation, as has been demonstrated for miR-155 and miR-146a (274-276). Interestingly, overexpression of miR-511 has also been shown to protect against LPS-induced endotoxemia in SPRET/Ei mouse models, which show dominant resistance against TNF induced shock (233). Recently, global profiling studies of monocyte-derived macrophages and DCs identified several novel LPS-responsive miRNAs, including miR-511 and showed that overexpression of miR-511 increased TLR4 expression, thereby acting as positive regulator of TLR4 in DCs (263,277). In this study, miR-511-3p was downregulated in response to TLR4 stimulation with LPS and its expression was varied according to the LPS dosage. Nonetheless, the mechanisms leading to LPS-mediated downregulation of miR-511-3p in human DCs are not fully understood. It is possible that downregulation of MR expression triggered by DC maturation with LPS (278,279), may drive suppression of miR-511-3p expression. Assessment of DC phenotypic expression following treatment with LPS showed that MR and DC-SIGN, which are highly expressed on immature monocyte-derived DC, are subsequently downregulated upon maturation. The functional and phenotypical changes that occur during DC maturation have been described (280,281) but generally involves reduced phagocytic activity and redistribution of surface receptors including those involved in antigen uptake (266,280).

This is in line with the data obtained showing downregulation of MR and DC-SIGN expression after DC maturation. Moreover, these changes correlated with up-regulation of the co-stimulatory molecules CD80, CD83, CD86 and HLA-DR for interaction with antigen-specific T cells. As observed, there were no changes to TLR4 surface expression before and after treatment with LPS.

Next, the effect of CLR engagement on miR-511-3p expression was also examined. The data obtained indicates that treatment of iDCs with mannan is able to upregulate miR-511-3p expression compared to untreated controls. However, mechanisms that promote miRNA expression and downstream functions following CLR engagement in DCs are currently unknown. Recently, it has been demonstrated that the CLR, CD69 is required for normal development of thymus-derived regulatory T cells by regulating miR-155 and its target, suppressor of cytokine signaling-1 (SOCS-1) (282). It is however likely that treatment with IL-4 may account for the increase in miR-511-3p expression as this induces MR expression in macrophages (273). Moreover, stimulation with mannan or sulphated galactose did not promote DC maturation. Conversely, there was a decrease in MR expression following treatment with the CLR ligands. Mannan in particular has been shown to inhibit binding to MR as well as DC-SIGN in competition studies by saturating these receptors (283,284), which could potentially account for the decrease in surface expression. However, it is unknown how mannan or SO_4-3-Gal-PA binding upregulates miR-511-3p expression in human DCs.

To achieve high effectiveness of exogenously introduced genetic material, optimization of transfection experiments are necessary, as failure results in undetectable effects in culture conditions. Transfection using cationic, liposome-based reagents are currently among the most frequently used and cost-effective methods for introducing genetic material (RNAi) into cells (285). However, its delivery particularly into primary cells

is thought to be challenging (286). Moreover, the effectiveness of transfection reagents is largely dependent on the cell type. For instance, some fibroblast, T cell, neuron and epithelial cell lines have shown resistance to commonly used cationic transfection reagents (287-289). Alternative approaches that have been demonstrated include the use of electroporation and virus-mediated delivery (290,291); however, these methods can be cytotoxic, disrupting cell function. Recently, delivery platforms including cationic polymers, inorganic nanoparticles, lipidoids and cationic protein-antibody fusions have been introduced that offer advantages in terms of stability, ease of preparation and cytotoxicity (292-295). In general, cationic lipids used as transfection reagents work by interacting with the negatively charged RNA/DNA molecules, forming lipoplexes through electrostatic forces that are eventually endocytosed by cells (296). In this study, the suitability of three lipid-based transfection reagents to deliver siRNA into primary monocyte-derived DCs was assessed with respect to the quantity of siRNA uptake and effect on cell viability. As shown, all three reagents promoted uptake of labelled siRNA, although the level of uptake varied according to the concentration of siRNA used. Lower transfection efficiency as shown with low siRNA concentration (25nM) leads to lower gene silencing, not as a result of siRNA quality, but because cells unaffected by transfection contribute to the overall observed target gene level. On the other hand, although the use of higher siRNA concentrations (100nM) resulted in a higher percentage of transfected cells, these also resulted in increased cell death.

In conclusion, it has been demonstrated that immature dendritic cells induce expression of miR-511-3p compared to $CD14^{+}$ monocytes; however, this upregulation of miR-511-3p expression is modulated depending on the nature of the stimulus, with DC maturation by LPS downregulating miRNA expression, whereas CLR engagement with

mannan upregulates miRNA expression. Furthermore, it has been demonstrated that HiPerFect transfection reagent provides optimal uptake of siRNA with minimal cell toxicity, serving as a useful tool for better understanding of the role of miR-511-3p in human dendritic cell phenotype and function.

Chapter 4: The Role of miR-511-3p in Modulating Dendritic Cell Phenotype and Downstream Immune Responses

Chapter 4: The role of miR-511-3p in modulating dendritic cell phenotype and downstream immune responses

4.1 Introduction

MicroRNAs are crucial players in mammalian gene regulation. Their role in fine-tuning gene expression, particularly through dysregulated expression has been reported in several human disorders including majority of cancers, cardiovascular, infectious and inflammatory diseases (208,228,297-300). More than 2000 miRNAs have been identified in the human genome primarily through sequence analysis and target prediction studies and each has the potential to regulate several hundreds of mRNAs (301). Of that number however, only few have been experimentally validated. Currently, majority of *in-vitro* studies rely on loss- or gain-of-function experiments, which involve manipulation of miRNA expression with antisense inhibitors or synthetic mimics (176,302). Loss-of-function approaches (i.e. genetic knockouts, sponges or oligonucleotide inhibitors) are widely preferred because their effect on target mRNA is observed by inhibiting a physiological concentration of the miRNA. This is achieved through binding of the synthetic miRNA to endogenous miRNA (via reverse complementarity), sequestering and making it unavailable for normal function (303,304). On the other hand, addition of synthetic exogenous miRNA may result in activation of undesirable cell responses or inhibition of non-physiological mRNA targets (305,306). Nonetheless, transfection with miRNA inhibitors or mimics, although transient, can provide useful information when culture conditions are optimised, ensuring adequate investigation of the endogenous functions within a cell.

Allergic diseases are typically characterized by the differentiation and expansion of allergen-specific Th2 cells, leading to the production of IgE antibodies and sensitization of mast cells (76,307). Allergy currently affects millions of people worldwide and

represents a huge burden in terms of morbidity and costs (308). Despite recent advances, the mechanisms leading to Th2-mediated inflammatory responses are still unclear. Allergic reactions are initiated by the recognition and uptake of allergens by antigen presenting cells (APCs), mainly dendritic cells (DCs) and culminate in Th2 cell polarisation and cytokine production (20). DCs have been shown to play a significant role in the induction and re-elicitation of Th2-driven responses during allergy (79,309,310), however the mechanisms underlying these events remain unclear.

Several studies have demonstrated that CLRs on DCs, such as the mannose receptor (MR) and DC-SIGN play a major role in uptake of glycosylated antigens and are vital in tailoring innate and adaptive immune responses to pathogens and allergens (18,311,312). Like other CLRs, MR and DC-SIGN possess at least one CRD with conserved motifs that determine their specificity to carbohydrate ligands (17,21,313). Consequently, ligation of CLRs with antigen induces downstream pathways in DCs leading to Th polarisation. Additionally, it is increasingly apparent that CLR activation is able to modulate TLR-induced signaling pathways, affecting NF-κB function and Th polarisation. Previously, our group demonstrated that MR in particular mediates allergen-driven Th2 adaptive immune responses following allergen uptake (24,314). This was evidenced by a reversal of Th2 to Th1 responses when MR-deficient DCs were exposed to Der p 1, the major allergen from house dust mite (HDM), in the presence of LPS. More recently, our group investigated the molecular basis of these events and have shown partial involvement of IDO and the aryl hydrocarbon receptor (AhR), which converge on components of the non-canonical NF-kb signaling pathway (116). IDO is a rate-limiting enzyme that mediates tryptophan metabolism and is expressed in a variety of cell types including DCs where it has been shown to control several immune regulatory processes (107,315). Furthermore, the finding that a subset

of DCs expressing IDO inhibit proliferation of T cells is indicative of IDO's involvement in the development of immune tolerance (316-318). However, given the role of MR in host defence and in modulating downstream events including IDO, it is important to gain further insight into regulatory mechanisms that impact MR expression and downstream DC function.

As previously mentioned, miR-511-3p has been shown to regulate the outcome of several human diseases as well as development and differentiation of immune cells particularly macrophages and DCs. Considering that miR-511 is embedded within the *MRC1* gene that encodes MR, it was hypothesized that it may play a role in regulating MR expression on human DCs, which would in turn modulate IDO activity and other downstream events leading to Th polarisation The previous chapter demonstrated that miR-511-3p is significantly induced in human dendritic cells and is modulated by LPS (TLR4) and CLR stimulation with specific ligands. This chapter examines the effect of miR-511-3p manipulation with antisense inhibitors and synthetic mimics on MR expression and overall Mo-DC phenotype and function particularly within the context of cytokine secretion, CLR induced modulation of IDO activity and downstream immune modulatory effects. Better understanding of how miR-511-3p modulates DC function will pave way for development of therapies against a range of inflammatory disorders.

4.2 Results

4.2.1 Transfection of human DCs with miR-511-3p inhibitors and mimics

In order to determine the effect of miR-511-3p expression on DC phenotype and function, DCs were differentiated from monocytes in the presence of specific inhibitors and mimics for miR-511-3p expression. Cell viability following miRNA transfection was determined using the Annexin V-FITC/PI staining kit (Figure 4.1).

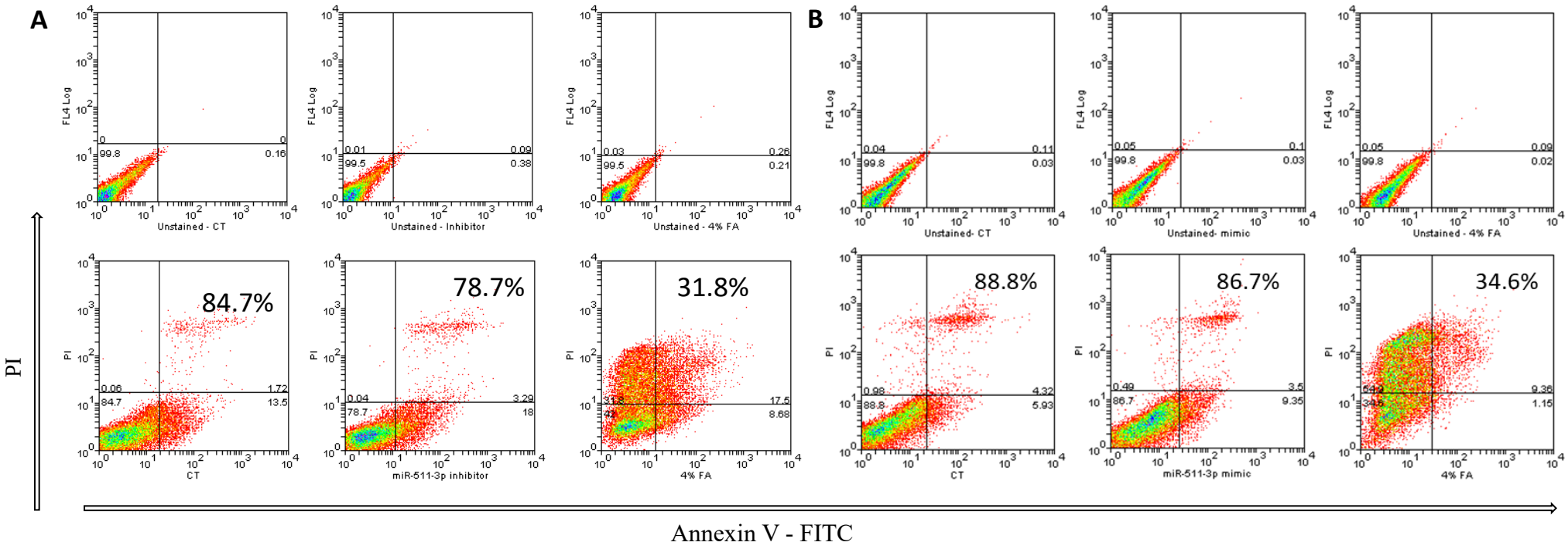
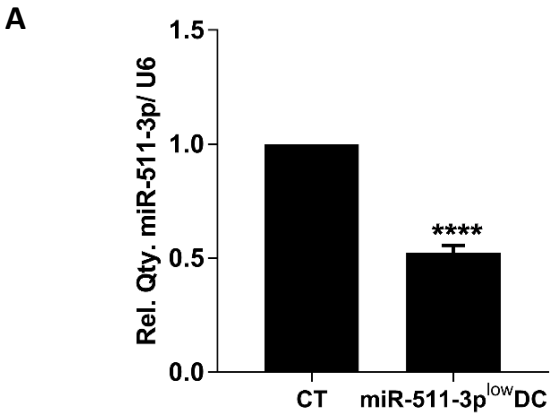


Figure 4.1: Examining apoptotic effect of miR-511-3p transfection on iDCs. Representative dot plots are shown for monocyte-derived DCs transfected with miR-511-3p A) inhibitors and B) mimics and harvested on day 6 for viability analysis with Annexin V apoptosis detection kit. Top panels represent unstained plots for scrambled controls (CT), inhibitor/mimic and formaldehyde treated positive controls. Cells were analysed by flow cytometry within 30 minutes of staining. In both A and B, representative data shown is from one independent experiment with a single donor out of a total of two donors.

The data obtained indicates that transfection with miR-511-3p inhibitors and mimics does not exert a significant influence on DC viability when used at 50nM concentration. As shown, more than 80% of cells remained viable after transfection with scrambled controls (negative) compared to 78% and 88% for inhibitors and mimics respectively. Additionally, cells in early apoptosis, displayed on the lower right quadrant were higher compared to necrotic cells, indicating that cell death may be due to factors other than miRNA treatment. As positive control, 4% formaldehyde solution was used, which clearly induced significant necrosis and cellular death in iDCs (Figure 4.1).

4.2.2 Changes in miR-511-3p expression levels affect MR and DC-SIGN expression in DCs

Next, surface marker expression for iDC was examined following treatment with miR-511-3p inhibitors and mimics to determine whether down or up regulation of miR-511-3p affects expression of key DC phenotypic markers. Transfection efficiency for introducing miR-511-3p inhibitors was above 90% (data not shown) and resulted in more than 50% downregulation of miR-511-3p expression (henceforth referred to as miR-511-3p^{low}) (Figure 4.2).



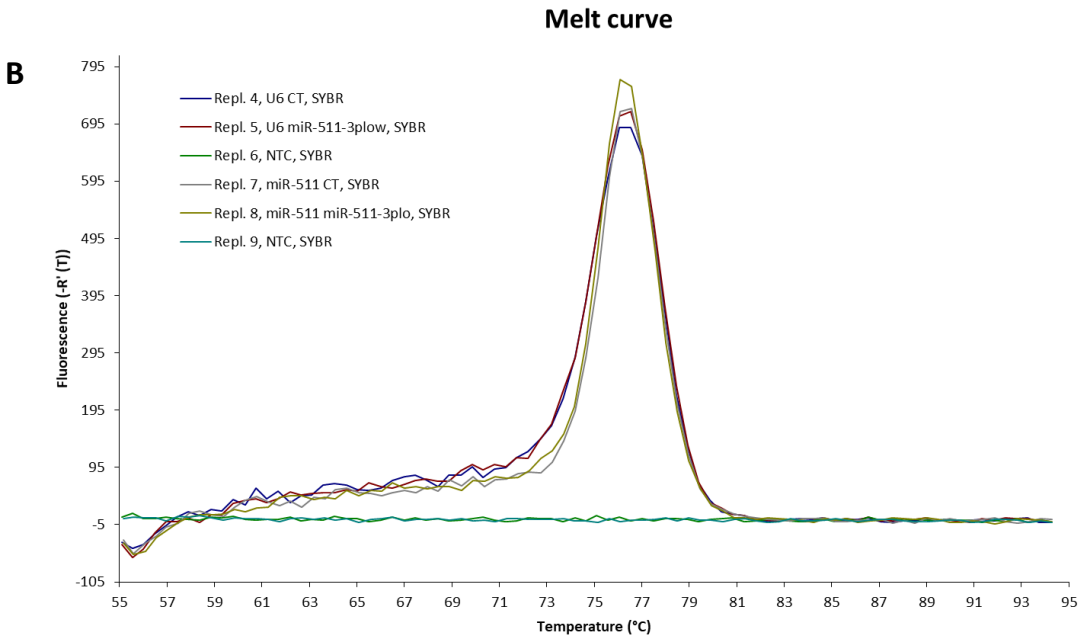
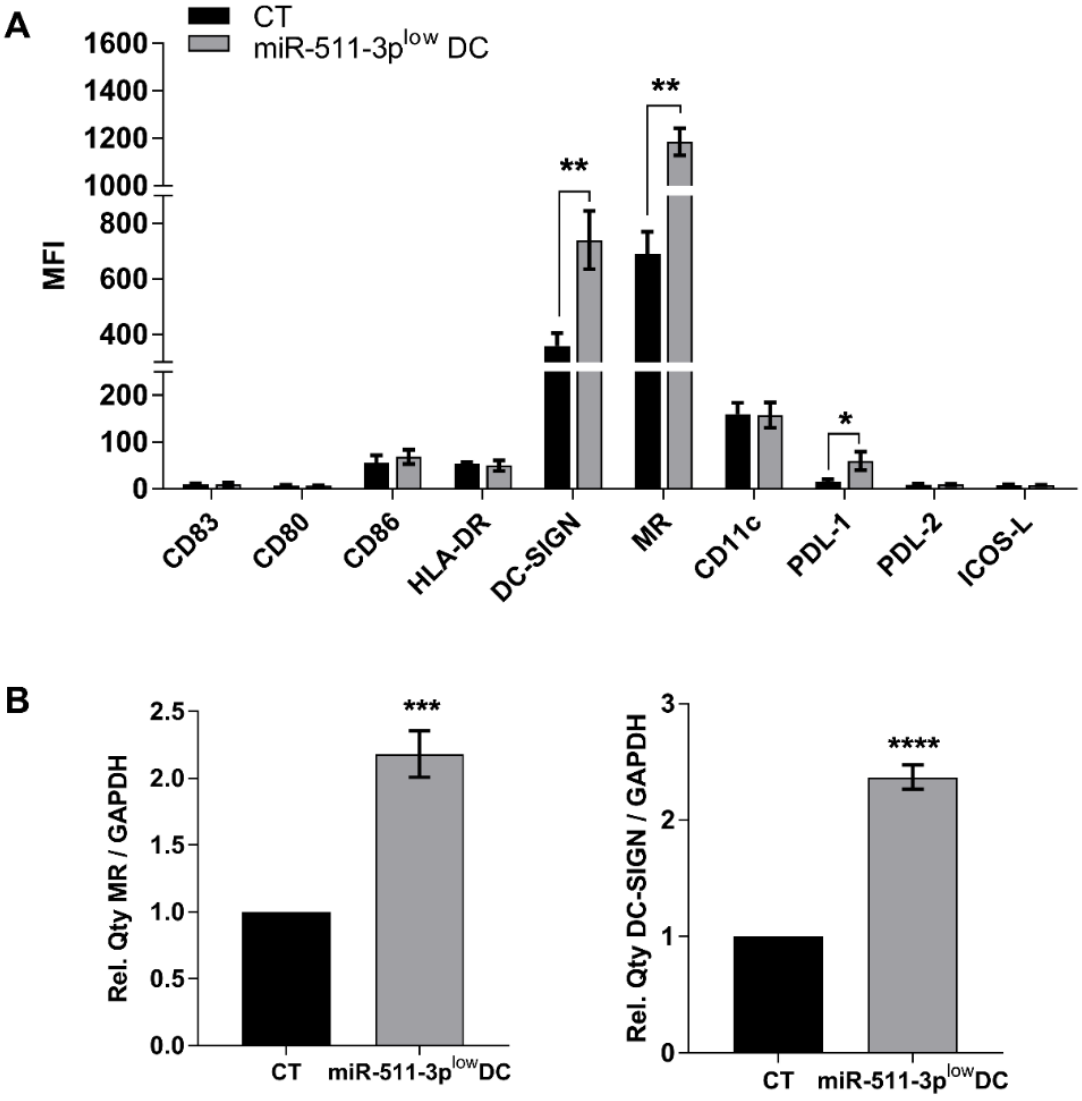


Figure 4.2: Silencing miR-511-3p expression in human DCs. A) qRT-PCR analysis of miR-511-3p expression in scrambled control (CT) and miRNA knockdown DCs. Monocytes were transfected with 50nM miR-511-3p inhibitor and negative control. Relative expression was normalised to RNU6. Data is represented as mean ± SD of three independent experiments with three biological replicates (donors), performed in triplicates. ****$p < 0.0001$ (Student's t-test). B) Representative melt curve analysis of qRT-PCR for analysis of miR-511-3p knockdown.

Interestingly, data obtained demonstrates for the first time that miR-511-3p^{low} DCs showed a significant increase in MR (CD206) and DC-SIGN (CD209) protein expression as measured by flow cytometry. This upregulation was also observed at the mRNA level where real time PCR analysis of the miR-511-3p^{low} cells showed a two-fold increase in MR and DC-SIGN mRNA levels compared to controls (Figure 4.3B). On the other hand, no changes were observed in expression of maturation (CD83) or co-stimulatory markers (CD80 and CD86), except PDL-1, which was significantly upregulated in the miR-511-3p^{low} cells (Figure 4.3).


A
CT
miR-511-3p^{low} DC
MFI
1600
1400
1200
1000
800
600
400
200
100
0
**
**
*
CD83
CD80
CD86
HLA-DR
DC-SIGN
MR
CD11c
PDL-1
PDL-2
ICOS-L
B
Rel. Qty MR / GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
***
CT
miR-511-3p^{low}DC
Rel. Qty DC-SIGN / GAPDH
3
2
1
0
****
CT
miR-511-3p^{low}DC

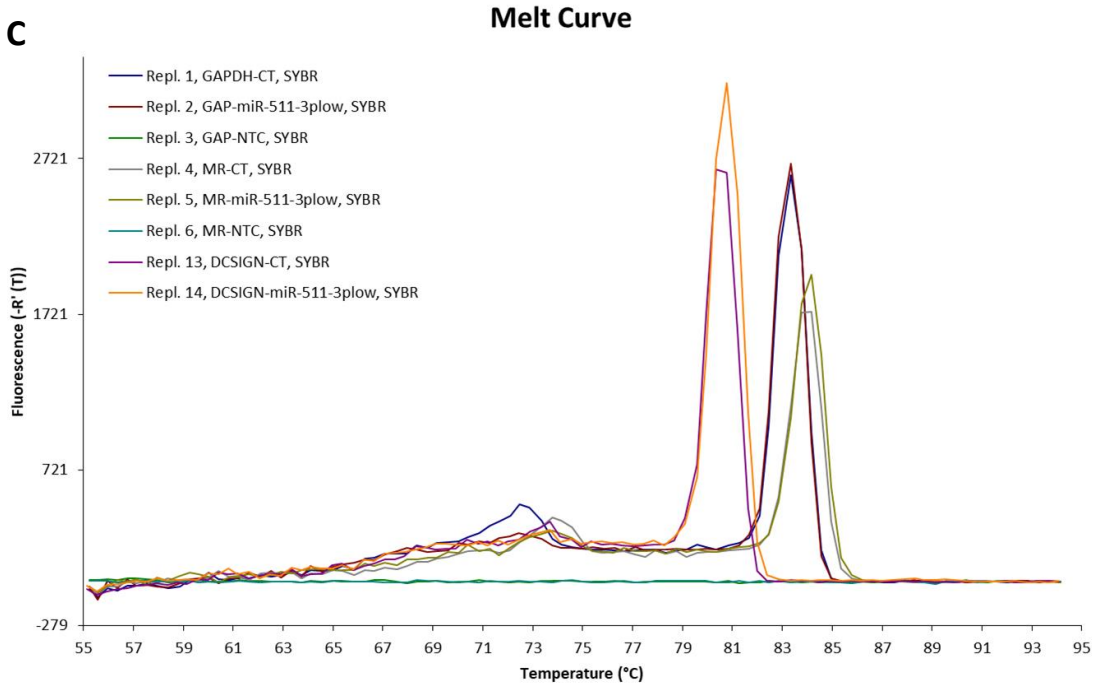


Figure 4.3: Flow cytometry and qRT-PCR analysis of miR-511-3p^{low} DC. A) Flow cytometry analysis of DC phenotypic markers following treatment with miR-511-3p inhibitors. Data is represented as mean ± SD of three independent experiments from three biological replicates*p < 0.05, **p < 0.01 (Student's t-test). Median fluorescence intensity (MFI) values are shown. B**)** Relative expression of MR and DC-SIGN mRNA in miR-511-3p^{low} DCs. Data is represented as mean ± SD of three independent experiment with three biological replicates (donors). ***p < 0.001, ****p < 0.0001 (Student's t-test). C) Representative melt curve analysis of qRT-PCR for MR and DC-SIGN expression respectively. Monocytes were transfected on day 0 with 50nM miRNA inhibitor and differentiated. Relative expression was assessed on day 6 and is normalised to GAPDH.

Additionally, there were no significant changes in the expression of the LPS receptor complex; CD14, TLR4 and MD-2 in miR-511-3p^{low} cells (Figure 4.4).

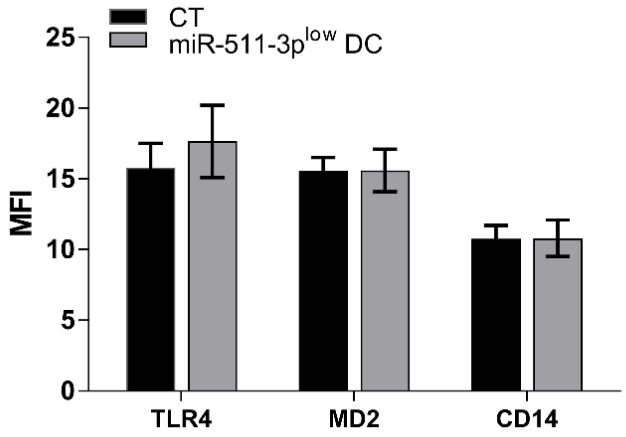


Figure 4.4: miR-511-3p^{low} DCs express normal levels of LPS receptor complex. Flow cytometry analysis for TLR4, CD14 and MD-2 surface expression. Data is represented as mean ± SD of three independent experiments with three biological replicates (donors). Cells were collected on day 6 and analysed. MFI values are shown.

Following the observed effect of miR-511-3p knockdown on MR and DC-SIGN, the effect of increased expression of miR-511-3p on DC phenotype was investigated. To this end, DCs were transfected with 50nM miRNA mimic, which resulted in a significant increase in miR-511-3p expression in DCs, (henceforth referred to as miR-511-3p^{hi}) (Figure 4.5). Interestingly, this upregulation resulted in a significant decrease in MR and DC-SIGN expression at both protein and mRNA levels (Figure 4.6B).

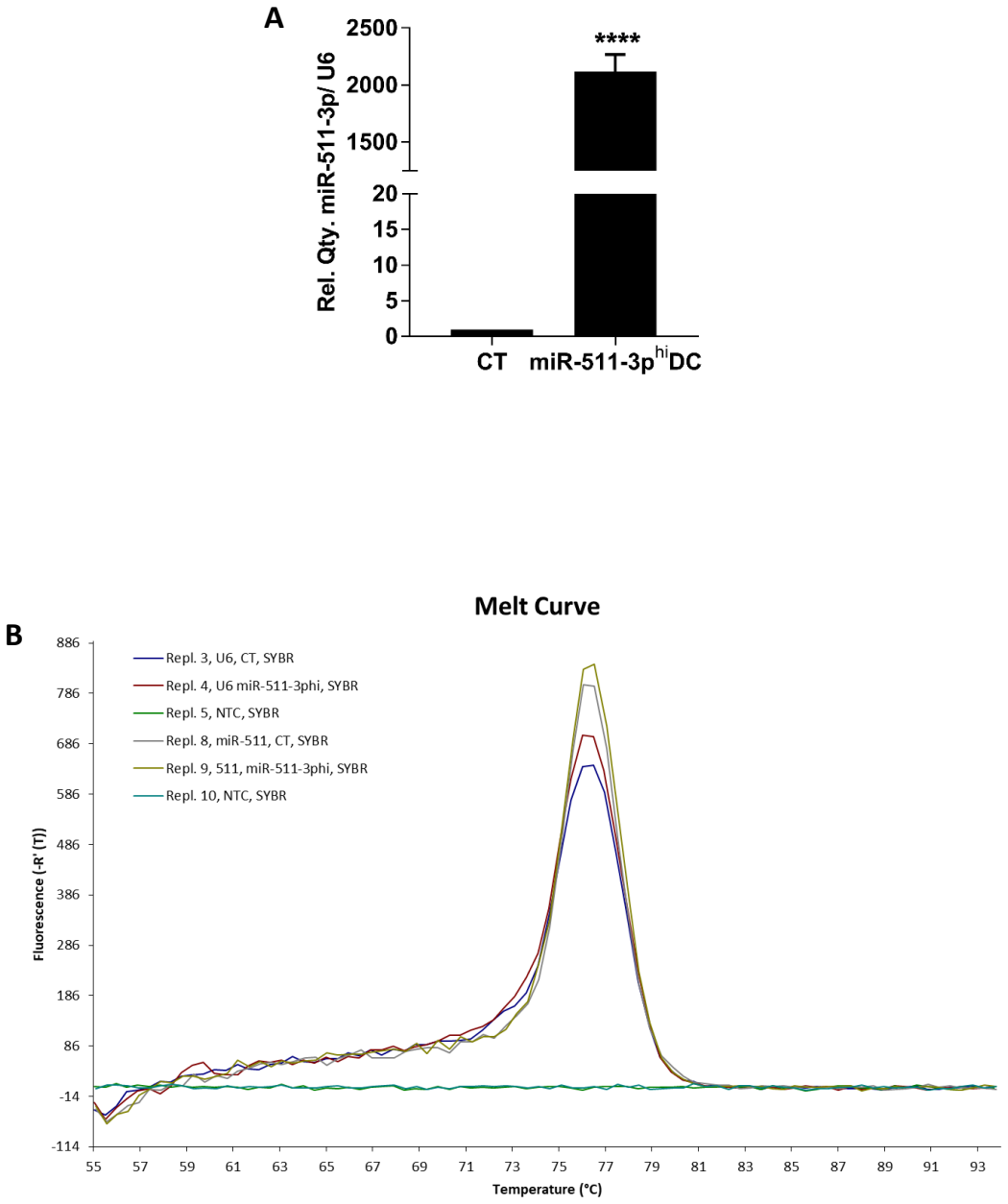


Figure 4.5: Overexpression of miR-511-3p in human DCs. A) qRT-PCR analysis of miR-511-3p expression in scrambled controls (CT) and miR-511-3p overexpressed DCs Data is represented as mean ± SD of two independent experiment with two biological replicates (donors) ****p < 0.0001 (Student's t-test). Monocytes were transfected with 50nM mimic and control. Overexpression was assessed on day 6. Relative expression was normalised to RNU6. B) Representative melt curve analysis of qRT-PCT for miR-511-3p overexpression.

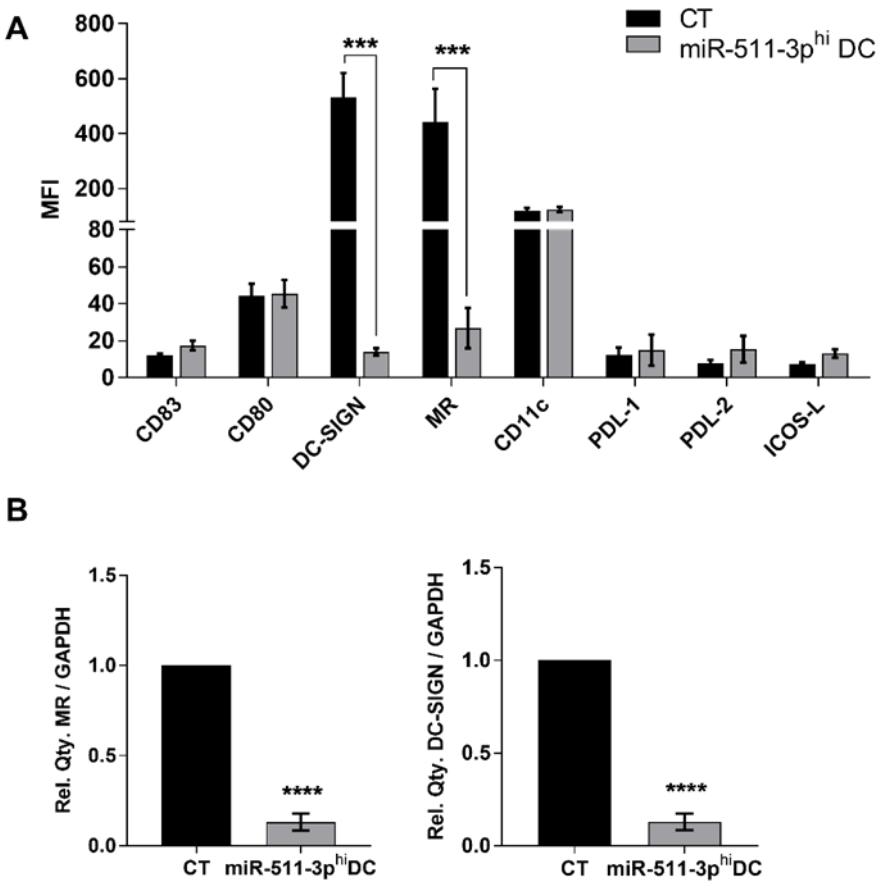


Figure 4.6: Flow cytometry and qRT-PCR analysis of miR-511-3p^{hi} DC. A) Flow cytometry analysis of DC surface markers following treatment with miR-511-3p mimics. Data is represented as mean ± SD of three independent experiments from three biological replicates. ***$p < 0.001$ (Student's t-test). MFI values are shown. B**)** Relative expression of MR and DC-SIGN mRNA in miR-511-3p^{hi} DCs. Data is represented as mean ± SD of one independent experiment with three biological replicates (donors). ****$p < 0.0001$ (Student's t-test). Monocytes were transfected on day 0 and overexpression was assessed on day 6. Data is normalised to GAPDH. .

Additionally, the expression of CD86 and HLA-DR showed significant increase in these cells, however there were no changes in any other surface markers tested. As with miR-511-3p^{low} DCs, there were no changes in expression of the LPS receptor complex (Figure 4.7).

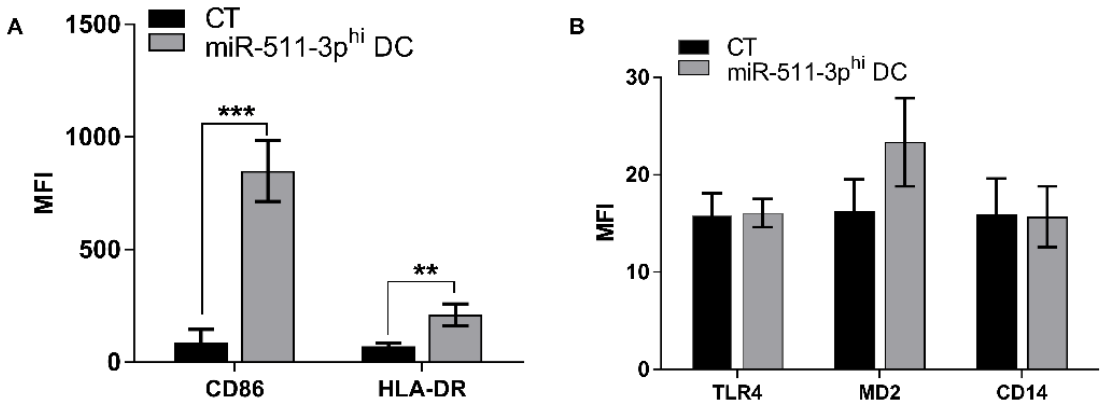


Figure 4.7: Surface marker expression in miR-511-3p^{hi} DCs. A) Flow cytometry analysis for CD86 and HLA-DR following miRNA overexpression. Data shown as mean ± SD of three independent experiments from three biological replicates. **p < 0.01, ***p < 0.001 (Student's t-test). MFI values are shown. B) MFI values for LPS receptor complex expression, TLR4, CD14 and MD-2; Data is shown as median fluorescence intensity (MFI). Data is shown as mean ± SD of three independent experiments from three biological replicates.

To better understand the impact of miR-511-3p up or downregulation on dendritic cell transcriptome, the differentially expressed genes (DEGs) between the two groups (miR-511-3p^{low} and miR-511-3p^{hi} DCs) as well as controls were analysed using Illumina gene expression arrays. Independent hierarchical clustering was visualised by a heat map (Figure 4.8), which revealed distinct groups of genes specifically upregulated or downregulated by each group (shown in red and blue blocks respectively).

Additionally, top 6 genes of interest in both conditions were visualised (Figure 4.8B) and is noteworthy that changes in MR and DC-SIGN protein levels as well as CD86 observed previously were in line with the preliminary transcriptome data. Furthermore, a volcano plot comparing the levels of gene expression between miR-511-3p inhibitor and mimic treatment was visualised. From Figure 4.9, there were 784 DEGs between both conditions, 417 of which were upregulated and 367 downregulated.

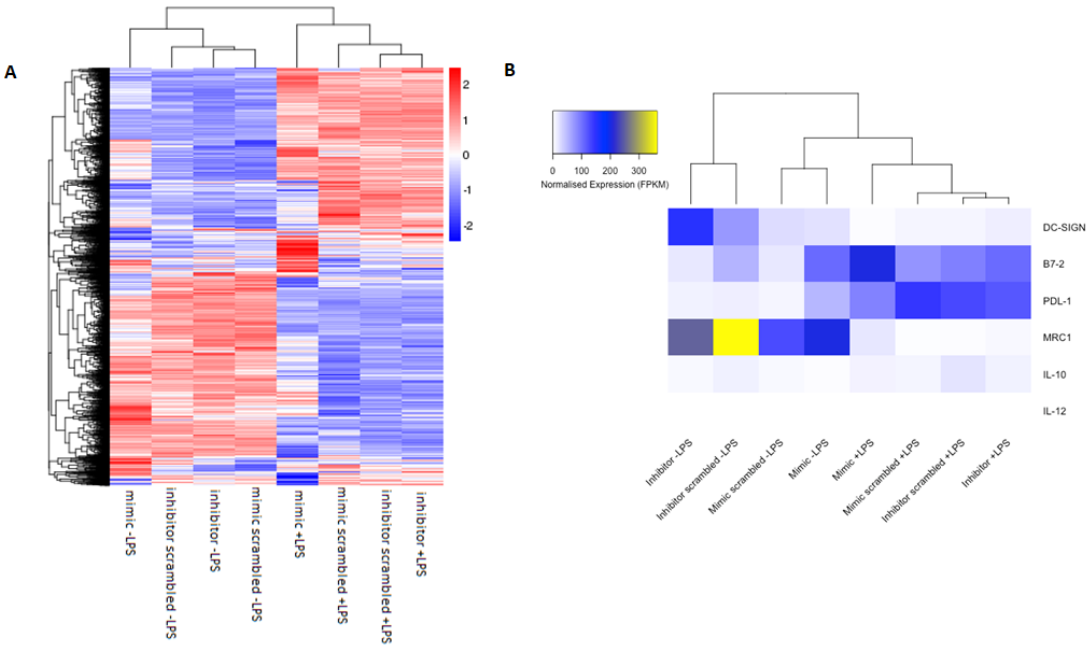


Figure 4.8: Heat maps of differentially expressed genes from preliminary RNA-seq data. A) Red and blue represents high and low expression genes respectively, indicated as log10 (FPKM+1) from large to small Data is from one independent experiment with a single independent donor B) Transcripts from top genes of interest matching mRNA expression with protein expression levels in observed data. Data is from one independent experiment with a single independent donor.

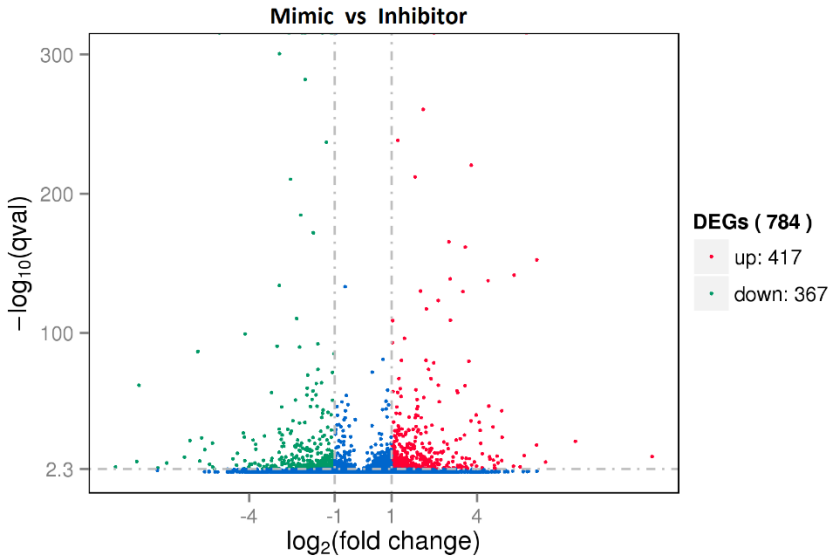
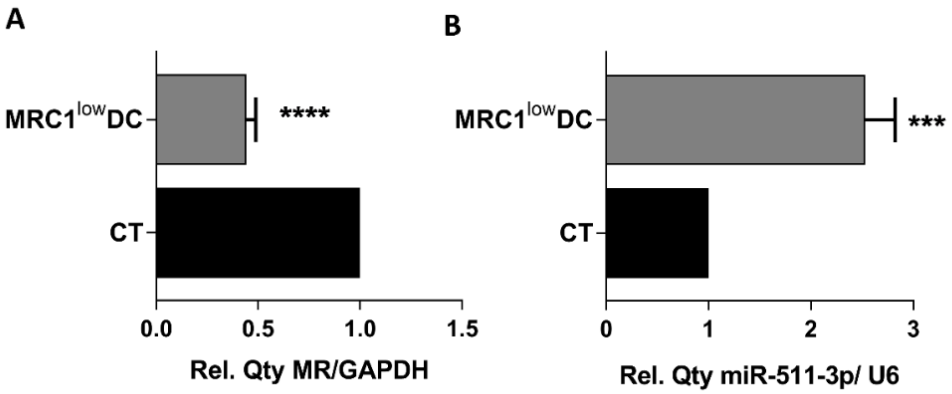


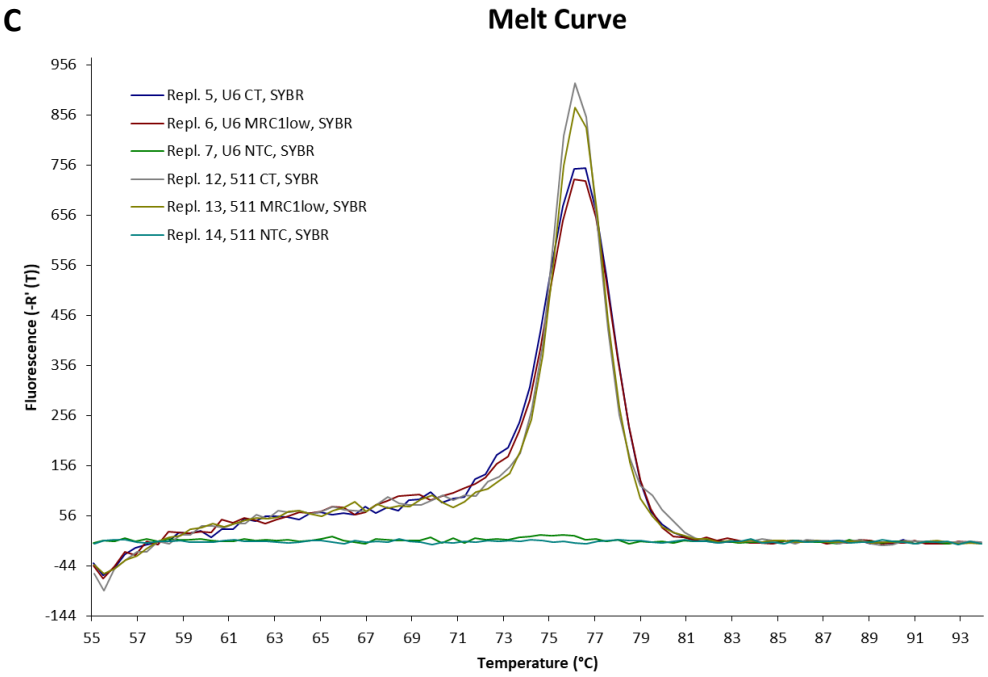
Figure 4.9: Overview of differential expression genes is displayed by volcano diagram. Volcano plot of differential gene expression between mimic and inhibitor treatment. The vertical axis (y-axis) corresponds to the mean expression value of log10 (q-value), and the horizontal axis (x-axis) displays the log2 fold change value. The red dots represent the up-regulated expressed transcripts; the green dots represent the transcripts whose expression down- regulatedThe threshold of differential expression genes is log2 (Fold Change)| > 1. Data represented is from one independent experiment with a single donor

4.2.3 Knockdown of *MRC1* upregulates miR-511-3p expression in human DC

In humans and mice miR-511-3p is embedded within the *MRC1* gene, and previous reports have suggested the existence of co-regulation between miR-511-3p and CD206/*mrc1* expression in M2-polarised tumour associated macrophages from mice (232). Considering the marked changes in MR expression in miR-511-3p^{low} cells in previous experiments, it was examined whether downregulation of *MRC1*, the gene controlling MR expression, would influence miR-511-3p expression in human DCs. Monocytes were differentiated into DCs in the presence of *MRC1* siRNA for 6 days and miR-511-3p expression was measured by qRT-PCR afterwards. *MRC1* knockdown (≥60%) was confirmed by measuring MR mRNA levels. Interestingly, it was found that

DCs showed a two-fold increase in miR-511-3p expression when *MRC1* was inhibited compared to transfected controls (Figure 4.10).





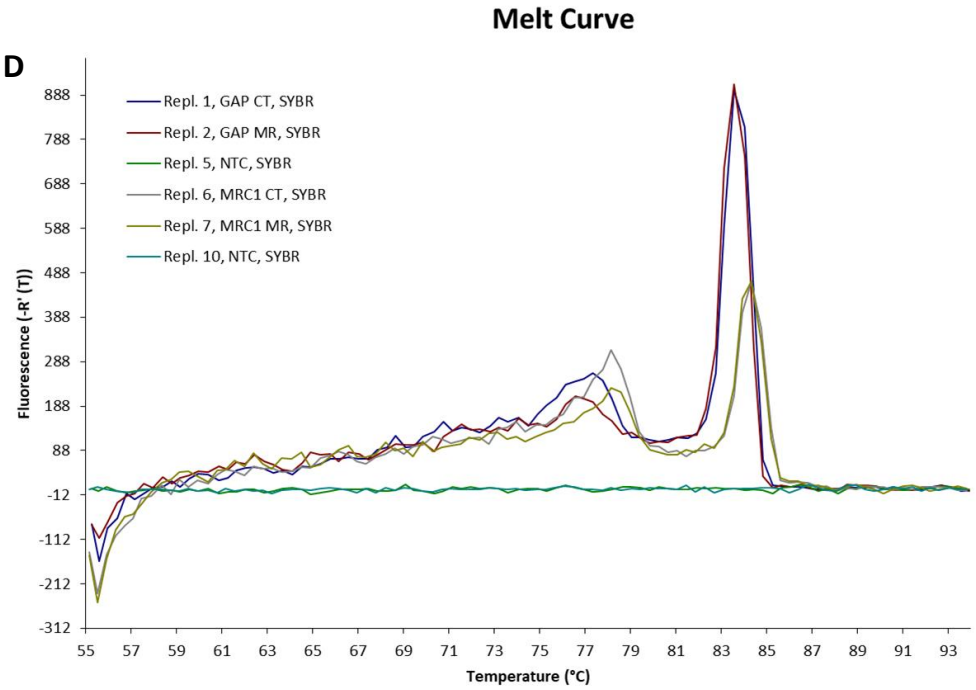


Figure 4.10 Knockdown of MRC1 upregulates miR-511-3p expression in human DCs A.) Gene silencing of *MRC1* in DCs. Data is represented as mean ± SD of two independent experiment with two biological replicates performed in triplicates. ****p < 0.0001 (Student's t-test). B) Relative expression of miR-511-3p in *MRC1*^{low} DC. Data is represented as mean ± SD of two independent experiment with two biological replicates performed in triplicates. ***p < 0.001 (Student's t-test). C and D) Representative melt curves of qRT-PCR for miR-511-3p and MR expression respectively are shown.

4.2.4 IDO activity is modulated by changes in miR-511-3p expression in DCs.

Our lab and others have previously shown that CLRs such as MR and DC-SIGN play key roles in allergen recognition and downstream events leading to Th2 development (23,24). In particular, mannan and selected glycosylated allergens, through MR engagement was shown to downregulate LPS-induced IDO activity in DCs, favouring Th2 polarisation (116). IDO mediates tryptophan metabolism and contributes to several immune regulatory processes, including the induction of regulatory T cells

(114,315,319). It was therefore investigated whether changes in MR and DC-SIGN expression levels following inhibition or enhanced expression of miR-511-3p would influence IDO activity in human DCs. Accordingly, miR-511-3p^{low} and miR-511-3p^{hi} cells were stimulated with 10µg/ml mannan in the presence and absence of LPS (0.1µg/ml) (Figure 4.11).

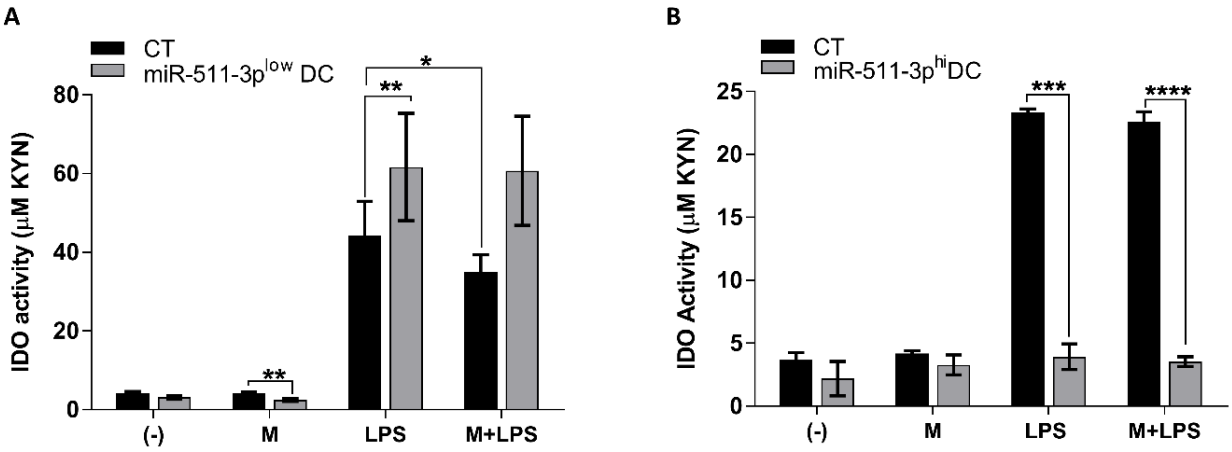


Figure 4.11: Modulation of miR-511-3p influences IDO activity. A) IDO activity in miR-511-3p^{low} DCs treated with mannan and LPS for 24 hours. Data is represented as mean ± SD of one independent experiment with three biological replicates (donors) performed in triplicates *p < 0.05, **p < 0.01 (2-way ANOVA with Tukey's post-hoc testing). B) IDO activity in miR-511-3phi DCs treated with mannan and LPS for 24 hours. Data is represented as mean ± SD of one independent experiment with three biological replicates (donors) performed in triplicates ***p < 0.001, ****p < 0.0001 (2-way ANOVA with Tukey's post-hoc testing). IDO activity was determined by colorimetric detection of KYN in culture supernatants.

As expected, a significant downregulation in IDO activity was observed when miR-511-3p^{low} cells were stimulated with mannan alone; however, in the presence of LPS, IDO activity was significantly upregulated, suggesting that miR-511-3p^{low} DCs could be potent suppressors of T cell responses, depending on the nature of the stimulus.

Conversely, miR-511-3p^{hi} cells showed no change in IDO activity when treated with mannan alone but showed a significant decrease in the presence of LPS (Figure 4.11B). Additionally, IDO activity was further examined in either MR^{low} or DC-SIGN^{low} DCs considering these receptors were affected by miR-511-3p expression. As shown, the absence of MR leads to an increase in IDO activity in mannan and M/LPS treated cells, whereas a decrease in DC-SIGN leads to a decrease in IDO activity (Figure 4.12)

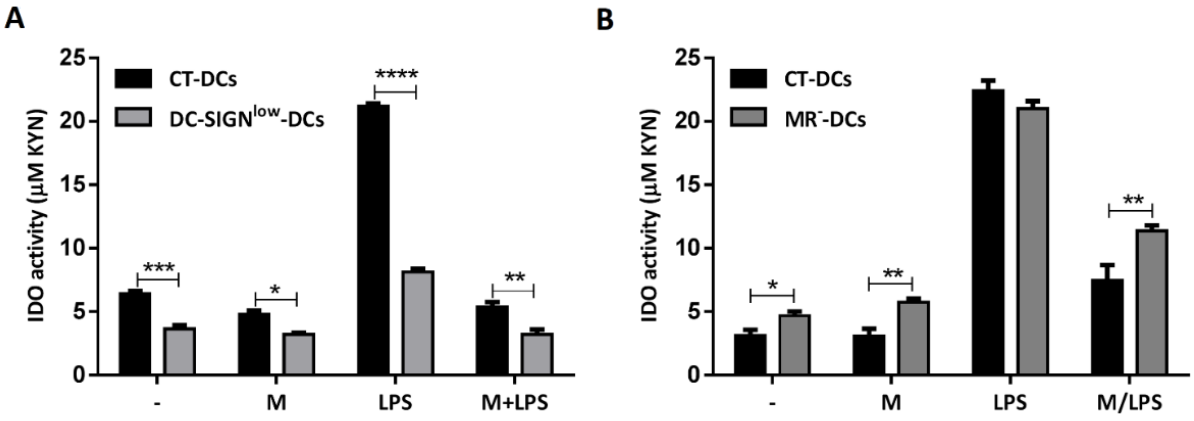


Figure 4.12. MR and DC-SIGN differentially regulate IDO activity in response to mannan in human DCs. A). IDO activity in CT and DC-SIGN^{low}-DCs stimulated with mannan (M) and LPS. Data is represented as mean ± SD of one independent experiment with two biological replicates (donors) performed in triplicates *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (2-way ANOVA with Tukey's post-hoc testing). B). IDO activity in CT and MR^{low}-DCs stimulated with mannan and LPS Data is represented as mean ± SD of one independent experiment with two biological replicates (donors) performed in triplicates *p < 0.05, **p < 0.01 (2-way ANOVA with Tukey's post-hoc testing). MR and DC-SIGN siRNA were the SMARTpool ON-TARGET plus siRNA from GE Healthcare. The ON-TARGET plus, non-targeting control siRNA was used as negative control.

4.2.5 Downregulation of miR-511-3p induces an anti-inflammatory DCs phenotype.

Depending on the nature of signals received, DCs secrete a variety of cytokines that drive downstream immune responses. Having examined the impact of miR-511-3p on expression of DC surface markers, the effect of miR-511-3p modulation on production of IL-10, IL-6, TNF-α, IL-12 and IL-1β in DCs, after treatment with LPS was investigated. A significant increase was observed in IL-10 and a decrease in IL-6 and IL-12p70 production in the miR-511-3p^{low} cells after treatment with LPS (0.1µg/ml) (Figure 4.13A). Although TNF-α and IL-1β production was downregulated, this did not reach statistical significance.

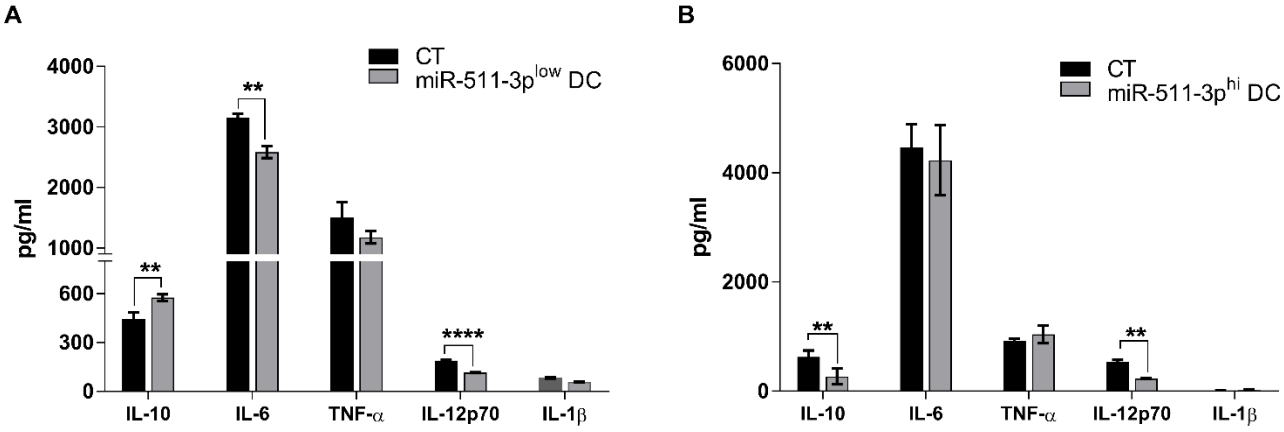


Figure 4.13: Cytokine secretion is modulated by miR-511-3p expression in human DCs. A) Cytokine production by miR-511-3p^{low} DCs stimulated with 0.1µg/ml LPS for 24 hours Data is represented as mean ±SD of two independent experiments with three biological replicates performed in duplicates. **p < 0.01, ****p < 0.0001 (Student's t-test). B) Cytokine production by miR-511-3p^{hi} DCs stimulated with 0.1µg/ml LPS for 24 hours. Data is represented as mean ±SD of two independent experiments with three biological replicates performed in duplicates. **p < 0.01 (Student's t-test). Culture supernatants were collected and examined by ELISA

In contrast to miR-511-3p^{low} cells, 1L-10 production in miR-511-3p^{hi} cells was decreased following stimulation with LPS. Also TNF-α and IL-1β showed a slight increase, however, this was not significant (Figure 4.13B). Interestingly, IL-12p70 showed a significant decrease in miR-511-3p^{hi} cells similar to the pattern observed for miR-511-3p^{low} cells, whereas no change was seen in IL-6 production. Furthermore, there was a significant increase in expression of PDL-1 on miR-511-3p^{low} cells, an observation which was absent in miR-511-3p^{hi} cells (Figure 4.14). No significant changes in cytokine production was observed in either knockdown or overexpressed conditions after treatment with mannan only (data not shown).

Principal component analysis (PCA) of the RNA-seq transcriptome data was done to compare the expression pattern of genes between miR-511-3p knockdown and overexpressed cells treated with or without LPS. As summarised in Figure 4.15A, three distinct clusters were formed with mimic treatment (M_511_L and M_511) clustering separately from inhibitor treatment and controls. Additionally, LPS treated miR-511-3p^{low} DCs as well as controls, clustered separately from unstimulated cells. Moreover, as shown from the dendrogram (Figure 4.15B), there were similarities in gene expression between unstimulated and LPS treated cells between the two conditions tested. It is however likely that major differences in gene expression would be found in miR-511-3p^{hi} DCs, which also exhibit similarities with inhibitor and controls hence clustering separately. From Figure 4.16, treatment with LPS may account for changes in gene ontology (GO) analysis of biological processes and molecular function

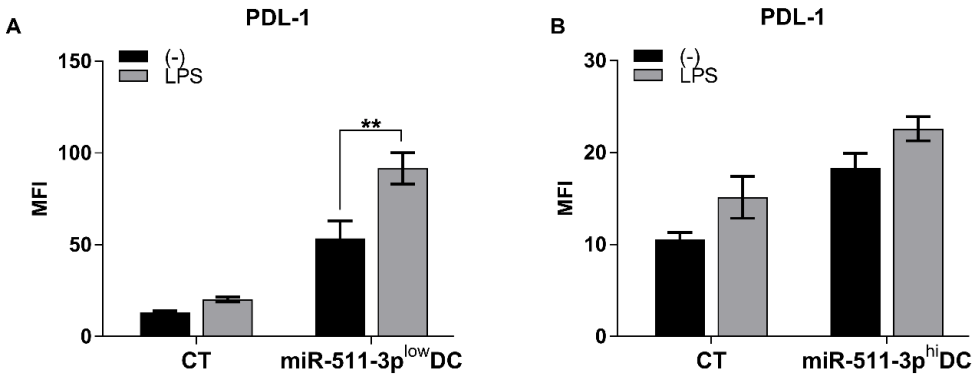


Figure 4.14: PDL-1 marker expression is modulated by changes in miR-511-3p expression in human DCs. A) PDL-1 expression by miR-511-3p^{low} DCs stimulated with 0.1µg/ml LPS for 24 hours. Data is represented as mean ±SD of three independent experiments with three biological replicates. **p < 0.01 (Student's t-test). B) PDL-1 expression by miR-511-3p^{low} DCs stimulated with 0.1µg/ml LPS for 24 hours Data is represented as mean ±SD of three independent experiments with three biological replicates performed in duplicates. MFI values are shown.

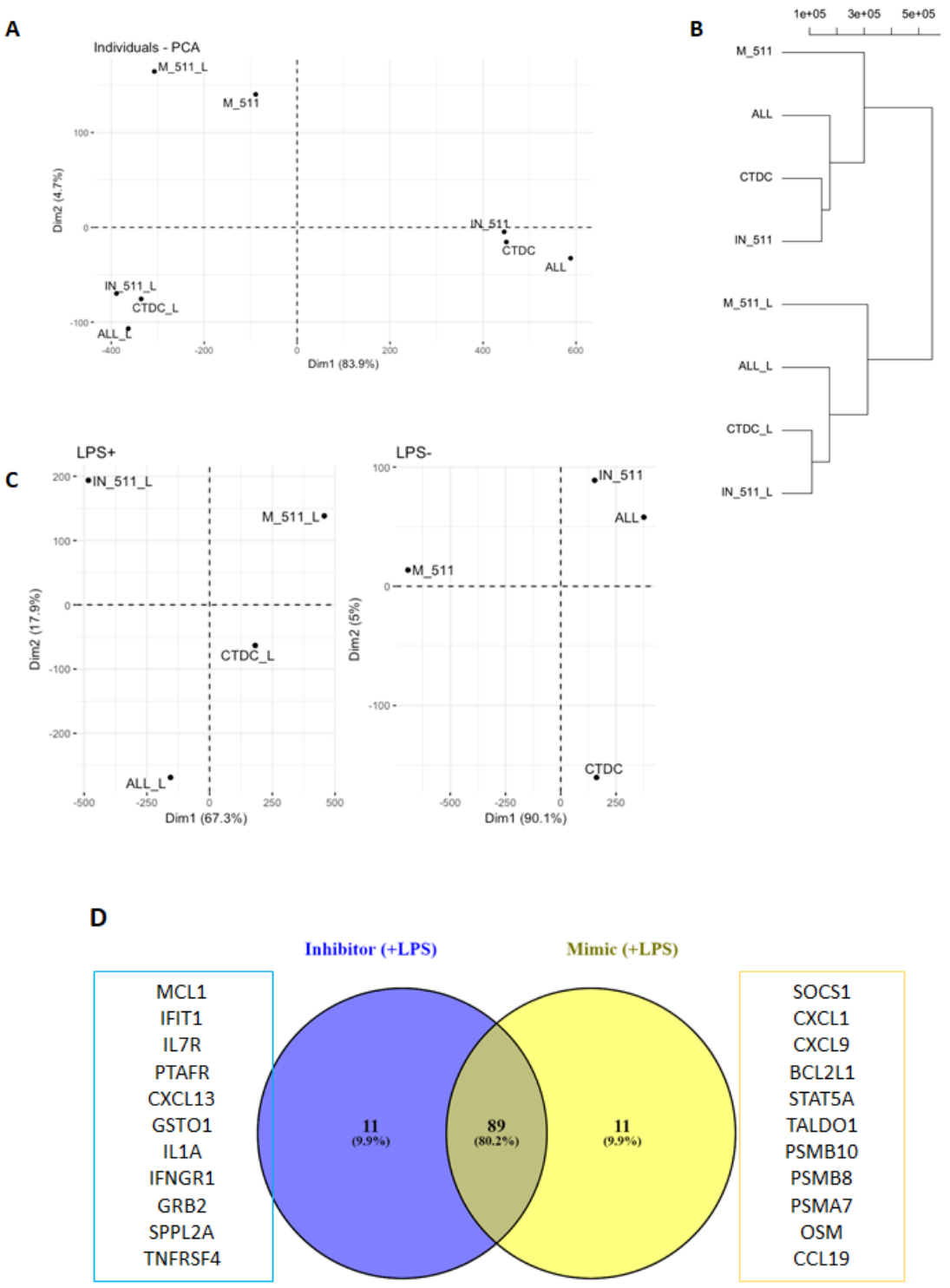


Figure 4.15: Principal Component Analysis and hierarchical clustering of normalised expression (FPKM). A and B) Treated miR-511-3p conditions and controls form three major clusters, with mimic clustering differently to inhibitor. C) LPS (+/-) is key in driving clustering of genes following miR-511-3p treatment. D) Top DEGs between mimic and inhibitor following LPS treatment. In each case, data represented is from one independent experiment each with a single donor.

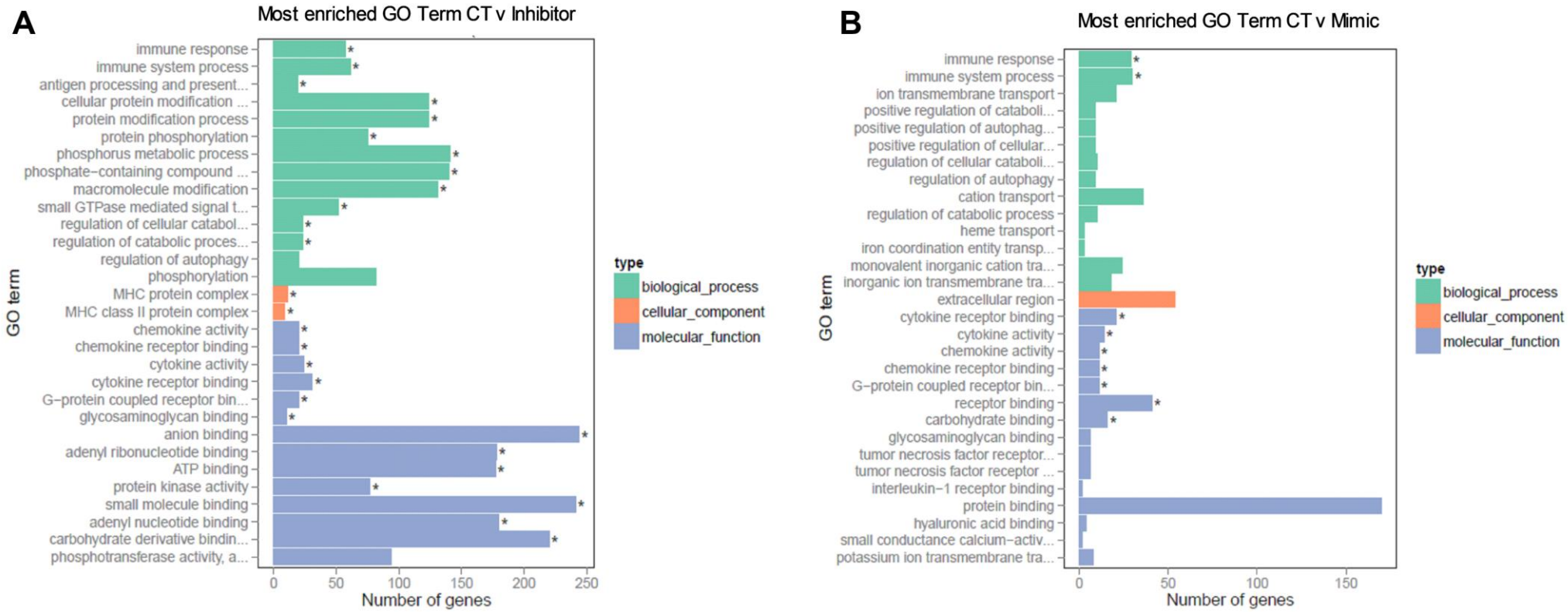


Figure 4.16. Histograms for differential GO Enrichment Analysis. The histograms display distribution of differential gene expression between miR-511-3p A) knockdown and B) overexpressed DCs in biological process, cellular component, and molecular function for the top 30 significantly enriched GO term. Vertical axis represents GO term and horizontal axis represents the number of the differential expression genes. In both A and B, data represented is from one independent experiment each with a single independent donor.

4.2.6 Changes in miR-511-3p expression in human DCs could modulate DC-induced T cell polarisation

Activation of T cells by DCs promote rapid induction of T cell proliferation and differentiation into distinct Th subsets such as Th1, Th2, Th17 and Treg, which facilitate various aspects of immunity. It was therefore examined whether up or down regulation of miR-511-3p expression in DCs affected T cell polarisation in autologous DC-T cell co-culture experiments. Immature miR-511-3p^{low} or miR-511-3p^{hi} DCs were stimulated with 0.1µg/ml LPS for 24 hours before co-culture with CD3^{+} autologous pan T cells in complete medium containing human AB serum. Control samples did not receive LPS. T cell responses were examined after 7 days of co-culture by measuring the level of cytokines produced in culture supernatants using the Human T cell response array from RayBio® as a screening platform.

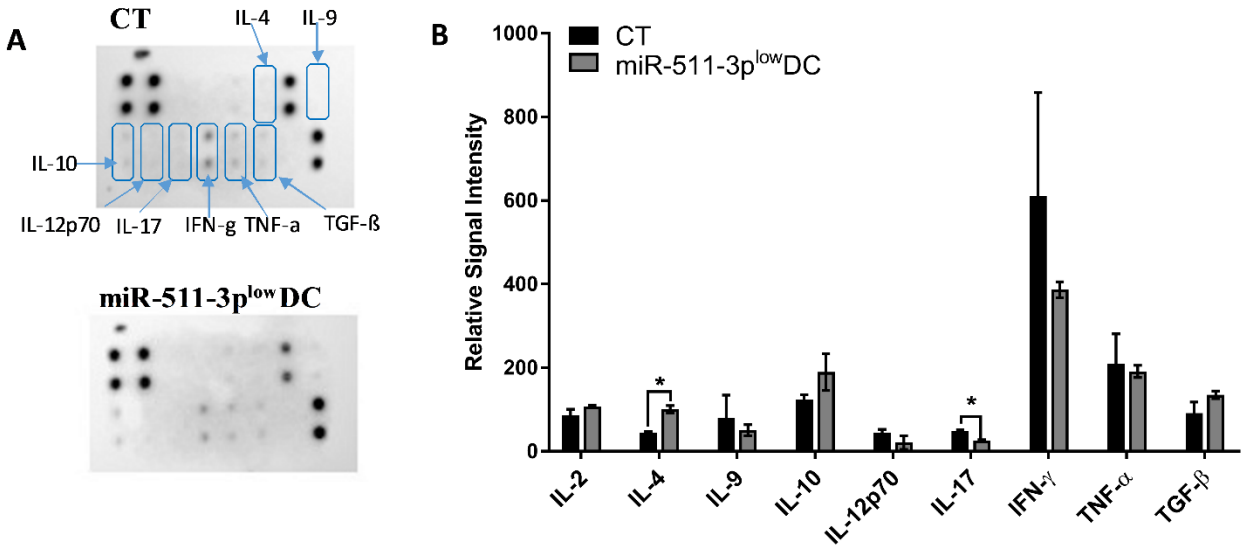


Figure 4.17: T cell cytokine responses from co-cultures with miR-511-3p^{low} DCs A) Array images of T cell cytokine responses from co-culture with miR-511-3p^{low} DCs. DCs were treated with 0.1µg/ml LPS before co-culture with autologous CD3^{+} T cells. Representative images shown are from one independent experiment with a single donor out of a total of two biological replicates (donors). B) Quantitative analysis of cytokine signal intensities from respective DC-T cell co-cultures. Data is shown as mean ±SD of

two independent experiments with two biological replicates performed in duplicates *p < 0.05 (Ordinary one-way ANOVA with Tukey's post-hoc testing)

Purity of isolated T cells was measured by flow cytometry. Interestingly, our data shows a significant increase in IL-4 and a decrease in IL-17 cytokine production from T cells co-cultured with miR-511-3p^{low} DCs, suggesting a bias towards Type 2 T cell responses (Figure 4.17). Additionally, IL-10 and TGF-β were also increased, whereas IFN-γ was decreased but these were not statistically significant. T cells co-cultured with miR-511-3p^{hi} DCs in contrast showed a significant decrease in TGF-β and IL-9 production. Although IL-4 and IL-10 were decreased, these changes did not reach statistical significant (Figure 4.18).

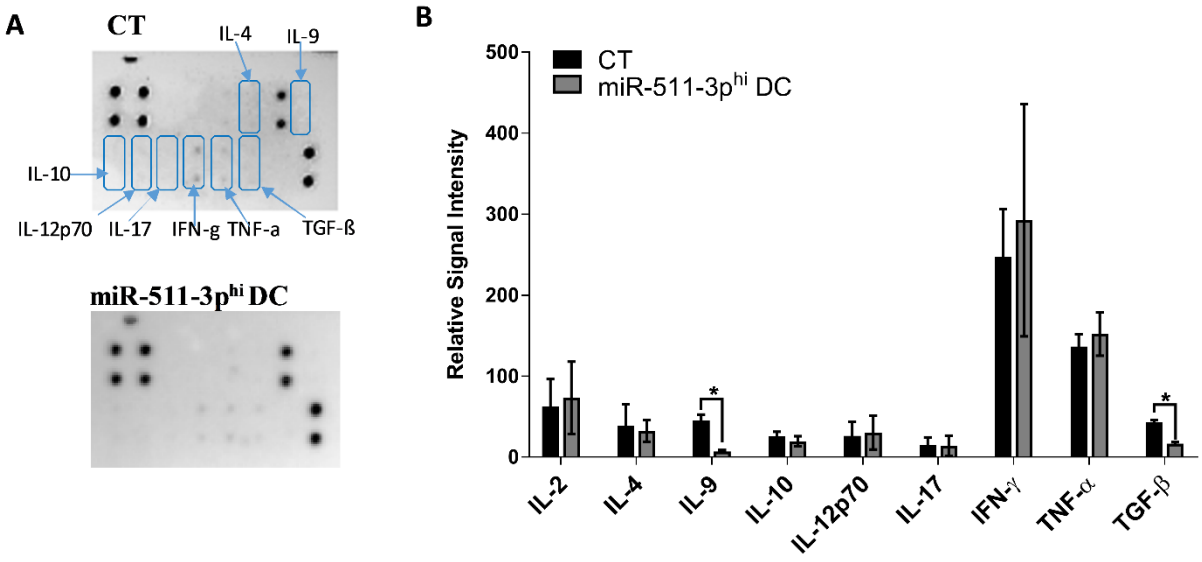


Figure 4.18: T cell cytokine responses from co-cultures with miR-511-3p^{hi} DCs A) Representative array images of T cell cytokine responses from co-culture with miR-511-3p^{hi} DCs. DCs were treated with 0.1µg/ml LPS before co-culture with autologous CD3^{+} T cells. Representative images shown are from one independent experiment with a single donor out of a total of two biological replicates (donors). B) Quantitative analysis of cytokine signal intensities from respective DC-T cell co-cultures. Data is shown as mean ±SD of two independent experiments with two biological replicates

performed in duplicates. *$p < 0.05$ (Ordinary one-way ANOVA with Tukey's post-hoc testing)

4.3 Discussion

Determining the biological importance of individual miRNAs has proven challenging recently, despite their apparent roles in regulating gene expression. The functions of specific miRNA molecules have so far been inferred from studies in cultured cells or animal models that utilise antisense inhibitors to disrupt target sequences or synthetic mimics to enhance expression. These studies have enabled crucial understanding of the roles miRNAs play in processes such as cell development and proliferation, apoptosis, homeostasis and immune response (320-322), as well as implicated them in the regulation of human health and disease. In this study, the role of specific inhibitors and mimics on miR-511-3p expression in human DCs was determined, particularly within the context of surface expression and downstream function.

Data obtained from this study has shown for the first time that transfection with miR-511-3p inhibitors in human DCs resulted in a significant increase in MR as well as DC-SIGN (CD209) protein levels, which also correlated with an increase in mRNA levels as measured by qRT-PCR. This is particularly interesting considering in mice, *Mrc1* is co-regulated with miR-511 as previously shown (232). Generally, intronic miRNAs are generated using their host transcription machinery and should be co-ordinately expressed with their respective host mRNA, (139). However, intronic miRNAs can also be transcribed from independent promoters and there have been reports of discordant expression between host genes and their intronic miRNAs (144,323-325), which could explain why host gene and miRNA expression are not always correlative. Also, the use of synthetic antisense inhibitors, which sequester the endogenous miRNA, making it unavailable for normal function (303,304), could account for observed changes in MR and DC-SIGN expression. Moreover, it was shown that downregulation of *MRC1* in human DCs resulted in an increase in miR-511-3p expression (Figure 4.10). The data

obtained also showed a decrease in MR and DC-SIGN protein and mRNA levels following miRNA overexpression, further highlighting the likely existence of discordant expression between *MRC1* and miR-511-3p.

In a string of previous studies, our lab has shown that MR and DC-SIGN play a central role in the uptake of clinically relevant allergens and in modulating downstream allergic inflammation and Th2 cell polarisation (22-24,98,116). It is therefore reasonable to assume that the crosstalk between miR-511-3p, MR and DC-SIGN could have significant implications in response to allergens. This is in line with recent data showing MR modulates macrophage polarisation and allergic inflammation through miR-511-3p in a mouse model of allergic sensitisation (235). The lack of *Mrc1* (*Mrc1*$^{-/-}$ mice) in these studies significantly reduced allergen uptake as well as miR-511-3p expression in lung macrophages and exacerbated allergic inflammation, suggesting that allergen uptake via MR may promote miR-511-3p expression (235). However, the present study found a reciprocal antagonistic relationship between MR and miR-511-3p in human DCs, which brings to light the complex differences that may exist between cell types (i.e. macrophages and DCs) as well as species, with MR expression for instance present on chromosome 10p12 in humans, while present on chromosome 2.2 in mice (225).

The data obtained is further supported by results from IDO activity analysis. As previously indicated IDO catalyses tryptophan (TRP) metabolism and participates in several immune-regulatory processes via induction of regulatory T cells (326-328). Recently, our lab showed that different airborne allergens, through MR engagement are able to downregulate LPS induced IDO activity in DCs thereby affecting Th polarisation (116). Similarly, the present study shows that mannan, a complex carbohydrate, is able to significantly downregulate IDO activity in miR-511-3p^{low} DCs; but has no effect on LPS-induced IDO activity. This may be due to the increased

expression of MR in the miR-511-3p^{low} cells. However, in the presence of LPS, miR-511-3p^{low} cells showed increased IDO activity, which supports the notion that miR-511-3p^{low} DCs may exert an anti-inflammatory role. Moreover, GO analysis of biological processes in the preliminary RNAseq data indicates a regulation of cellular catabolic processes by miR-511-3p^{low} DCs, which was absent in miR-511-3p^{hi} conditions (Figure 4.15). Additionally, it was demonstrated that MR and DC-SIGN can have opposing effects on IDO activity, whereby DC-SIGN ligation with mannan (in MR^{low} DCs) favours induction of IDO activity (Figure 4.12). This could potentially account for the observed induction of IDO activity in mannan and LPS treated cells. However, it is worth confirming the roles of MR or DC-SIGN by inclusion of double knockdown populations (miR-511-3p and either MR or DC-SIGN), to determine the overall contribution of either receptors to miR-511-3p induced modulation of IDO activity in human DCs.

Nevertheless, our data indicates significant changes in DC phenotype and function following transfection with miR-511-3p inhibitors and mimics. As shown, it is clear that knockdown of miR-511-3p promotes the establishment of an anti-inflammatory DC phenotype characterised by increased IL-10 production and decrease in IL-12p70 and IL-6. Immune cells such as macrophages and DCs rapidly respond to LPS-induced inflammation through pro-inflammatory cytokine production, which are then downregulated following induction of anti-inflammatory signals and resolution of inflammation. Moreover, it was shown that miR-511-3p^{low} cells treated with LPS significantly increase in PDL-1 expression. Several studies have demonstrated a key role for the transmembrane protein PDL-1 (329,330) and IL-10 secretion (331,332) in promoting immune suppression. These include studies from our group showing that PDL-1 upregulation and IL-10 secretion following LPS conditioning, promotes

development of human DCs with anti-inflammatory properties (333). PDL-1 belongs to the B7 family of costimulatory molecules that also includes B7-1 (CD80), B7-2 (CD86), as well as PDL-2; and T cell engagement (via PD-1) with each of these co-stimulatory receptors can induce inhibitory and in some cases stimulatory signals. The data obtained in this study therefore strongly suggests that the induction of PDL-1 and IL-10 following knockdown of miR-511-3p may promote immune suppression during inflammation. In line with this, the T cell co-culture data indicates that miR-511-3p^{low} DCs may promote Th2-type responses, evidenced by the production of IL-4 in co-culture supernatant. Post-transcriptional regulation by miRNAs is vital for controlling numerous immunological events including the development and function of effector T cells. For instance, the miR-17~92 cluster was shown to be important for Th1 polarisation evidenced by the lack of IFN-γ and T-bet in miR-17~92-deficient mice (334). Recently, the miR-21~27~24 family cluster has been shown to limit Th2 responses by directly and indirectly targeting IL-4 and GATA3 (335). In the present study, a significant increase in IL-4 and decrease in IL-17 production from T cells was observed in co-culture with miR-511-3p^{low} DCs, which may suggest a bias towards Th2 phenotype. Although IL-10 was increased this was not significant (Figure 4.17). The bias towards Th2 phenotype, driven by miR-511-3p^{low} DCs, is also backed by the finding that TGF-β is significantly reduced in T cells co-cultured with miR-511-3p^{hi} DCs, which although not conventional, may indicate suppression of regulatory T cells.

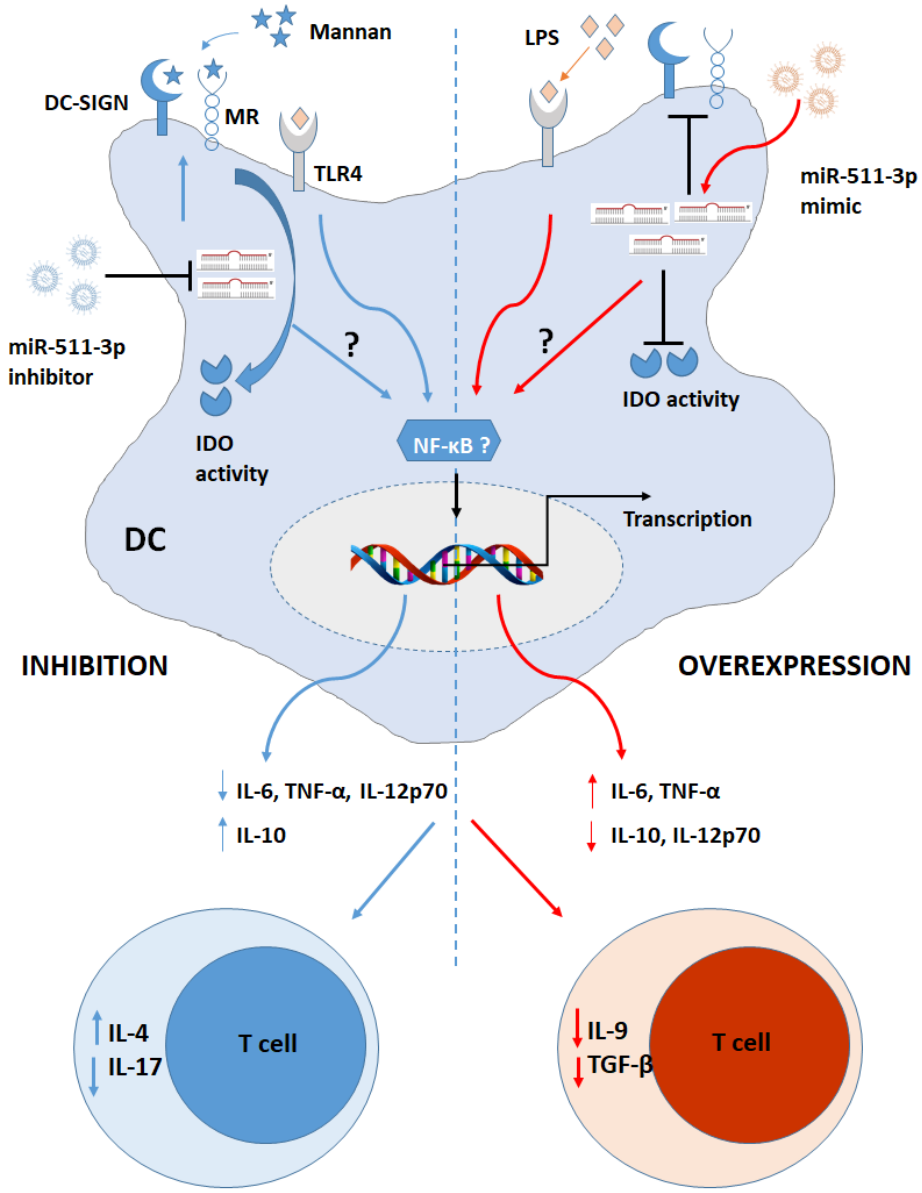


Figure 4.19: Schematic representation of miR-511-3p induced modulation of DC function. miR-511-3p mediated regulation of MR and DC-SIGN expression impacts IDO activity as well as cytokine production following treatment with mannan and LPS in human monocyte-derived DCs and affects cytokine production in autologous T cell co-cultures.

In conclusion, the present study has shown that manipulation of miR-511-3p expression in human DCs plays a key role in regulating DC phenotype and function partly through regulating MR and DC-SIGN expression. Additionally, miR-511-3p knockdown may

contribute towards induction of anti-inflammatory DC phenotype and promote immune suppression at least within the context of LPS induced inflammation. These data provides new insight into miR-511-3p mediated immune regulation, and could pave way for rational design of new therapies against inflammatory diseases.

Chapter 5: The Role of miR-511-3p in Modulating PPARγ Activity in Human Dendritic Cells

Chapter 5: The role of miR-511-3p in modulating PPARγ activity in human DCs

5.1 Introduction

The innate immune system rapidly responds to endotoxin exposure through the induction of acute inflammatory responses (336). Endotoxins such as lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria, can induce potent inflammatory responses through the release of an array of mediators such as cytokines (e.g. interleukin (IL)-6 and tumour necrosis factor (TNF)-α), chemokines (e.g. CXCL8) and growth factors (e.g. transforming growth factor (TGF)-β1). However, when uncontrolled, inflammatory responses can lead to tissue damage and chronic inflammatory diseases (337-339). Dendritic cells (DCs) are the most efficient antigen presenting cells (APCs) capable of initiating and maintaining primary immune responses (340), and are key players in regulating inflammatory responses (341). DCs are highly sensitive to even low concentrations of LPS in the environment, and detect the presence of LPS (for instance during bacterial infection) via the innate conserved Toll-like receptor 4 (TLR4). This triggers downstream signaling through adaptor proteins to activate the NF-κB and AP-1 pathways, which co-ordinate expression of genes required to initiate or control inflammation (339,342).

Recently, the role of the peroxisome proliferator-activated receptor γ (PPARγ) in modulating inflammatory responses has been of particular interest. PPARγ is a lipid-activated transcription factor expressed in a variety of cell types including DCs and macrophages, where it regulates genes associated with adipogenesis, lipid metabolism and inflammation (117,343,344). For instance, PPARγ activation was shown to result in trans-repression of pro-inflammatory genes by targeting signal-dependent activation of transcription factors and proteins such as NF-κB (345). In DCs, PPARγ regulates various processes including maturation and migration, activation, antigen presentation

and cytokine production (124,126,127). Moreover, PPARγ activation in DCs in the presence of LPS resulted in decreased expression of the pro-inflammatory cytokine IL-12 (128), suggesting that PPARγ ligands may promote anti-inflammatory immune responses. Studies by us and others have also demonstrated a key role for the tryptophan metabolizing enzyme, indoleamine 2,3-dioxygenase (IDO) in controlling LPS-mediated inflammatory responses via promotion of tolerogenic DC phenotype and a decrease in Th1 immune responses (326,333,346,347). Interestingly, the recent finding that PPARγ promotes IDO activity and subsequent immune suppression in the tumour microenvironment highlights the complexity of PPARγ-induced regulatory mechanisms(348). Nevertheless, the overall immunosuppressive effect of PPARγ and the underlying mechanisms particularly during LPS-induced inflammatory responses in human DCs are not fully understood.

MicroRNAs (miRNAs) are a class of gene regulators that bind the 3'UTR of target genes and cause translational inhibition or mRNA degradation (144,255). Due to their role in regulating different biological processes (i.e. development and differentiation, homeostasis and immune activation), miRNAs represent crucial regulators in human health and disease. Recently, miR-511-3p (located at the -'3 end of the mature miR-511 strand) has been identified as an important regulator of human DC and macrophage development and function. For example, miR-511 was shown to be a key regulator of TLR4 expression in human macrophages and DCs (263). Additionally, transcriptional products associated with inflammation and wound healing in alternatively activated macrophage (AAM), were found to be altered by miR-511 expression (234). Interestingly, PPARγ has also been highlighted as a putative target of miR-511 activity, affecting human myeloid cell differentiation and function (263,349) however the mechanisms underlying these events are not yet known. Here, it was hypothesized that

changes in miR-511-3p expression could impact PPARγ mRNA levels and transcriptional activity within the context of LPS-mediated inflammatory responses in human DCs. Therefore potential link between miR-511-3p expression and PPARγ activity was investigated and how this influences LPS-mediated inflammatory responses in human DCs. Better understanding of the mechanisms regulating PPARγ activity could pave way for rational design of therapies against a range of inflammatory disorders.

5.2 Results

5.2.1 PPARγ activity in DCs is induced by RSG but downregulated by LPS.

A number of natural (e.g. 15d-PGJ_2) as well as synthetic agonists (also known as thiazolidinediones - TZDs) have been previously shown to induce PPARγ activation in a variety of cell types (350,351). Here, immature monocyte-derived DCs (DCs) were initially treated with LPS and the synthetic PPARγ agonist rosiglitazone (RSG) to determine the influence of these ligands on PPARγ activity. PPARγ activity was assessed by measuring expression of one of its target genes (i.e. *FABP4*) by qRT-PCR. It was found that RSG upregulates PPARγ activity; however, LPS stimulation inhibited PPARγ activity either alone or in the presence of RSG (Figure 5.1A). Subsequently, it was determined whether ligand-activation of PPARγ by RSG could modulate surface marker expression of DCs. To examine this, DCs were treated with LPS for 24 hours in the presence or absence of low dose RSG. As expected, DCs showed an increase in expression of maturation and activation markers CD83, CD86 and PDL-1 respectively and a decrease in the c-type lectins, mannose receptor (MR) and DC-SIGN following LPS stimulation (Figure 5.1B). Notably, RSG stimulation significantly reduced expression of CD83 in RSG/LPS cells, suggesting that DC maturation or activation status could be compromised by PPARγ activation. Lastly, it was examined whether RSG treatment could modulate LPS-induced proinflammatory cytokine production by DCs. Interestingly, the production of TNF-α was significantly reduced in RSG/LPS treated cells; however in the presence of the PPARγ antagonist GW9662, cells showed a slight increase in TNF-α production (Figure 5.1C) suggesting that PPARγ is able to downregulate LPS-induced TNF-α production in DCs. Although IL-6 was reduced in RSG/LPS conditions, this was not significant.

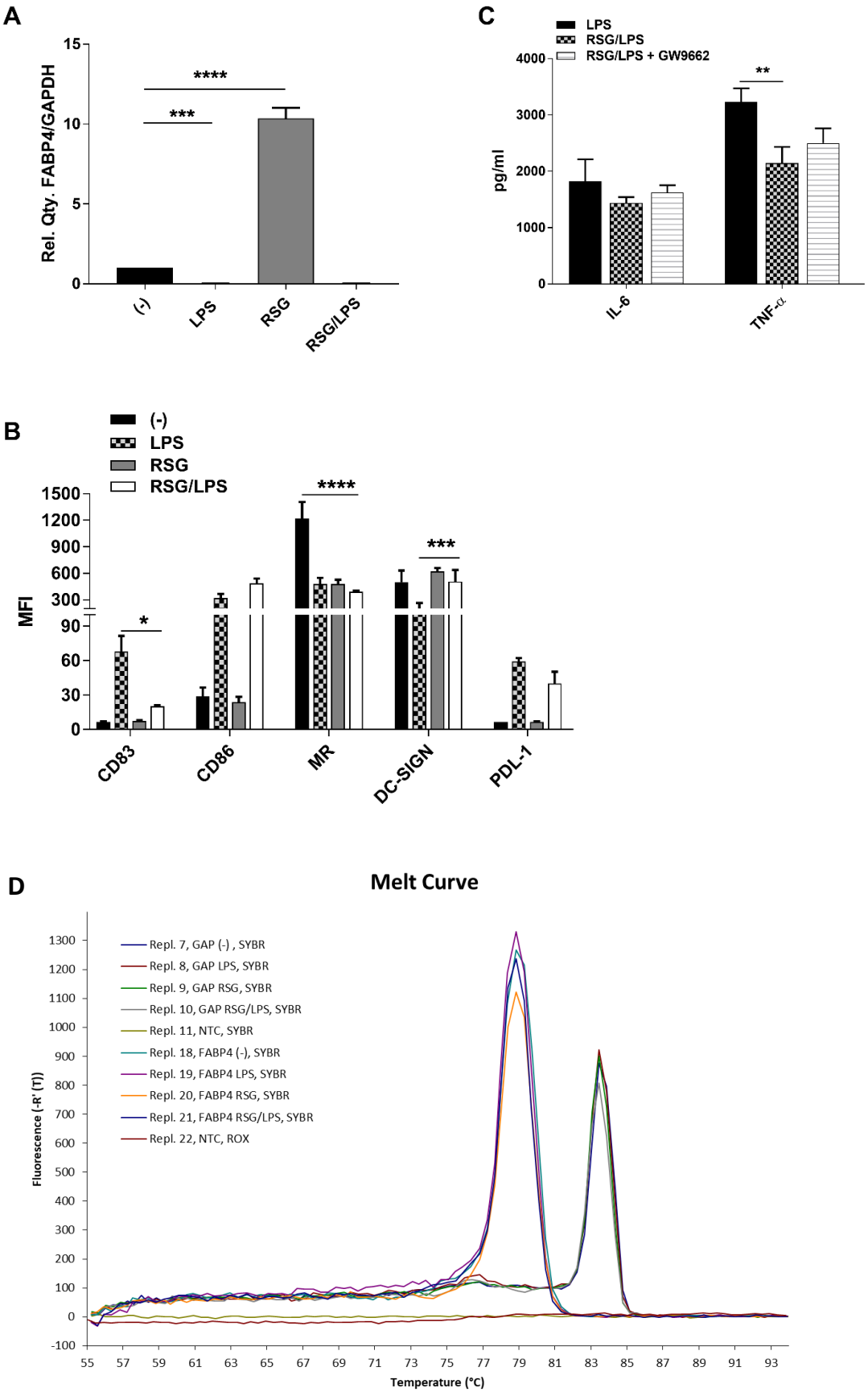

A
Rel. Qty. FABP4/GAPDH
15
10
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(-)
LPS
RSG
RSG/LPS
C
LPS
RSG/LPS
RSG/LPS + GW9662
pg/ml
4000
3000
2000
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0
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IL-6
TNF-α
B
(-)
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RSG
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1500
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CD83
CD86
MR
DC-SIGN
PDL-1
D
Melt Curve
Repl. 7, GAP (-) , SYBR
Repl. 8, GAP LPS, SYBR
Repl. 9, GAP RSG, SYBR
Repl. 10, GAP RSG/LPS, SYBR
Repl. 11, NTC, SYBR
Repl. 18, FABP4 (-), SYBR
Repl. 19, FABP4 LPS, SYBR
Repl. 20, FABP4 RSG, SYBR
Repl. 21, FABP4 RSG/LPS, SYBR
Repl. 22, NTC, ROX
Fluorescence (-R' (T))
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
55
57
59
61
63
65
67
69
71
73
75
77
79
81
83
85
87
89
91
93
Temperature (°C)

Figure 5.1: PPARγ activation by rosiglitazone modulates DCs phenotype. A) Immature monocyte-derived DCs were stimulated with 0.1µg/ml LPS in the presence or absence of 5µM rosiglitazone (RSG) for 24 hours. PPARγ activity was determined afterwards by measuring *FABP4* expression by qRT-PCR. Data is normalised to GAPDH. Data is expressed as mean ± SD from one independent experiment with three donors. ***p < 0.001, ****p < 0.0001 (one way ANOVA with Tukey's post-hoc testing) B) Flow cytometry analysis of DC surface markers following treatment with RSG and LPS. Representative median fluorescence intensity (MFI) values are shown. Data is expressed as mean ± SD from two independent experiments with two donors *p < 0.05, ***p < 0.001, ****p < 0.0001 (one way ANOVA with Tukey's post-hoc testing) C) IL-6 and TNF-α production is affected by RSG and GW9662 in LPS treated cells Data is represented as mean ± SD of a single experiment with two independent donors. **p < 0.01 (2-way ANOVA with Tukey's post-hoc testing). D) Melt curve analysis of qRT-PCR for *FABP4* expression

5.2.2 IDO activity is downregulated by PPARγ in human DCs

Induction of the tryptophan metabolizing pathway by IDO mediates immunosuppression in response to a variety of stimuli including IFN-γ and TNF-α (113,352). Under certain conditions, IDO induction can also be mediated through an IFN-γ-independent mechanism, such as presence of LPS during bacterial infection (333,353). In order to test whether the immune-modulatory effect of PPARγ activation influences LPS-induced IDO activity, immature DCs were treated with LPS in the presence and absence of RSG. IDO activity was assessed by colorimetric determination of kynurenine (KYN) produced in culture supernatant. As summarised in Figure 5.2, RSG alone had no effect on IDO activity but significantly downregulated LPS-induced IDO activity. Additionally, treatment with GW9662 in RSG/LPS conditions caused a slight decrease in IDO activity compared with RSG/LPS alone, however this was not

significant (Figure 5.2A). As indicated in the LIVE/DEAD stain, RSG or GW9662 had no significant effect on cell viability (Figure 5.2B and C).

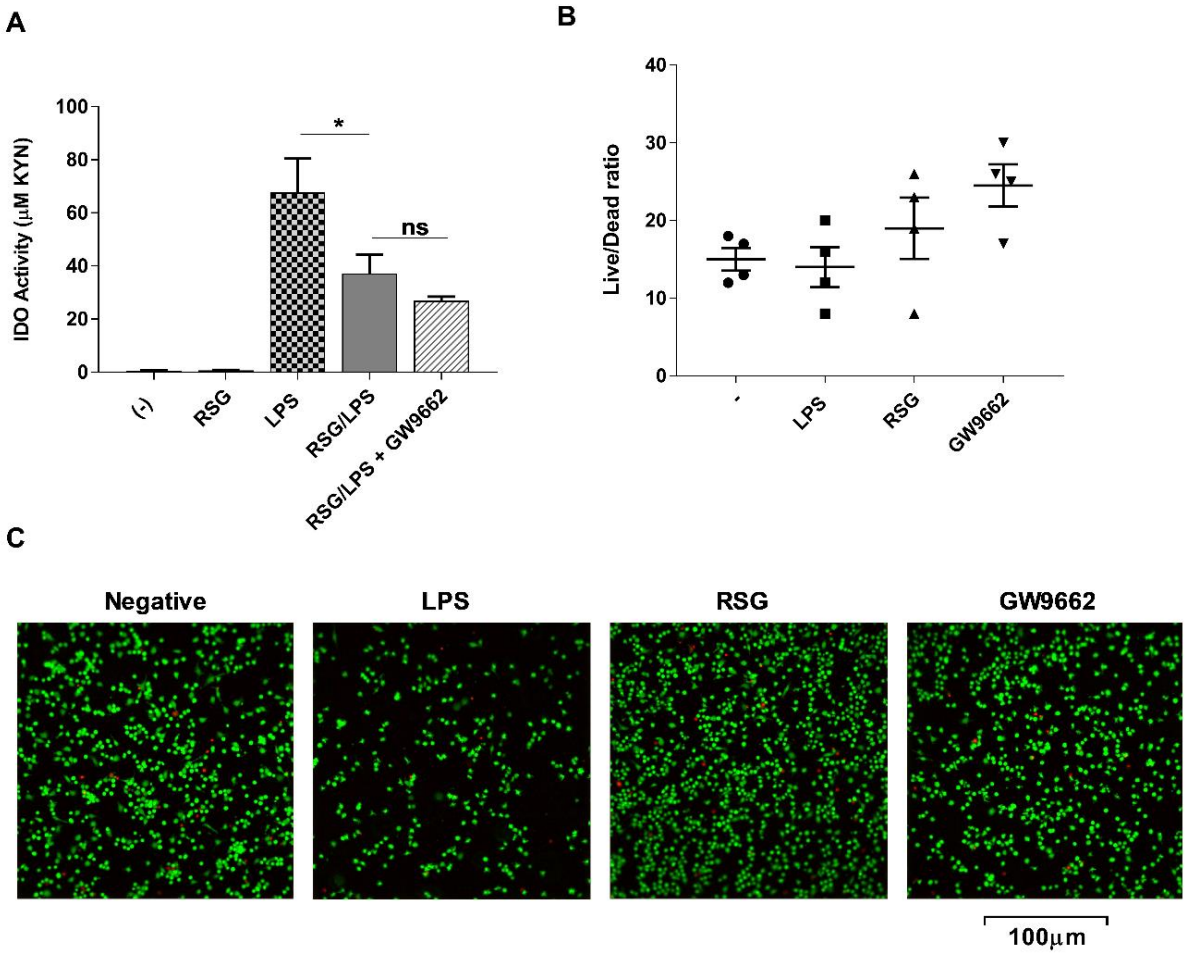
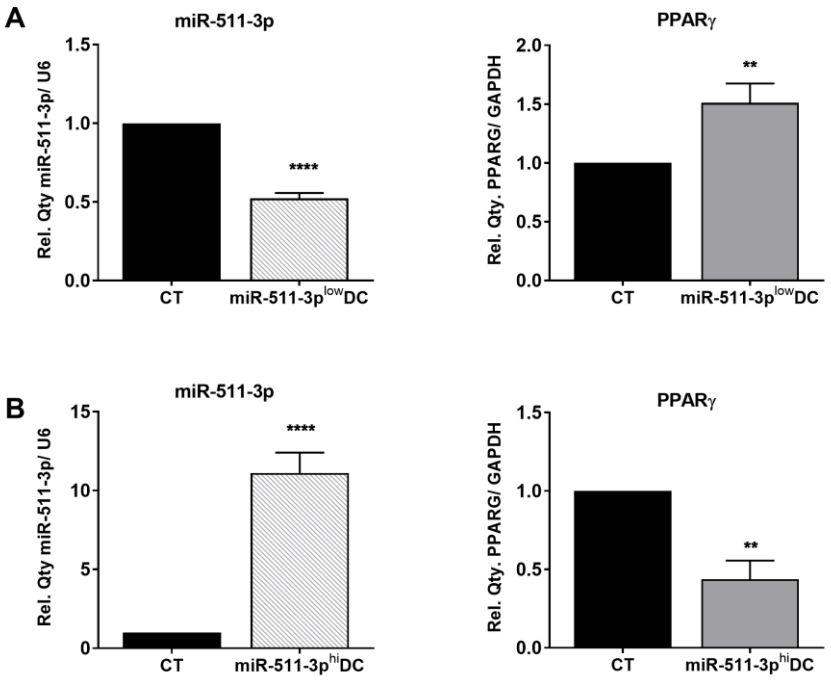


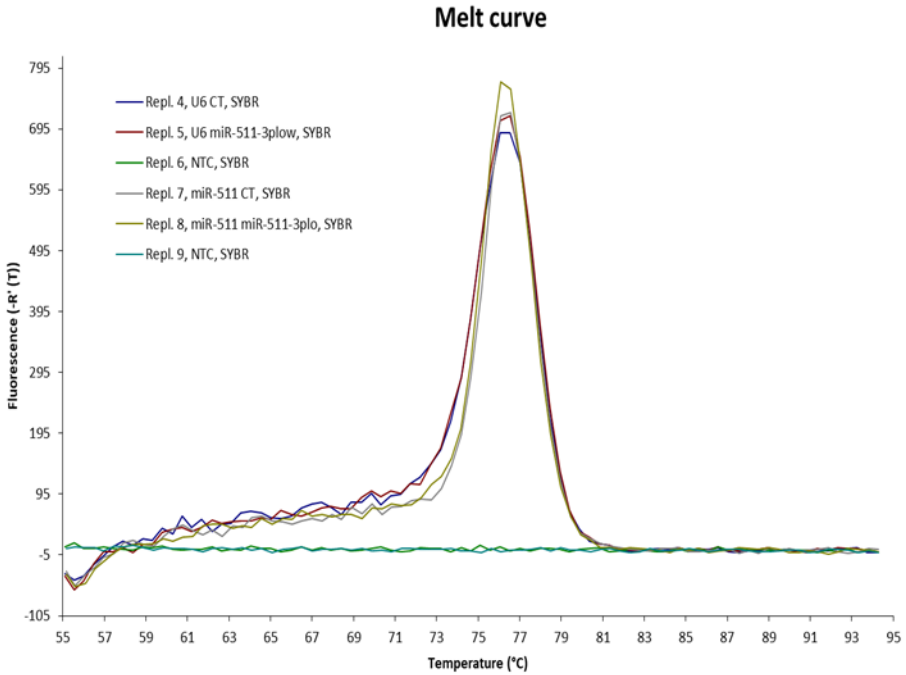
Figure 5.2: IDO regulation by human monocyte-derived dendritic cells. IDO activity in human DCs treated with 5μM PPARγ agonist RSG and 0.1μg/ml LPS for 24 hours in the presence or absence of 5μM PPARγ antagonist GW9662. Culture supernatant was collected and examined for IDO activity. Data is represented as mean ± SD of two independent experiments with two independent donors. *$p < 0.05$, (ordinary one-way ANOVA with Tukey's post-hoc testing. B and C) Ratio of Live/Dead cells in unstimulated DCs and cells treated with LPS, RSG or GW9662. Live/Dead ratios are from one representative experiment with a single donor out of a total of two independent donors and experiments. Live and dead cells are represented by green and red fluorescence respectively. Live and dead cell count was done in 4 different fields of view in each image. Data points represent cell count in each field of view ± SEM

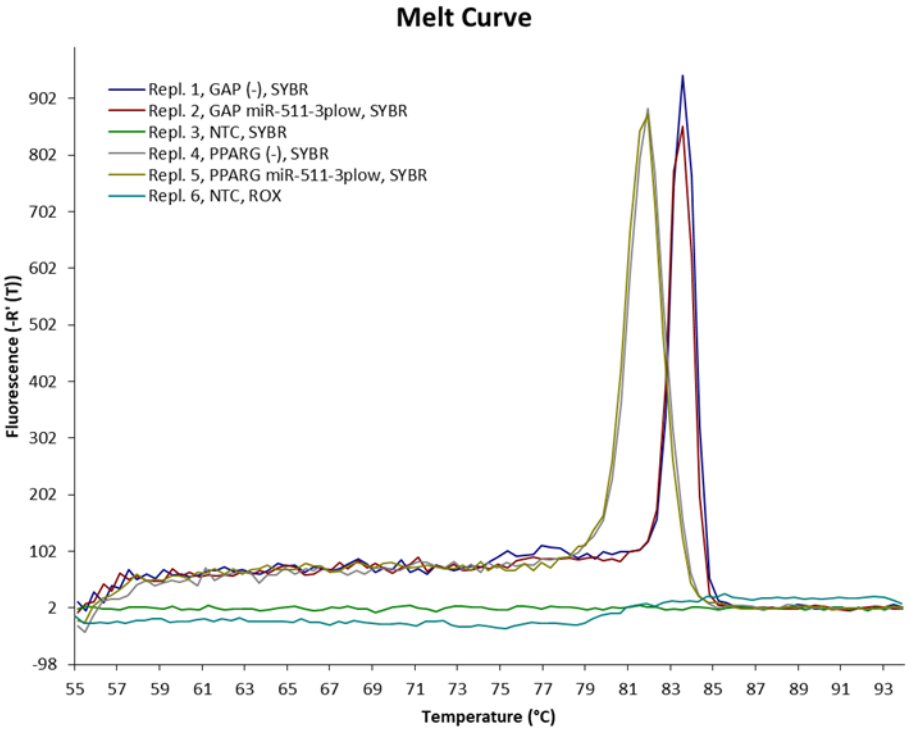
5.2.3 Changes in miR-511-3p expression modulate PPARγ activity in human DCs

To determine whether the regulatory role of PPARγ on DC phenotype and function demonstrated above is influenced by miR-511-3p, monocyte-derived DCs were differentiated in the presence of miR-511-3p specific inhibitors and mimics, which resulted in about 50% downregulation or an increase in miR-511-3p expression respectively (Figure 5.3A). Subsequently, the expression of PPARγ was measured by qRT-PCR. Accordingly, the data obtained indicates for the first time that knockdown of miR-511-3p (henceforth referred to as miR-511-3p^{low}), resulted in an increase in PPARγ mRNA levels; whereas a decrease in PPARγ mRNA expression was seen in miR-511-3p overexpressed cells (i.e. miR-511-3p^{hi}) (Figure 5.3B).



C


Melt curve
Repl. 4, U6 CT, SYBR
Repl. 5, U6 miR-511-3plow, SYBR
Repl. 6, NTC, SYBR
Repl. 7, miR-511 CT, SYBR
Repl. 8, miR-511 miR-511-3plo, SYBR
Repl. 9, NTC, SYBR
Fluorescence (-R' (T))
795
695
595
495
395
295
195
95
-5
-105
55 57 59 61 63 65 67 69 71 73 75 77 79 81 83 85 87 89 91 93 95
Temperature (°C)


Melt Curve
Repl. 1, GAP (-), SYBR
Repl. 2, GAP miR-511-3plow, SYBR
Repl. 3, NTC, SYBR
Repl. 4, PPARG (-), SYBR
Repl. 5, PPARG miR-511-3plow, SYBR
Repl. 6, NTC, ROX
Fluorescence (-R' (T))
902
802
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Temperature (°C)

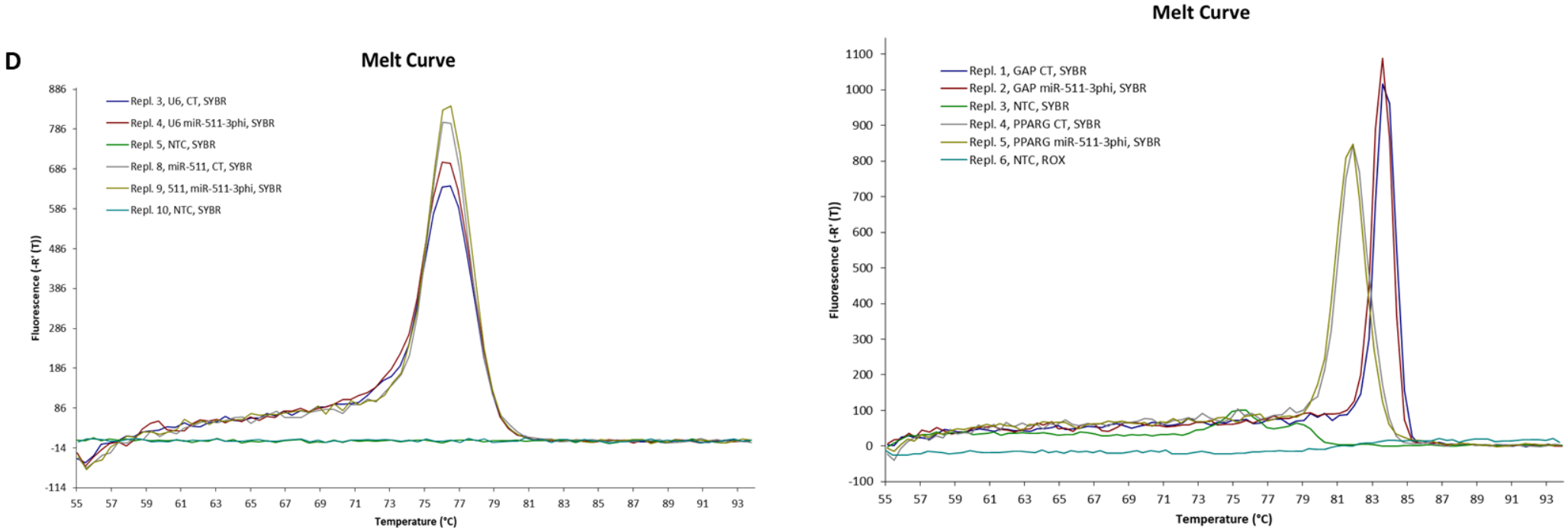
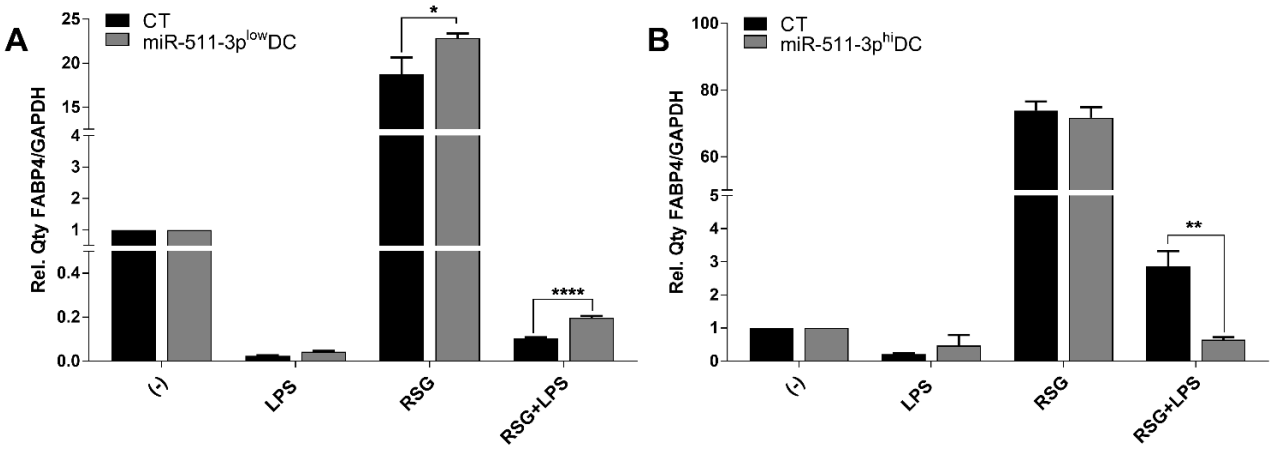


Figure 5.3: Analysis of PPARγ mRNA levels in response to up or downregulation of miR-511-3p expression. qRT-PCR of miR-511-3p and PPARγ expression respectively in A) miR-511-3p^{low} and B) miR-511-3p^{hi} DCs. Monocytes were differentiated in the presence of 50nM miR-511-3p specific inhibitors and mimics. Gene expression was assessed on day 6 and is normalised to U6 and GAPDH for miRNA and mRNA respectively. Data in A and B is expressed as mean ± SD of three independent experiments with three donors **p < 0.01, ****p < 0.0001. Representative melt curve of qRT-PCR for miR-511-3p and PPARγ expression respectively in C) miR-511-3p^{low} and D) miR-511-3p^{hi} DCs

Next the effect of LPS and RSG treatment on *FABP4* expression in the miR-511-3p transfected cells was examined. Interestingly, the data obtained shows that PPARγ activity is significantly increased in miR-511-3p^{low} DCs compared to controls, following RSG stimulation alone, and in the presence of LPS (Figure 5.4A), which supports the notion that inhibition of miR-511-3p may promote PPARγ activity and suppression of LPS-induced inflammation. In line with this observation, a decrease in PPARγ activity was seen in miR-511-3p^{hi} DCs treated with RSG alone, and in the presence of LPS (Figure 5.4B).



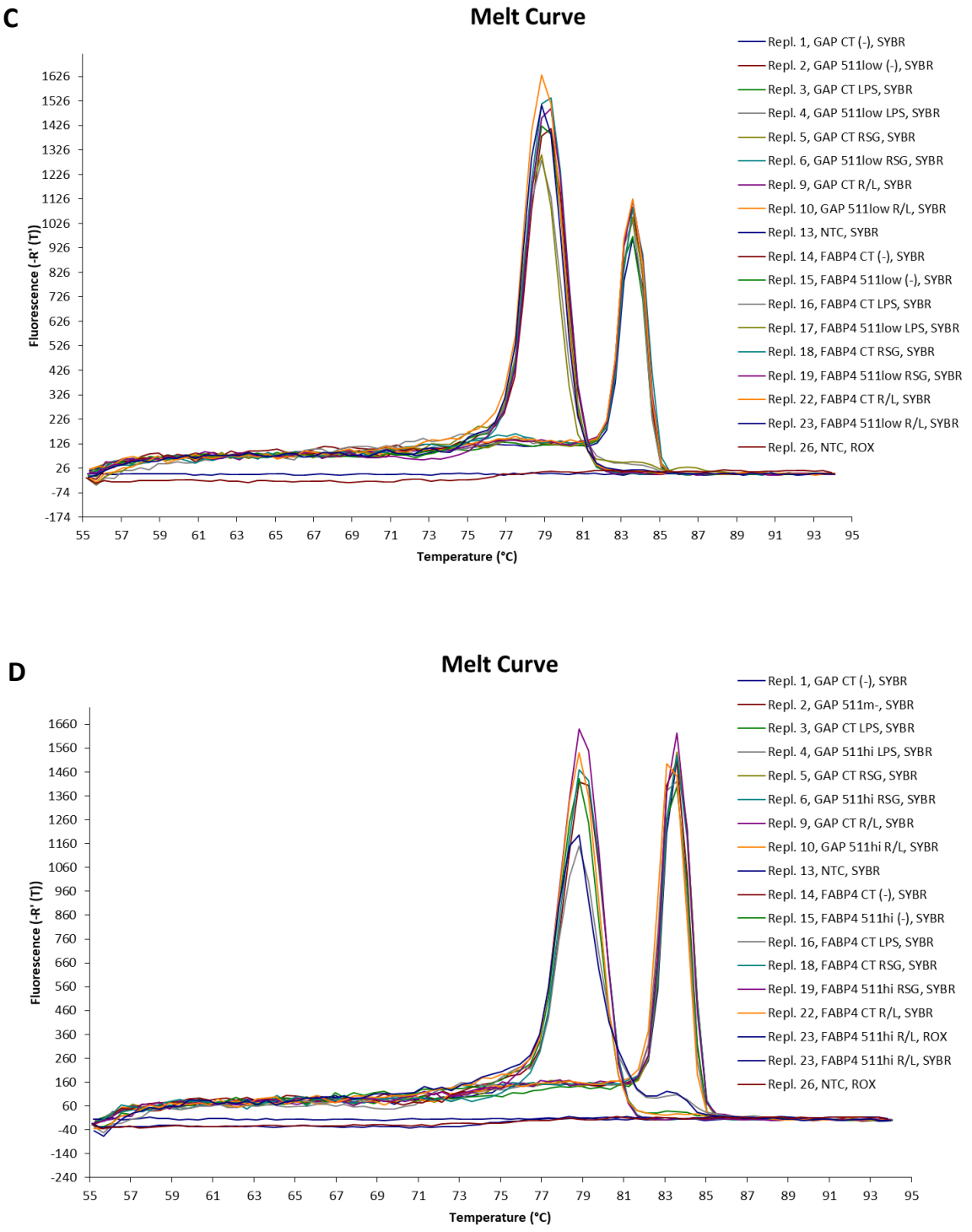


Figure 5.4: PPARγ activity is modulated by miR-511-3p expression. A) *FABP4* expression in miR-511-3p^{low} DCs treated with 5uM RSG and 0.1ug/ml LPS. Data is normalised to GAPDH and expressed as mean ± SD from one independent experiment with three independent donors. *$p < 0.05$, ****$p < 0.0001$ (Student's T-test). B) *FABP4* expression in miR-511-3p^{hi} DCs treated with 5uM RSG and 0.1ug/ml LPS Data is expressed as mean ± SD from one independent experiment with three independent

donors. **p < 0.01 (Student's T-test). Monocyte derived DCs were transfected with 50nM miR-511-3p inhibitor and mimic for 6 days. Representative melt curve analysis of *FABP4* qRT-PCR for C) miR-511-3p^{low} and D) miR-511-3p^{hi} DCs.

5.2.4 PPARγ activation modulates cytokine production by miR-511-3p transfected DCs

Having examined the changes in PPARγ activity by miR-511-3p transfected DCs, the levels of pro- and anti-inflammatory cytokines (IL-6 and IL-10) produced by the miR-511-3p^{hi}and miR-511-3p^{low} DCs were examined to determine whether this was influenced by PPARγ activation. DCs transfected with miR-511-3p mimics or inhibitors were treated with RSG with or without LPS for 24 hours. As shown in Figure 5.5A, RSG alone or in the presence of LPS stimulation significantly induced IL-6 production in miR-511-3p^{hi} DCs whereas no changes were seen in miR-511-3p^{low} DCs. In contrast, IL-10 production was significantly increased in miR-511-3p^{low} DCs treated with RSG, whereas no changes were seen in miR-511-3p^{hi} cells (Figure 5.5B).

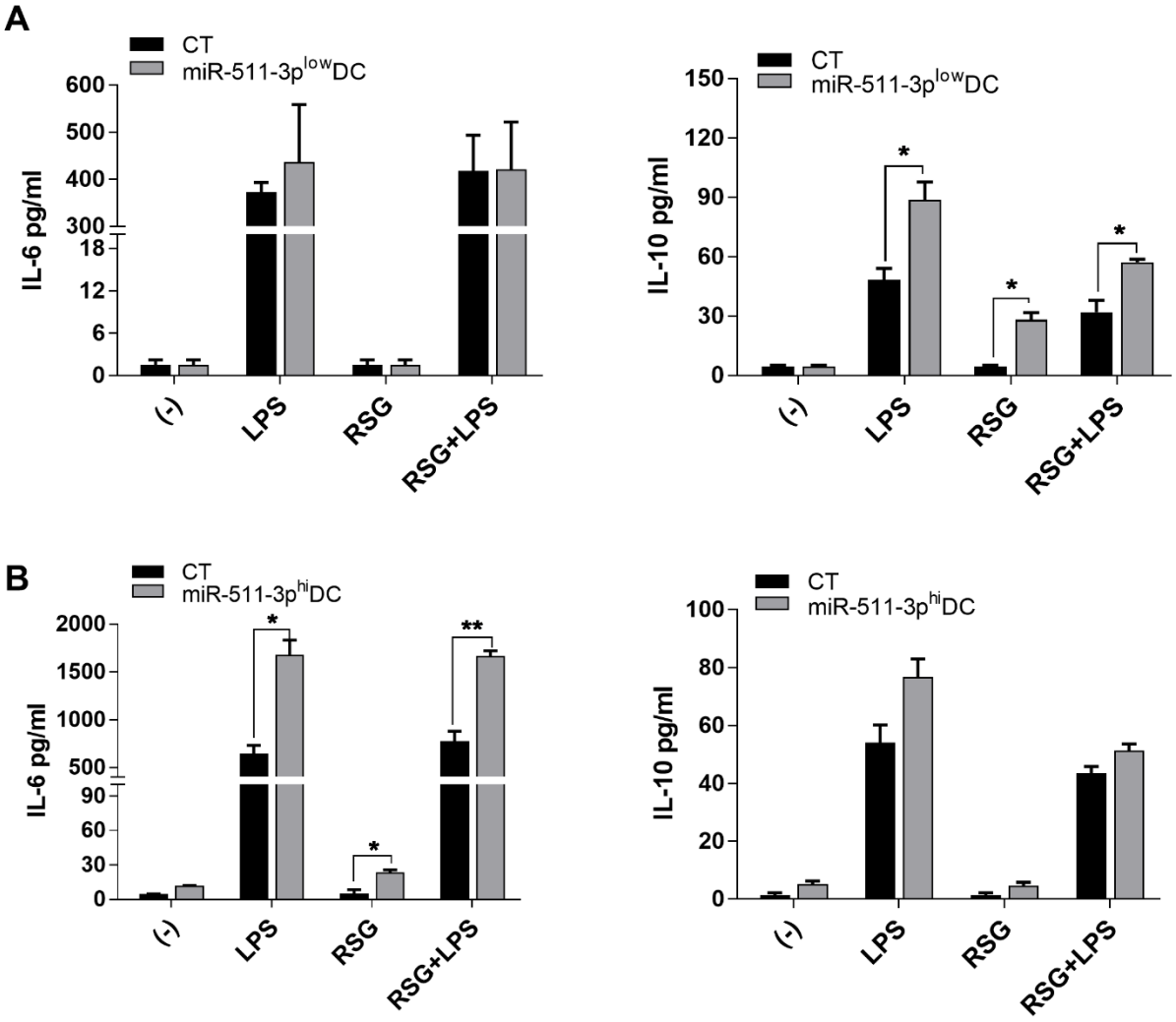


Figure 5.5: Cytokine secretion is modulated by PPARγ activity in miR-511-3p transfected DCs. A) IL-6 and IL-10 cytokine production by miR-511-3p^{low} DCs stimulated with 5µM RSG and 0.1µg/ml LPS for 24 hours. Data is represented as mean ± SD of three independent experiments with three independent donors *$p < 0.05$ (two-way ANOVA with Tukey's post-hoc testing). B) IL-6 and IL-10 cytokine production by miR-511-3p^{hi} DCs stimulated with 5µM RSG and 0.1µg/ml LPS for 24 hours. Culture supernatants were collected cytokine levels were quantified by ELISA. Data is represented as mean ± SD of three independent experiments with three independent donors *$p < 0.05$, **$p < 0.01$ (two-way ANOVA with Tukey's post-hoc testing).

5.3 Discussion

The PPAR receptors, which belong to the nuclear hormone receptor superfamily, were originally identified as key players in controlling oxidation of lipids and fatty acids. Among the three members, PPARγ is essential for controlling adipocyte differentiation and glucose metabolism (354). Currently, several reports have highlighted the importance of PPARγ agonists as treatment against inflammatory and other diseases (355-357). For instance, synthetic PPARγ agonists, (TZDs) were used in phase II trials for treatment of ulcerative colitis and was shown that disease progression was often accompanied by decreased PPARγ expression; however the underlying mechanisms were not defined (357). MicroRNAs are crucial players in mammalian gene regulation and their role in fine-tuning gene expression, particularly through dysregulated expression has been reported in several infectious and inflammatory diseases. Recently, miR-511-3p has emerged as an important regulator of immune responses, including aspects of DC biology, however their role in regulating PPARγ activity is not known. In the present study, the role of miR-511-3p in modulating PPARγ activity in human DCs was investigated mainly within the context of LPS-induced inflammation.

In line with previous reports, RSG induced PPARγ activity (Figure 5.1); however this increased activity was completely abrogated by LPS. Two isoforms of PPARγ have been identified (PPARγ1 and 2) and studies show that both isoforms are downregulated in monocyte and macrophage cell lines following treatment with LPS (358). However, prolonged exposure to LPS in these studies enabled recovery of PPARγ mRNA levels to almost basal levels after 24 hours (358). Similarly, in a rat model of sepsis, infusion of low-dose LPS was shown to significantly decrease hepatic PPARγ protein levels, but administration of the LPS binding agent (polymyxin B) reduced plasma endotoxin level and prevented PPARγ downregulation in the septic animals (359), thus highlighting the

complex mechanisms underlying the regulation of PPARγ during LPS-induced inflammation.

Several reports have indicated that induction of the IDO pathway of tryptophan metabolism contributes towards immune modulatory events (107,109). Within the context of inflammation, IDO induction leads to the suppression of T cell effector responses (360) possibly through generation of Tregs, which help dampen the immune response associated with inflammation. A role for LPS in IDO induction has been demonstrated by our group and others (116,361), which explains why IDO activity is increased following LPS treatment. However, following treatment with RSG, IDO activity was significantly reduced in the presence of LPS (RSG/LPS) compared to LPS alone. No changes were found after treatment with the PPARγ antagonist. Recently, it has been demonstrated that fatty acid oxidization by PPARγ in melanomas positively regulates the enzymatic activity of IDO in DCs, by promoting a switch towards a tolerogenic state to enable immune evasion (348). In contrast, these observations indicate that PPARγ activation may negatively regulate IDO in DCs within the context of LPS induced inflammation.

Interestingly Tserel et al, using TargetScan and a combination of different algorithms highlighted *PPARG* as a putative target of miR-511 activity (263). As shown (Figure 5.3) overexpression of miR-511-3p, significantly downregulated PPARγ expression by qRT-PCR. Recently, miRNAs have been shown to mediate pathways decreasing PPARγ expression during the onset of inflammation. For instance miR-27b was shown to decrease PPARγ1 mRNA level, as mutation or deletion of the miR-27b start site completely abolished reduction of luciferase activity in THP1 macrophages (358). Additionally, they showed that miR-27b inhibitors prevented LPS-dependent PPARγ1 mRNA reduction in a concentration-dependent manner. Several other studies have

demonstrated the role of miRNAs in regulating PPARγ expression and activity (362,363). For instance, upregulation of miR-34a and miR-34c promote activation of human hepatic stellate cells (HSCs) by targeting PPARγ to repress its expression, thereby promoting liver fibrosis (364).

The anti-inflammatory effect of PPARγ ligands in regulating immune responses is well documented. In particular, high dose 15d-PGJ2 or TZD (PPARγ agonists) treatment of monocytes and macrophages was shown to inhibit secretion of pro-inflammatory cytokines (IL-6, TNF-α and IL-1β) (351) and suppress IFN-γ-dependent inducible nitric oxide synthase (iNOS), gelatinase B and scavenger receptor A genes (350,365). Additionally, it has been shown that IL-10, which is produced under both pro- and anti-inflammatory conditions, is induced by RSG in experimental models of colitis and Parkinson's disease (366,367); however agonists such as 15d-PGJ_2 and pioglitazone were shown to reduce IL-10 expression in a lung sepsis model (368). In a cell reporter model, overexpression of PPARγ was shown to block TLR4/NF-κB signaling thereby preventing LPS-induced inflammation (369). These studies therefore suggest that PPARγ and signalling molecules/pathways controlling its expression or activity may serve as a target for anti-inflammatory therapy. As shown in Figure 5.4, miR-511-3p^{low} DCs significantly induce PPARγ activity (*FABP4*) following treatment with RSG compared to controls, suggesting that miR-511-3p expression may inhibit PPARγ activity. In line with that, PPARγ activity was reduced in RSG treated miR-511-3p^{hi} DCs compared to controls. This observation is further supported by the cytokine data, where miR-511-3p^{hi} DCs significantly promote IL-6 production following LPS and RSG treatment. This could be the result of PPARγ inhibition by miR-511-3p^{hi} cells. Similarly, the anti-inflammatory cytokine IL-10 is upregulated in miR-511-3p^{low} cells

compared to controls and miR-511-3p^{hi} DCs (Figure 5.5), which further highlights PPARγ as a key player in controlling LPS-induced inflammation.

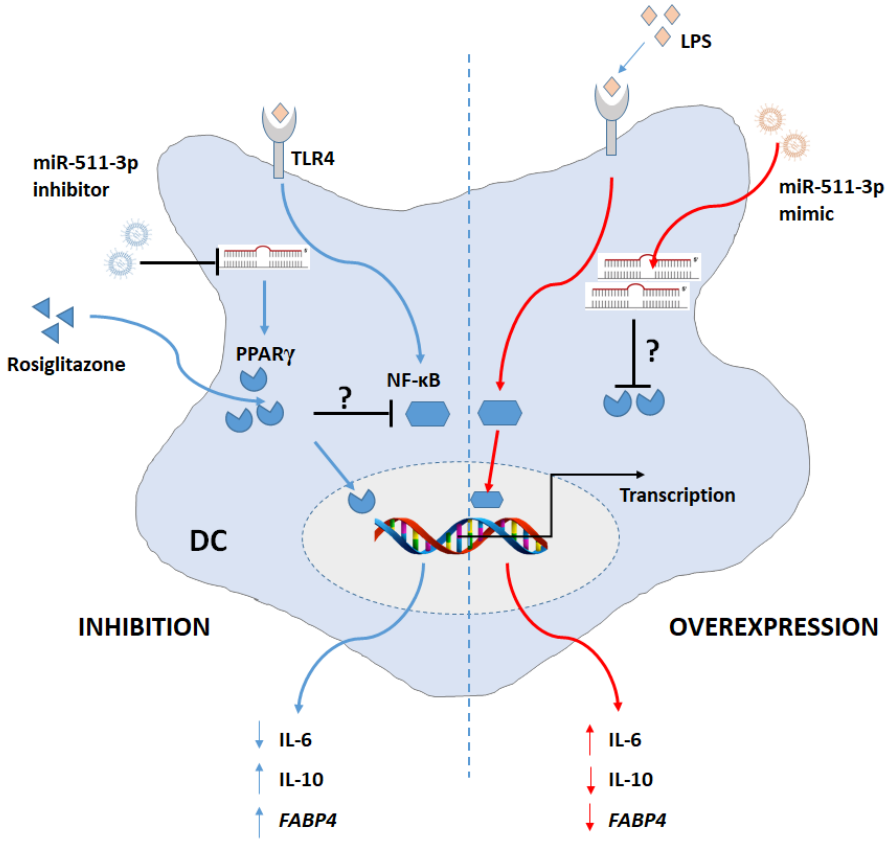


Figure 5.6: Schematic representation of the relationship between miR-511-3p and PPARγ in modulating human DCs function. Downregulation of miR-511-3p promotes of PPARγ expression and activity (FABP4) and subsequently suppresses LPS-induced inflammation most likely through inhibiting NF-κB. In contrast, overexpressing miR-511-3p reverses the effects of PPARγ and promotes transcription of pro-inflammatory genes.

Collectively, these data highlight the potential immune-regulatory role of miR-511-3p on PPARγ activity in human DCs. The roles of PPARγ and its ligands in immune regulation are beginning to be elucidated, and this transcription factor is emerging as a key player in various stages of the resolution of inflammation.

Chapter 6: The Role of miR-511-3p in Modulating the NF-kB Pathway in Human Dendritic Cells

Chapter 6: The role of miR-511-3p in modulating NF-kB pathway in human DCs

6.1 Introduction

As an immediate response following pathogen recognition, cells of the innate immune system secrete cytokines and other mediators that contribute towards the inflammatory process to aid clearance of the pathogen. Historically, these responses are divided into phylogenetically conserved signaling events, through which changes elicited in the extracellular milieu alter gene expression at the cellular level. A high number of transcriptional changes that occur as well as the associated gene products, are mediated by the NF-κB transcription factor (342).

In mammals, the NF-κB family consists of five subunits, each of which contains a conserved Rel homology: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel. The first two are synthesized as large precursors that are further processed post-transcriptionally into the p50 and p52 subunits respectively (342,370). Under steady state conditions, NF-κB proteins are sequestered in the cytoplasm by specific inhibitors known as IκBs. Activation occurs through a diverse array of chemical agents as well as cellular stress conditions including bacterial LPS and viral pathogens as well as growth factors and cytokines (371). In the process, NF-κB proteins form numerous homo- and/or heterodimers depending on the nature of the specific response. For instance, the p50 and p52 homodimers have been shown to function as transcriptional repressors whereas dimers containing RelA or c-Rel are activators. RelB on the other hand exhibits a more regulatory flexibility, acting both as a repressor and an activator (372). Two major pathways for NF-κB activation have been described (Figure 6.1) namely the canonical (or classic) pathway, which involves IκB kinases (IKKβ and IKKγ/ NEMO), required for activation of dimers such as p50/c-Rel or p50/RelA; as well as the non-canonical (or alterative) pathway, which requires IKKα alone for

complexes inhibited by p100. Both pathways can be activated by an overlapping set of signals, and both can also inhibit or activate an overlapping set of target genes as demonstrated for TNF-α (373,374). A third (or atypical) pathway has also been described, which unlike the former or latter, is activated by stress signals and mediated by the ataxia telangiectasia mutated (ATM) checkpoint kinase (375).

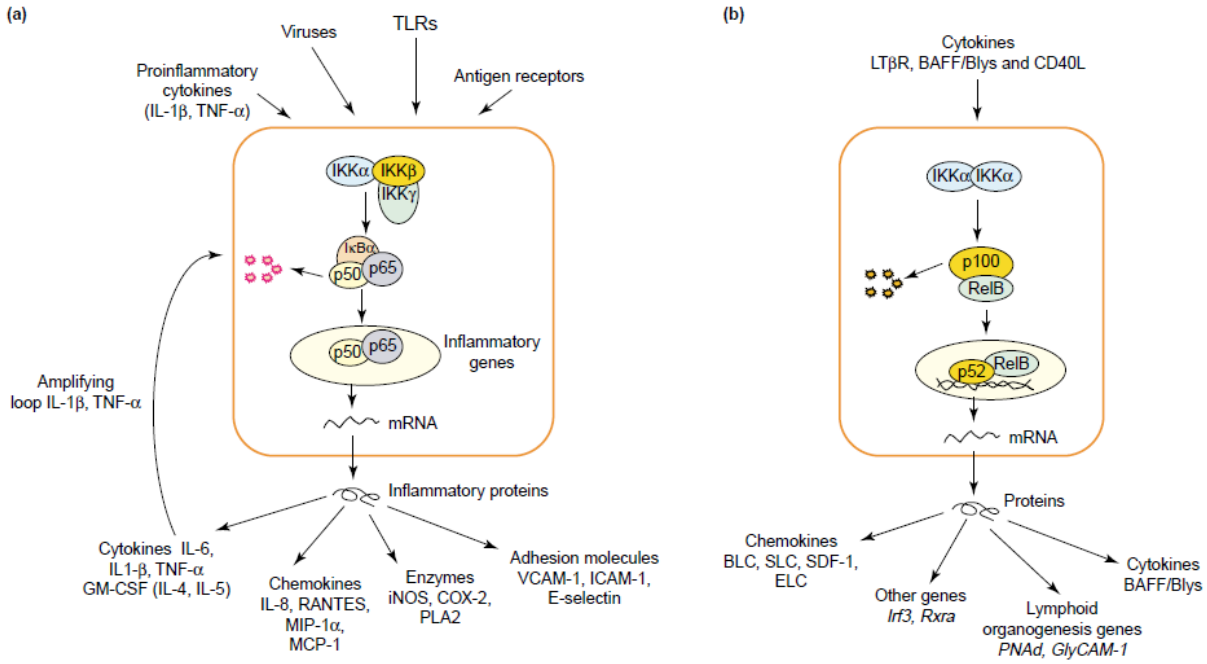


Figure 6.1: Classical and Alternative pathway of NF-κB activation. (A) The classical pathway is triggered by LPS, TNF-α or TLRs. This leads to activation of IKKβ in the IKK complex, which can then phosphorylate and degrade IκBα. NF-κB dimers can then translocate to nucleus and transcribe target genes. (B) The alternative pathway is triggered by BAFFR, CD40L or LTβR, which leads to activation of IKKα. IKKα can then phosphorylate p100 resulting in its subsequent proteasomal processing to p52. The p52-RelB heterodimers then activate transcription of target genes. Adapted from (372).

Several studies have demonstrated the role of miRNAs in regulating immune responses through the NF-κB pathway (376-378). For instance, miR-15a, miR-16 and miR-223 were shown to target IKKα mRNA; however, a decrease in these miRNAs during monocyte-macrophage differentiation increases IKKα protein levels and contributes

towards p52 production (379). As shown by others, miR-146 acts as an important negative regulator of immune responses by targeting NF-κB activation following exposure to LPS or TNF-α (274,380,381). Additionally, the TNF-α-induced protein 3 (TNFAIP3, also known an A20), a critical inhibitor of NF-kB signaling, is inhibited by miR-125a/b to promote NF-κB activation (382). Regulation of NF-κB activation by miRNAs has also been reported for disease conditions particularly cancer, with key players targeting proteins such TAK1 and MyD88 in pancreatic adenocarcinoma (miR-143 and miR-940 respectively), IKK complex in ovarian cancer (miR-199a) and TRAF6 in hepatocellular cancer (miR-429) (383-386). Within the context of allergic inflammation, miRNAs have also been shown to regulate the NF-κB pathway. For example, RNA profiling of peripheral blood from house dust mite (HDM) sensitized asthmatic patients showed a downregulation of miR-625, miR-22 and miR-513 compared to healthy controls. Interestingly, these miRNAs were shown to target genes including *CBL, PPARGC1B* and *ESR* that belong to the PI3K-AKT and NF-κB signaling pathways and resulted in lower levels of inflammatory cytokines (IFN-γ, TNF-α, IL-12, and IL-10) in plasma from the asthmatic patients (387).

As summarised above, NF-κB is a key player in regulating inflammatory gene products during an immune response. It was hypothesised that considering changes in miR-511-3p expression demonstrated in previous chapters, affects human DC phenotype and function (i.e. cytokine secretion and IDO enzyme activity) within the context of inflammation, it is likely that components of the NF-κB pathway may be modulated by changes in miR-511-3p expression. Therefore, it was examined whether changes in miR-511-3p (and its downstream effect on CLR expression), can modulate LPS-induced activation of the NF-κB pathway in human DCs.

6.2 Results

6.2.1 The NF-κB pathway is modulated by changes in miR-511-3p expression.

To better understand how changes in miR-511-3p expression might affect components of the NF-κB pathway following LPS stimulation, (in the presence and absence of mannan) a reverse phase protein array (RPPA) approach was employed. This platform enables measurement of protein expression as well as post-translational modifications across a set of tissue or cell samples and represents a useful way of picturing signal transduction pathways in normal or diseased cells (388).

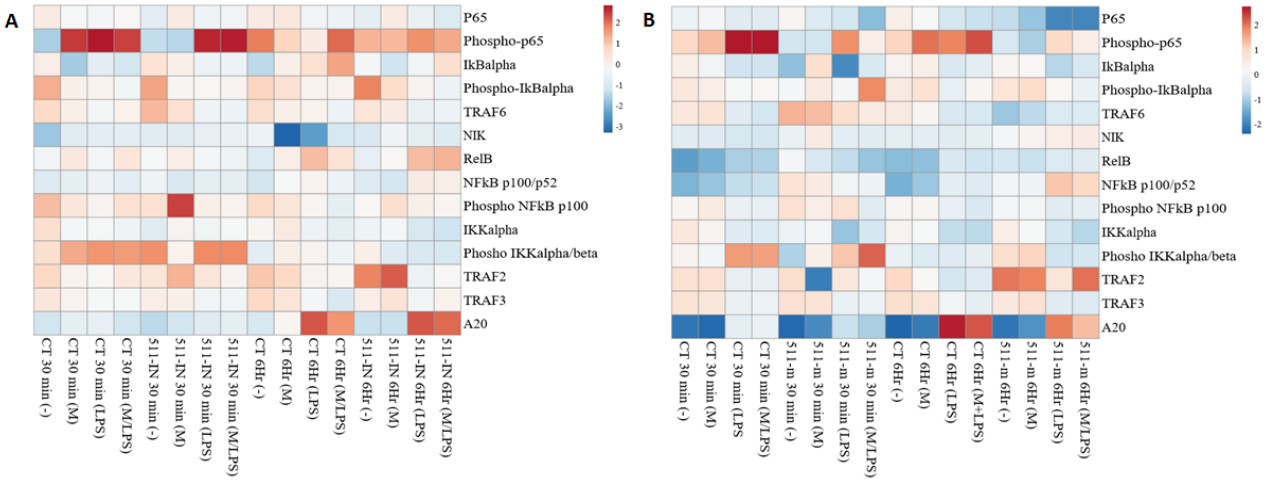


Figure 6.2: The NF-κB pathway is modulated by changes in miR-511-3p. A) Heat map illustrating the relative expression of NF-κB proteins at 30 minutes and 6 hours in miR-511-3p^{low} DCs stimulated with mannan and LPS. Data is from one independent experiment with two biological replicates and two technical replicates for each donor. B) Heat map illustrating the relative expression of NF-κB proteins at 30 minutes and 6 hours in miR-511-3p^{hi} DCs stimulated with mannan and LPS. Data is from one independent experiment with two biological replicates and two technical replicates for each donor.

To achieve this, a panel of 14 proteins from both the canonical and non-canonical NF-κB pathways was evaluated at two different time points (30 minutes and 6 hours) using β-actin as a reference control. The heatmap (Figure 6.2) summarises relative abundance

of proteins, from low (blue) to high (orange-red) expression in both miR-511-3p knockdown and overexpressed cells. Rapid induction of proteins such as phospho-p65 (pP65), pIKKab and pNF-κBp100/p52 was seen at earlier time point for both conditions following LPS stimulation, whereas others such as TRAF6 and pIKbalpha were highly induced in miR-511-3p^{hi} cells alone. It is noteworthy that levels of these proteins after 30 minutes were higher in the miR-511-3p^{low} cells compared to miR-511-3p^{hi} cells particularly for pP65 and pIKKab, but differences were not significant (Figure 6.3).

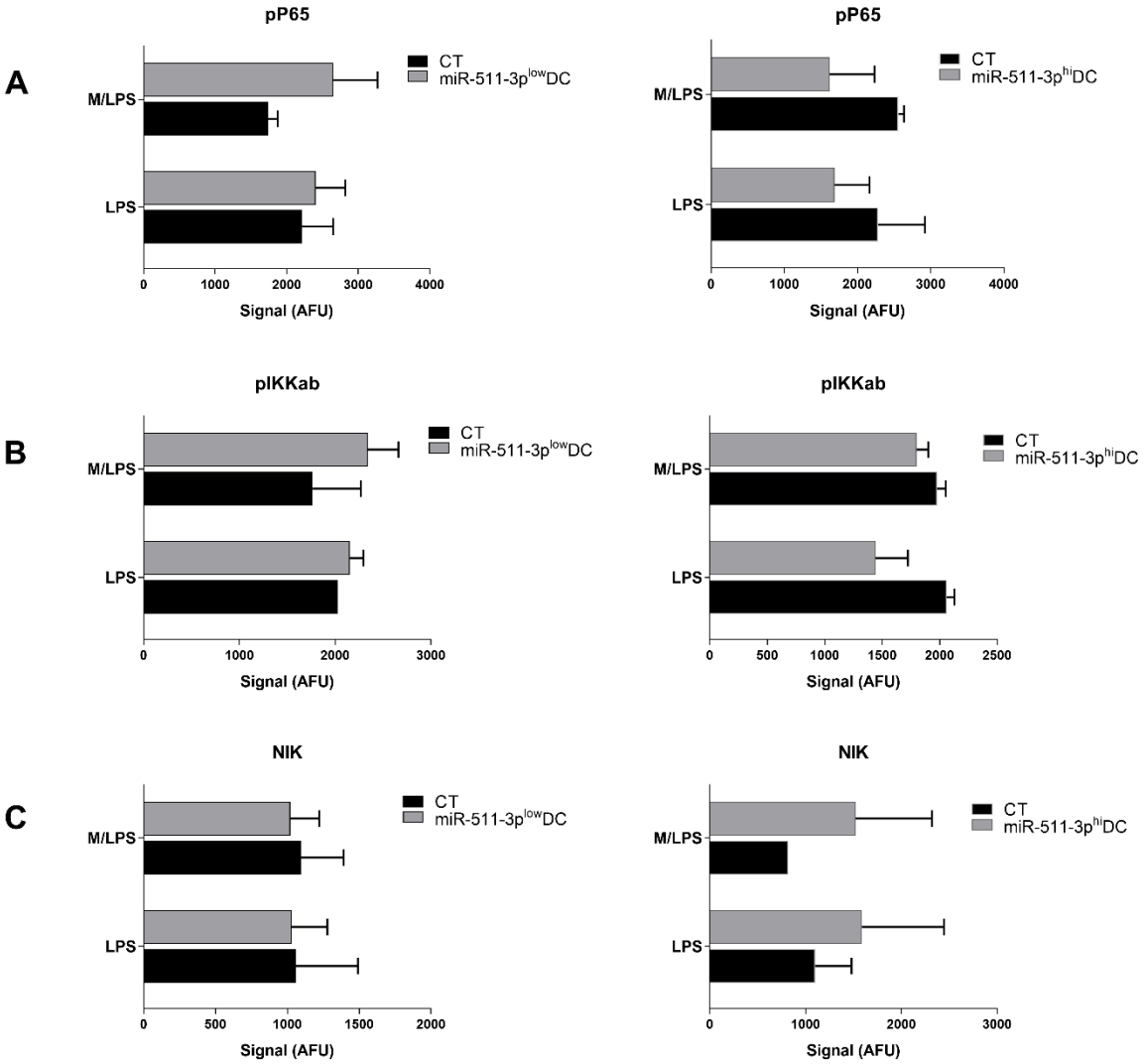


Figure 6.3: NF-κB pathway is modulated by changes in miR-511-3p expression. Protein expression of A) phospho-P65 B) pIKKab and C) NIK between miR-511-3p^{low} and miR-511-3p^{hi} DCs stimulated with LPS alone, and mannan followed by LPS

(M/LPS). For each condition (A, B and C), data is expressed as mean ± SD of one independent experiment with two biological replicates and two technical replicates for each donor. Fluorescent signals are represented as AFT (arbitrary units) with β-actin normalisation.

On the other hand, the NF-κB inducing kinase (NIK), which activates the non-canonical pathway, was highly induced in miR-511-3p^{hi} compared to knockdown cells after 30 minutes and 6 hours, although the difference was not significant. In most cases, mannan alone did not induce pathway activation in controls or transfected cells (data not shown) but showed similar levels of expression in the presence of LPS (M/LPS) compared with LPS alone (Figure 6.3).

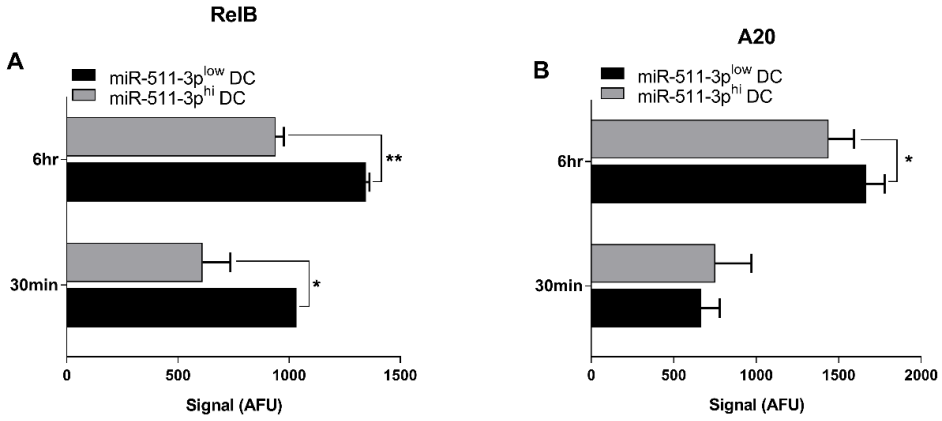


Figure 6.4: NF-κB pathway is modulated by changes in miR-511-3p expression. A) Protein expression of RelB between miR-511-3p^{low} and miR-511-3p^{hi} DCs stimulated with LPS alone after 30 minutes and 6 hours. Data is expressed as mean ± SD of one independent experiment with two donors and two technical replicates for each donor. *p < 0.05, **p < 0.01 (Student's T-test). B) Protein expression of A20 between miR-511-3p^{low} and miR-511-3p^{hi} DCs stimulated with LPS alone after 30 minutes and 6 hours Data is expressed as mean ± SD of one independent experiment with two donors and two technical replicates for each donor. *p < 0.05 (Student's T-test). Fluorescent signals are represented as AFU with β-actin normalisation.

Interestingly, there was a significant increase in RelB expression (an indicator of non-canonical pathway activation) in miR-511-3p^{low} DCs compared to overexpressed conditions at early time point (30 minutes) and after 6 hours following LPS stimulation (Figure 6.4). A similar trend was also seen for A20 inhibitor between both conditions after 6 hours of LPS treatment, suggesting that the NK-κB pathway may be significantly inhibited in miR-511-3p^{low} cells at later time points compared to overexpressed conditions.

6.3 Discussion

Nuclear factor-κB is a crucial transcription factor required for optimal expression of a diverse set of genes including cytokines, adhesion molecules, growth factors and immune receptors (389). Cytokines such as TNF-α, IL-6, IL-1β and IL-8, involved in the generation of acute inflammatory responses, are controlled by NF-κB (341). Activation of this pathway is however tightly regulated by inhibitory proteins (e.g. IκB) that sequester NF-κB in the cytoplasm. Phosphorylation of IκBs liberates active NF-κB, which then migrates and binds specific promoters in the nucleus to activate transcription (342). Increasing evidence suggests that microRNAs are key regulators of this multi-layered system and therefore function to keep a check on immune responses and inflammation (390-392).

In the present study, a protein microarray approach was used to study the effect of miR-511-3p manipulation on components of the NF-κB pathway. As shown by others, targeting the p65/p50 dimers, (the most abundant form of NF-κB dimers) (393), represents an important strategy to modulate inflammatory genes. In this regard, miRNAs such as miR-9, upregulated in PMNs, was shown to inhibit NF-κB after LPS stimulation (394). From the data obtained, treatment with LPS alone or in the presence of mannan (M), promotes rapid expression of pP65 and pIKbalpha, suggesting that the canonical pathway is strongly induced in both miR-511-3p knockdown and overexpressed conditions by TLR4 activation. Although expression was higher in miR-511-3p^{low} DCs compared to overexpressed cells and controls, this was not statistically significant (Figure 6.3). Interestingly, the cross-talk between receptors on DCs such as CLRs and TLR4 has been implicated in modulating TLR-induced NF-κB activation. For instance, MR was shown to promote *Pneumocystis*-mediated P65 nuclear translocation in the presence of LPS, whereas in the presence of MR siRNA, NF-κB

activation was decreased (395). Likewise, components of *C. albicans*, which interact with the MR was shown to promote NF-κB activation via modulation of TLR2 and TLR4 (396), further highlighting the involvement of MR in modulating the NF-κB pathway. Recently, miR-23b was shown to target the NF-κB inducing kinase (NIK) to suppress NF-κB activation and mediate immunosuppression by promoting production of IL-10 and IL-4 in a mouse model of sepsis (397). In the present study, NIK expression was increased following overexpression of miR-511-3p, compared to miR-511-3p^{low} cells and controls, although this was not statistically significant (Figure 6.2). It is therefore likely that increased expression of miR-511-3p may promote alternative pathway activation of NF-κB in human DCs via NIK.

In general, activation of the canonical NF-κB pathway induces inflammation, however, it has been reported that RelB, a member of the alternative NF-κB pathway can induce potent anti-inflammatory responses (398). For instance, mice deficient in RelB exhibited increased inflammation in the lungs and kidneys (399,400), and RelB expression was shown to suppress lung inflammation by inhibiting cigarette smoke extract-induced PGE_2 production in mice (401). As described in Figure 6.4, miR-511-3p^{low} DCs showed a significant increase in RelB expression at the earlier and later time points compared to miR-511-3p^{hi} cells, which suggests that miR-511-3p knockdown cells may promote immunosuppression in the context of inflammation. In a previous study, overexpression of RelB was also shown to modulate IL-1β-induced production of PGE_2 and Cox-2 in human lung fibroblasts partly through miR-146 expression (402). Other mechanisms have been demonstrated, which appear to limit inflammatory responses through suppression of NF-κB. For instance, exposure to endotoxins, or cytokines such as TNF-α and IL-β can induce production of IL-10 to suppress proinflammatory cytokines. In line with this, the TNF-α induced protein 3 (TNFAIP3)

or A20, which is expressed in cells such as DCs and macrophages has been reported to negatively regulate NF-κB-mediated inflammation by inhibiting potent inflammatory responses induced by TNF-α signaling to maintain homeostasis. Several miRNAs have been shown to target A20 to inhibit its function (382,403-405). For example, Zhao et al recently showed that forced expression of miR-221 following LPS treatment, promoted IL-6 and TNF-α cytokine production and enhanced NF-κB as well as MAPK activation (406). Additionally, they showed that miR-221 exerted its pro-inflammatory effects by targeting A20 expression in macrophages both *in vitro* and *in vivo*, thus highlighting the importance of A20 in controlling inflammatory responses. In the present study, downregulation of miR-511-3p in human DCs resulted in a significant increase in A20 expression after 6 hours of incubation, compared to overexpressed cells and controls, which suggests A20, may exert its inhibitory effect at a later time point after encounter with pathogen, to mediate immune suppression. Additionally, the data indicates that overexpression of miR-511-3p may inhibit A20 function, and ultimately promote NF-κB activation. Xuan et al also showed that mice with DC-specific deletion of A20 died within 6 hours after injection with low-dose LPS, whereas A20 expressing mice survived.

In conclusion, data presented in this chapter demonstrate that miR-511-3p may play an important role in regulating activation of NF-κB in human DCs by modulating expression of key components such as NIK, RelB and A20 in the context of LPS-mediated inflammation. In particular, knocking down miR-511-3p promotes increased expression of RelB and A20, which are relevant for resolving inflammatory responses. Further studies are however needed to understand the molecular mechanisms through which miR-511-3p mediates regulation of these components.

Chapter 7: General Discussion

Chapter 7: General discussion

The immune system provides a well-orchestrated response against a myriad of pathogenic infections including bacteria, viruses, fungi and parasites (407). The molecular signatures typically associated with the invading organism are first recognised by innate immune receptors (i.e. PRRs) on cells such as macrophages and DCs, which initiate a cascade of signaling events leading to transcription of genes necessary for eliminating the pathogen. Given the complexity of these events, it is apparent that highly regulatory mechanisms exist to ensure generation of appropriate responses against the foreign invader (408). One line of such regulatory events that has emerged over the last decade is the role of microRNAs (miRNAs). Since discovery of the first miRNA, *lin-4* in *C. elegans*, several hundreds of miRNA have been identified in humans, which are predicted to regulate expression of nearly 60% of all protein coding genes (409). As a result, miRNAs are now recognised as powerful regulators of cellular and biological events and crucial controllers of inflammatory and other disease-related processes (212-214).

Recently, miR-511 has emerged as an important regulator of human diseases particularly cancer (227-230), however its role in regulating inflammatory disorders such as allergy as well as DC function is not well understood. Therefore, the main aim of this study was to investigate the role of miR-511-3p (located at the 3' end of mature strand) in modulating human DC function. This was particularly focussed on examining the influence of miR-511-3p up or down regulation using synthetic mimics or antisense inhibitors on DC phenotype and function, within the context of LPS-induced cytokine production, effect on key immune modulatory (i.e. IDO and PPARγ activity) and signaling (i.e. NF-κB) molecules, as well as downstream T cell polarization. Better

understanding of how miR-511-3p modulates human DC function could pave the way for rational design of therapies against inflammatory disorders.

Initial experiments were aimed at evaluating the expression profile of miR-511-3p in human DCs following treatment with TLR4 and CLR agonists. Compared to $CD14^{+}$ monocytes, miR-511-3p was clearly induced in immature Mo-DC. Mechanisms that have been demonstrated to promote expression of miR-511-3p in cells such as macrophages (particularly from mice) include treatment with IL-4 (234,273), which in addition to GM-CSF are key drivers of Mo-DC differentiation *in vitro*. Interestingly, IL-4 also promotes *mrc1* gene expression (which encodes MR) in macrophages from mice, and the finding that miR-511-3p is embedded within the *mrc1/CD206* gene in both humans and mice suggests the involvement of IL-4 in promoting miR-511-3p expression in Mo-DCs. Intriguingly, surface expression of MR and another C-type lectin receptor namely DC-SIGN on Mo-DCs were modulated following up or downregulation of miR-511-3p expression, with suppression of miR-511-3p (i.e. miR-511-$3p^{low}$ DCs) promoting an increase in MR and DC-SIGN protein and mRNA expression; and vice-versa with an increase in miR-511-3p expression (i.e. miR-511-$3p^{hi}$ DCs).

In mice, studies show that miR-511-3p and the *mrc1* gene are co-regulated as with most intronic miRNAs (232), however this is not always the case, with a third of all intronic miRNAs predicted to have independent promoters as previously reported by Kondo et al and others (324,325,410). Additionally, according to TargetScan version 7.2, which predicts mRNA targets of miRNA on the basis of complementarity to miRNA seed regions, miR-511-3p does not directly target MR or DC-SIGN mRNA but may exert off target effects on both receptors, thus further highlighting the complexity of regulatory mechanisms between intronic miRNAs and their respective host mRNAs.

Treatment of Mo-DCs with the TLR4 agonist, LPS on the other hand downregulated miR-511-3p expression and there have been reports indicating that miRNAs regulated by LPS treatment target genes that contribute to an inflammatory phenotype. For example, the protein kinase Akt1, which is activated by LPS, was shown to positively regulate Let-7e and miR-181 in mouse macrophages; however, macrophages lacking Akt1 ($Akt1^{-/-}$) showed heightened responsiveness to LPS signaling due to a decrease in Let-7e expression, resulting in endotoxin tolerance. Overexpression of let-7e on the other hand, restored sensitivity and tolerance to LPS *in vitro* and *in vivo* (411). Generally, LPS mediates an inflammatory response and is a key promoter of DC maturation, a process that induces changes in expression of key receptors such as HLA-DR, CD86, CD40 and PDL-1 required for interaction with naïve T cells in lymphoid organs (280). These changes also include downregulation of receptors required for antigen capture such as MR and DC-SIGN (278,279) and may account for suppression of miR-511-3p expression following exposure to LPS. Nevertheless, mechanisms leading to decrease in miR-511-3p expression following treatment with LPS are not fully known. Conversely, treatment with mannan promoted upregulation of miR-511-3p whereas sulphated galactose (SO_4-3-Gal-PAA), which binds the cysteine rich domain of MR, downregulated miR-511-3p expression in DCs with exception of 10µg/ml treatment which had no effect. Nevertheless, the underlying mechanisms are not fully understood. Considering the observed changes to Mo-DC surface marker expression following up or downregulation of miR-511-3p expression as well as the effect of TLR and CLR engagement on miR-511-3p expression in DCs, subsequent experiments were aimed at evaluating functional responses in DCs associated with changes in miR-511-3p expression.

Indoleamine 2,3-dioxygenase (IDO) plays a central role in promoting immune suppression by inhibiting Th1 cells and promoting the development of Tregs. The role of IDO in modulating the outcome of diseases such as allergy, cancer and autoimmunity has been demonstrated by our group and others. For instance, our group has shown, in the context of allergy that MR or DC-SIGN ligation with glycosylated antigens can have opposing effect on IDO activity, whereby MR ligation promotes a decrease in IDO activity and DC-SIGN ligation promotes an increase. Several allergens are highly glycosylated and can interact with various CLRs on DCs such as MR and DC-SIGN. It is likely that these receptors may antagonise each other in allergen recognition and differ in downstream pathways leading to IDO modulation. Following the observed changes in expression of MR and DC-SIGN in miR-511-3p$^{low\ /hi}$ DCs, their effect on IDO activity was investigated. TargetScan prediction software showed that miR-511-3p does not target the IDO1 mRNA in humans; nonetheless it is likely that changes observed in IDO activity following treatment with mannan and LPS may be due to the changes in MR and DC-SIGN induced by miR-511-3p modulation. Hence, miR-511-3p may exert its influence on MR and DC-SIGN via binding to other targets. For instance, increased expression of miR-155 was shown to downregulate the PU.1 transcription factor and was associated with decreased transcriptional activity of the DC-SIGN promoter, ultimately affecting receptor function (412). Moreover, immature Mo-DCs express high levels of MR and DC-SIGN, which could explain the significant downregulation of IDO activity in miR-511-3p^{low} cells after treatment with mannan alone, since these cells express even higher levels of MR and DC-SIGN. Similarly, miR-181a, which is known to promote B and T cell lineage development, was shown to enhance IDO1 and IL-6 mRNA but not protein expression in mesenchymal stem cells (MSCs), to regulate the immunosuppressive properties of MSCs by targeting

components of the MAPK signaling pathway such as JNK and p38 respectively (413). On the other hand, miRNAs such as miR-153 was shown to directly target and bind the 3'UTR of IDO1 in solid tumours, highlighting the potential of miR-153 in therapies against tumours. Taken together, these observations highlight involvement of miR-511-3p regulation in controlling IDO partly through MR and DC-SIGN.

In addition to IDO activity, cytokine production and expression of surface markers such as PDL-1 influence the immune stimulatory or immune regulatory capacity of human DCs. It was therefore determined how LPS stimulation of miR-511-3p$^{low\ /hi}$ DCs influence production of a panel of pro- and anti-inflammatory cytokines. As indicated, treatment with mannan alone had no effect on cytokine production. Nevertheless, CLRs have been shown to modulate TLR-induced signaling particularly TLR4. For example, MR ligation with ManLAM was shown to inhibit IL-12 production after challenge with LPS (414). Data contradicting CLR modulation of TLR pathways however indicates that ligation of DC-SIGN with fucosylated antigens from *Schistosoma mansoni* can rather promote pro-inflammatory cytokine production (415), suggesting the differing effects of MR or DC-SIGN ligation on DC function. In this study, the levels of proinflammatory cytokines IL-6 and IL-12p70 were significantly reduced in miR-511-3p^{low} cells, compared to controls which could in turn impact polarisation of Th17 and Th1 cells respectively. In line with this, there was a significant reduction in IL-17 production in T cell co-cultures with miR-511-3p^{low} DCs treated with LPS. Additionally, IFN-γ production was also reduced, although not statistically significant, further highlighting the impact of miR-511-3p knockdown on T cell function. Several miRNAs such as miR-146, miR-148 family and miR-21, have been demonstrated to function as negative regulators of DC signaling following antigen uptake and are able to modify the activation of T cells (416). The anti-inflammatory cytokine IL-10, as well

as expression of the PDL-1 surface marker, both of which promote regulatory T cell (Treg) differentiation and function (332), were significantly increased in the miR-511-3p^{low} DCs. Although T cells showed an increase in IL-10 production after co-cultures with miR-511-3p^{low} DCs, this was not significant. In contrast to these observations, there was a decrease in IL-10 as well as IL-12p70 production in the miR-511-3p^{hi} DCs, whereas no significant changes were seen in TNF-α or IL-6 production. The effect of these changes was reflected in co-culture of miR-511-3p^{hi} DCs with T cells, where a significant decrease was seen in TGF-β production by the T cells highlighting the potential of miR-511-3phi DCs to promote a pro-inflammatory Th phenotype. Interestingly also, IL-10 was decreased whereas IFN-γ production was increased, although these changes were not significant. It is therefore likely that changes induced by miR-511-3p modulation play a key role in regulating DC function.

In order to further understand how changes in miR-511-3p expression might affect signaling events in DCs following treatment with mannan and LPS, a reverse-phase protein array approach was used to investigate the expression changes of a panel of 14 proteins that are components of the NF-κB pathway. Treatment with mannan alone had no significant impact on expression of proteins in miR-511-3p$^{low/hi}$ DCs; however the levels of phosphorylated P65, pIKKab and NIK after treatment with LPS or M/LPS were up or downregulated accordingly compared to controls. Even so, the contribution of mannan to the observed changes in protein expression is likely influenced by LPS in M/LPS conditions. On the other hand, CLRs such as DC-SIGN for instance have been shown to modulate TLR-induced activation of NF-κB by activating the serine and threonine kinase Raf-1 leading to an increase in production of IL-10 (417). Our group has also shown that MR engagement with mannan downregulates RelB expression after TLR-4 induced NF-κB activation resulting in decreased IL-12 production by DCs

(116). Interestingly, downregulation of miR-511-3p expression in this study resulted in a significant increase in expression of RelB and A20 following LPS or M/LPS treatment. In another study, RelB was also shown to be a direct target of miR-146a, which decreased the inflammatory responses of Ly-6^{hi} and $CD14^{lo}$ $CD16^{+}$ monocytes in mice and humans respectively, following LPS challenge (418). The changes in expression of key NF-κB proteins indicated above coupled with the ability of miR-511-$3p^{low}$ DCs to increase IL-10 production and suppress pro-inflammatory cytokine release highlights the involvement of miR-511-3p knockdown in promoting anti-inflammation.

Lastly, the role of miR-511-3p in regulating PPARγ activity in human Mo-DCs was investigated. PPARγ, a ligand activated transcription factor involved in adipocyte differentiation and lipid metabolism has been shown exhibit immune modulatory properties by controlling inflammation, particularly in macrophages. Unlike IDO however, PPARγ is putatively targeted by miR-511-3p as demonstrated by Tserel et al (263). Additionally, the PPARγ coactivator 1 alpha (*PPARGC1A*) is also targeted by miR-511-3p as predicted by TargetScan. Within the context of LPS-induced inflammation, knockdown of miR-511-3p promoted expression of PPARγ mRNA as well as IL-10 production following treatment with PPARγ agonist rosiglitazone (RSG), whereas there was a downregulation in IL-6 production. These changes were however reversed in DCs treated with miR-511-3p mimics. In a different study, upregulation of miR-27b in macrophages following LPS exposure was also shown to directly target PPARγ to promote Th1 differentiation and cytokine secretion (358). Interestingly, a number of potential links between PPARγ and c-type lectins, particularly the mannose receptor (MR) have also been suggested. These include data showing that interaction between MR and Man-LAM from *Mycobacterium tuberculosis* can induce PPARγ activation, which promotes intracellular survival of the bacterium (419). Considering

that modulation of miR-511-3p expression was associated with changes in MR and DC-SIGN expression as well as changes in IDO activity, it is likely that miR-511-3p may play a role in regulating DC function through PPARγ. Additionally, MR expression is upregulated by PPARγ activity in alternatively activated macrophages (AAM) in the lung and enhances apoptotic cell recognition and phagocytosis (420). Administration of GW9662 was shown to reverse enhanced phagocytosis and reduced proinflammatory cytokine expression (420). Similarly, in this study stimulation of Mo-DCs with GW9662 was seen to reverse the effects of RSG on LPS-induced pro-inflammatory cytokine production. Furthermore, others have shown that Th2 cytokines such as IL-13 and IL-4 can induce MR expression through PPARγ activation in DCs and murine macrophages (421,422). It is therefore likely that changes in MR expression particularly following knockdown of miR-511-3p may contribute to the overall regulatory effect on PPARγ activity in human DCs.

In summary, this study has demonstrated the role of miR-511-3p in regulating the phenotype and function of human monocyte-derived DCs. As shown, miR-511-3p is highly expressed in DCs compared to $CD14^+$ monocytes. Changes in miR-511-3p expression however affect the expression of key DC markers particularly MR and DC-SIGN, which in turn modulate downstream IDO activity. In addition, cytokine secretion and downstream T cell function was affected by miR-511-3p modulation. Furthermore, the changes in MR and DC-SIGN expression induced by miR-511-3p expression modulate components of both the canonical and alternative NF-κB pathway. Lastly, this study has shown that miR-511-3p may affect the anti-inflammatory properties of Mo-DCs by targeting the expression and activity of PPARγ. These observations highlight the complexity of miR-511-3p induced regulation of human DCs and could pave way for design of therapies against inflammatory disorders.

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