

Identification of Novel Interactions between MicroRNAs and Pattern-Recognition Receptor Signalling in Dendritic Cells



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Abstract

Dendritic cells are equipped with a range of different pattern-recognition receptors (PRR) aimed at recognizing foreign pathogens. Recent evidence has suggested that PRR signalling regulates the expression of microRNAs (miRNAs), important post-transcriptional regulators of gene expression, which have been shown to fine-tune innate immune responses.

This thesis describes the discovery of miR-650, a novel PRR-responsive miRNA that is down regulated in monocyte-derived dendritic cells (DCs) on PRR stimulation. Chapter 4 describes the characterisation of miR-650 expression in DCs matured by exposure to a variety of different pathogen-derived ligands, or during Influenza A virus infection. When correlating the level of miR-650 to the induction of DC activation markers on the cell surface, an inverse correlation was observed, suggesting a relationship between miR-650 down regulation and the effective dose of the ligand.

Work presented in this thesis further explores the potential function of miR-650 by using a multi pronged approach encompassing computational biology, genome-wide expression profiling and individual reporter assays, to gain insight into the gene networks regulated by miR-650. While Chapter 5 focuses on the identification and confirmation of individual miR-650:target interactions, Chapter 6 investigates both direct as well as secondary effects exerted by miR-650 on a global level.

The work in these two chapters identifies a number of novel miR-650 targets and suggests a dual role for miR-650 in the innate immune response. Firstly, it is shown that miR-650 directly regulates a group of interferon-stimulated genes with known antiviral activity. Supporting its role in antiviral host defence, miR-650 is also shown to directly target components of the autophagic machinery, and even more importantly, down regulation of miR-650 induces autophagosome formation. Secondly, identified targets also include negative regulators of innate signalling suggesting that, in addition to its antiviral function, PRR-mediated down regulation of miR-650 expression may also provide a negative feedback loop controlling inflammatory responses. Notably, miR-650 displays reciprocal target regulation with miR-155, a well studied miRNA with established functions in the innate immune system, thus suggest-

ing cooperativity between the two miRNAs.

The original aim of this thesis was to examine the effect of HIV-1 infection on the global miRNAome of DCs using a genome-wide profiling method. However, as outlined in Chapter 3, the data generated suggest that HIV-1 infection has little or no impact on miRNA expression. Further work is needed to establish if this represents deliberate immune evasion by HIV-1, or just indicates the limits of the methodology employed.

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List of Abbreviations

3' UTR	three prime untranslated region
A	adenine
Ab	antibody
ABI	Applied Biosystems
AGO1	argonaute
AP-1	activating protein - 1
APC	antigen presenting cell
APOBEC3G	apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G
Atg	autophagy-related protein
ATP	Adenosine triphosphate
AZT	azidothymidine
BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1
BCG	bacillus Calmette-Gurin
Bcl-2	B-cell lymphoma 2)
Bcl-XL	B-cell lymphoma-extra large
BDCA	Blood dendritic cell antigen
BIR	baculovirus inhibitor of apoptosis protein repeat
BSA	Bovine serum albumin
C	cytosine
CA	capsid
CARD	Caspase recruitment domains
CCR	C-C chemokine receptor
CD	cluster of differentiation
Cdc42	Cell division control protein 42
cDNA	complementary DNA
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
cRNA	complementary RNA
CRUK	Cancer Research UK
CUTL1	cut-like 1 (Drosophila)
CXCL10	chemokine (C-X-C motif) ligand 10
CXCR	C-X-C chemokine receptor
Cy	cyanine
DAMP	danger-associated molecular pattern
DAP12	ITAM-bearing signaling subunit
DC	dendritic cell
DC	dendritic cell

DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
EBSS	Earle's balanced salt solution
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EMCV	Encephalomyocarditis Virus
Env	HIV-1 envelope protein
ER	endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Fetal Calf Serum
FWD	forward
G	guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gbp	guanylate binding protein
GCH1	GTP cyclohydrolase 1
GFP	green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
HA	hemagglutinin
HAART	Highly Active Anti-Retroviral Therapy
HAU	hemagglutinin unit
HCV	Hepatitis C Virus
HIV-1	Human Immunodeficiency Virus - 1
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HSV	Herpes simplex virus
IFISS	ink jet in-situ synthesis
IFIT2	interferon-induced protein with tetratricopeptide repeats 2
IFITM1	interferon induced transmembrane protein 1
IFN	interferon
IGVL	immunoglobulin lambda variable
IL-10	interleukin 10
IL-12	interleukin 12
IL-4	interleukin-4
IL-6	interleukin 6
IL-8	interleukin 8
IN	integrase
ING4	inhibitor of growth family, member 4
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
IRF3	Interferon regulatory factor 3
ISG	Interferon Stimulated Gene
ISGF3	Interferon-stimulated gene factor 3

ISRE	interferon-stimulated response element
ITAM	immunoreceptor tyrosine-based activation motif
iTraq	Isobaric tags for relative and absolute quantitation
JAK	Janus Kinase
kb	kilobase
KS	Karposis sarcoma
LAP	LC3-associated phagocytosis
LB	luria bertani
LC3	Light chain 3
LCMV	Lymphocytic Choriomeningitis
LIR	leukocyte immunoglobulin-like receptor
LPS	lipopolysaccharide
LRR	leucine-rich repeats
LTR	Long terminal repeat
M tropic	macrophage tropic
MA	matrix
MACS	Magnetic-activated cell sorting
MAPK	mitogen activated protein kinase
MDA5	melanoma differentiation associated gene 5
MDCK	Madin-Darby canine kidney
MDDC	monocyte-derived dendritic cell
MDP	muramyl dipeptide
MEF	mouse embryonic fibroblasts
MHC	major histocompatibility complex
miRNA	microRNA
MMTV	Mouse mammary tumor virus
MOI	multiplicity of infection
MRE	miRNA response element
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
Mtpn	myotrophin
MxA	myxovirus (influenza) resistance A
MyD88	Myeloid differentiation primary response gene (88)
NC	nucleocapsid
NDRG2	NDRG family member 2
NEB	New England Biolabs
NEDD8	neural precursor cell expressed, developmentally down-regulated 8
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NLRP3	NLR family, pyrin domain containing 3
NOD2	(nucleotide-binding oligomerization domain containing 2
NOS2	nitric oxide synthase 2
nt	nucleotide
NUB1	negative regulator of ubiquitin-like proteins 1
OGT	Oxford Gene Technology
P body	Processing body

p-TEFb	Positive Transcription Elongation Factor b
p16^{INK4a}	Cyclin-dependent kinase inhibitor 2A
p300	E1A binding protein p300
p53	protein 53
PAM₃CSK₄	Pam ₃ Cys-Ser-(Lys) ₄
PAMP	Pathogen-associated molecular pattern
PB2	polymerase basic protein 2
PBMC	peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCAF	P300/CBP-associated factor
PCR	Polymerase Chain Reaction
pDC	plasmacytoid dendritic cell
PDCD4	programmed cell death 4
PFA	paraformaldehyde
PHA	phytohemagglutinin
PHD-finger	plant homeodomain finger
PIC	pre integration complex
Pol	Polymerase
PR	protease
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
PRR	pattern-recognition receptor
PVDF membrane	polyvinylidene difluoride
PYD	pyrin domain
qRT-PCR	quantitative real-time PCR
REV	reverse
RIG-I	retinoic-acid-inducible gene I
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RLH	RIG-like helicase
RNA	ribonucleic acid
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute medium
RQ	relative quantification
rRNA	ribosomal RNA
RSV	Respiratory syncytial virus
RT	reverse transcription
S100B	S100 calcium binding protein B
SAGE	serial analysis of gene expression
SD	standard deviation
SDN1	small RNA degrading nuclease 1
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SH2	Src Homology 2
SILAC	Stable isotope labeling with amino acids in cell culture
siRNA	small interfering RNA
SIRPB1	signal-regulatory protein beta 1
snoRNA	small nucleolar RNA

SNP	Single-nucleotide polymorphism
snRNA	small nuclear RNA
SOCS	Suppressor of cytokine signalling
SOCS1	suppressor of cytokine signaling 1
ssRNA	single-stranded RNA
STAT	Signal Transducer and Activator of Transcriptio
SV40	Simian vacuolating virus 40
T	thymine
T tropic	T cell tropic
TAB1	TGF-beta activated kinase 1/MAP3K7 binding protein 1
Tab2	TGF-beta activated kinase 1/MAP3K7 binding protein 2
TAR element	trans-activation response element
Tat protein	trans-activating protein
TBS	Tris Buffered Saline
TCID50	50% Tissue Culture Infective Dose
Th	T helper cell
THBS1	Thrombospondin 1
TIR	Toll and interleukin-1 receptor
TIRAP	toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRBP	TAR RNA binding protein
Treg	T regulatory cell
TREX1	three prime repair exonuclease 1
TRIF	Toll/IL-1 Receptor Domain-Containing Adaptor Inducing IFN- beta
UV	ultraviolet
VSV	Vesicular stomatitis virus
Waf1	cyclin-dependent kinase inhibitor 1A
WHO	World Health Organization
WIMM	Weatherall Institute of Molecular Medicine
WT	Wild type
XRN-2	5'-3' exoribonuclease 2

Chapter 1

Introduction

1.1 The innate immune response

1.1.1 An introduction to the innate immune response

The innate immune system is an evolutionary conserved mechanism that provides rapid and effective protection against many invading pathogens. It consists of a variety of different components including anatomical barriers that are impermeable to most pathogens, soluble factors and innate immune cells residing at the portal of entry. These include phagocytic cells such as macrophages, neutrophils and dendritic cells which are able to engulf and ingest invading pathogens, as well as natural killer cells which are specialised in destroying infected cells. In addition, it includes a variety of cells including basophils, mast cells and eosinophils that are capable of releasing important inflammatory mediators (Figure 1.1).

Pathogen recognition by the innate immune system relies on the detection of highly conserved pathogenic motifs. Successful recognition leads to the activation of the innate immune system and drives a cascade of defense mechanisms with the aim of restricting or removing the invading pathogen. In the first instance a localised inflammatory response is established by the induction of a variety of inflammatory mediators, such as pro-inflammatory cytokines and chemokines. However, if this fails to control the spread of the pathogen an adaptive response becomes necessary. In these cases, specialised antigen-presenting cells of the innate immune system prime and guide adaptive responses, which are specifically tailored to the exact nature of the invading organism [Iwasaki & Medzhitov, 2010].

In addition to its role in host defense, the innate immune system also plays a key role in maintaining tolerance by effectively distinguishing self from non-self.

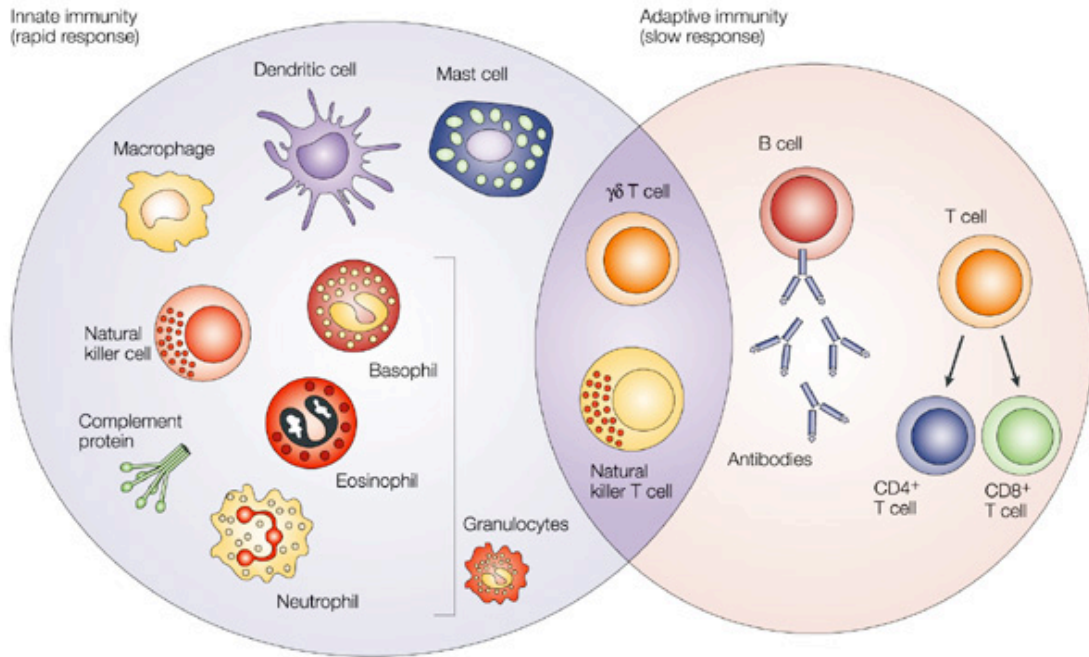


Figure 1.1: Cells of the innate immune system. The innate immune system provides the first line of defence against invading pathogens. It consists of a variety of components, including soluble factors and diverse cellular components, including macrophages, dendritic cells, mast cells, granulocytes and natural killer cells. Recognition by the innate immune system is essential for the activation of the adaptive immune response, which is slower, but also more specifically tailored to the invading pathogen. NK-T cells and a specific set of $\gamma\delta$ T cells are 'innate-like' cells which are located at the interface between the innate and adaptive immune system. Figure taken from [Dranoff, 2004].

1.1.2 Dendritic cells

Dendritic cells (DCs) were first discovered in the 1970s by Ralf Steinman as a novel haemopoietic-derived cell population in mouse spleen which have the ability to prime naive T lymphocytes [Steinman & Witmer, 1978]. DCs are now defined as professional antigen presenting cells (APCs) and can be divided into distinct subsets according to localisation, phenotype and immune function [Ju *et al.*, 2010]. They are equipped with highly efficient mechanisms to detect invading pathogens, capture, process and present exogenous or foreign antigen, and to activate naive T lymphocytes [Mellman & Steinman, 2001]. They are therefore not only important for activating the adaptive immune response but also control the quality of the response, by driving naive T cells into distinct effector cells. DCs also constantly take up self-antigen against which immune recognition should be avoided [Huang *et al.*, 2000] and therefore play an important role in establishing and maintaining central as well as peripheral

tolerance [Steinman & Nussenzweig, 2002]. Supporting their role in the protection against autoimmune disease, DC depleted mice show increased frequencies of CD4⁺ T cells in peripheral tissues and spontaneously develop autoimmunity, which among others is characterised by the onset of inflammatory bowel disease [Ohnmacht *et al.*, 2009]. Furthermore, a variety of genes involved in DC function have been shown to be associated with increased risk to autoimmune diseases, such as Crohn's disease [Hampe *et al.*, 2007].

1.1.3 The classical model of DC function

The life cycle of DCs can be described by the Langerhans cell paradigm, which was originally derived from a series of seminal papers on Langerhans cells, a special DC subset in the epidermis (Reviewed in [Banchereau, 2002]) (Figure 1.2). According to this two-stage model, DCs reside in peripheral tissues in an immature state dedicated to sampling their environment. Immature DCs are characterised by a high endocytic capacity and low-level expression of MHC and co-stimulatory molecules. Recognition of pathogenic components triggers a maturation process which is accompanied by a broad range of phenotypic and functional changes. In general, DC maturation is characterised by increased expression of MHC-peptide complexes and co-stimulatory molecules CD80, CD86 and CD40 on the cell surface as well as secretion of pro-inflammatory cytokines, such as IL-12 and TNF- α , and chemokines CCR2, CCR5 and CCR7a [Reis e Sousa, 2006]. Activated DCs migrate via the lymphatic vessels or the blood to the draining lymph nodes. During this process they transiently show increased ability to sample and process antigen, which is subsequently lost and replaced by a strong T cell stimulatory capacity [Reis e Sousa *et al.*, 1993]. DCs are involved in inducing both CD4⁺ and CD8⁺ T cell responses against antigens presented on MHC class I and MHC class II molecules, respectively. In addition, DCs have been shown to process endogenous antigen for presentation in the context of Class II, possibly through sampling autophagy vesicles [Schmid *et al.*, 2007]. Similarly, DCs present exogenous antigen to CD8⁺ T cells via Class I, a process referred to as cross-presentation [Kurts *et al.*, 2010].

Over the recent years it has become apparent that the maturation state of DCs, which is dependent on the nature of the pathogen, can drive the induction of a range of different T cell responses including Th1, Th2 and Th17 and, in some cases, also tolerogenic Treg cells. The nature of this response is partially due to the environmental signals to which the immature DC was exposed and therefore the nature of the pathogen. For example, different environmental signals have been shown to drive DCs towards becoming either Th1 or Th2 inducing, with exposure to helminthes leading to Th2 priming [Whelan *et al.*, 2000], and sensing of bacteria

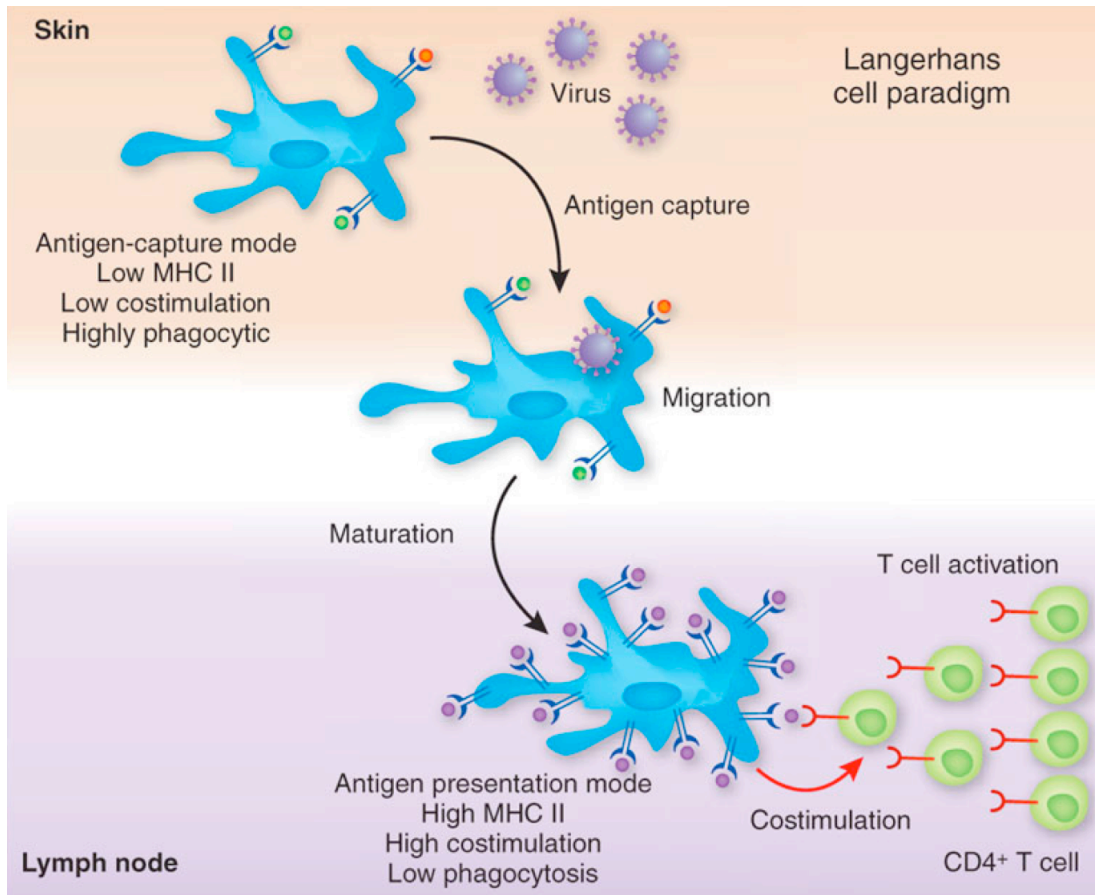


Figure 1.2: A schematic representation of the Langerhans cell paradigm. Immature DCs reside in the peripheral tissues where they capture exogenous antigen. After encountering a pathogen these DC migrate to the draining lymph nodes and mature by expressing MHC-antigen complexes and up regulating co-stimulatory molecules so they can activate naive T cells. Adapted from [Heath & Carbone, 2009].

primarily leading to a Th1 response [Vieira *et al.*, 2000]. Since then a number of studies have demonstrated that the wide variety of responses are driven by recognition of a combination of pathogenic motifs through specialised pathogen-recognition receptors which together dictate the maturation state of the DC [Huang *et al.*, 2001] [Pulendran, 2005]. However, DCs are not solely responsible for determining that type of adaptive T cell response that is mounted against the pathogen. Many pathogens secrete mimics of host cytokines which possess mainly immunosubvertive mechanisms. For example, the Epstein-Barr virus encodes BCVR1, an IL-10 homolog, produced to suppress the Th1 cellular immune response and turn the response to Th2, which is less effective [Hsu *et al.*, 1990].

1.1.4 Pattern-recognition receptors

Pathogen recognition by the innate immune system depends on germ-line encoded pattern-recognition receptors (PRRs) [Janeway, 1989]. These receptors have evolved to recognize a limited range of 'pathogen-associated molecular patterns' (PAMPs) (Table 1.1). PAMPs represent pathogenic components which are unique to microorganisms, allowing discrimination between self and non-self molecules. Importantly, PAMPs are essential for the physiology of the pathogen and therefore restrict the escape of the invading pathogen by evolutionary adaptation. Depending on the life cycle of the pathogen, recognition can occur in various cellular compartments, including the extracellular environment, endosome or cytoplasm [Kawai & Akira, 2010]. Consequently, expression of a particular PRR is confined to the cellular compartment in which it can gain access to its matching ligand (Figure 1.3).

Innate immune receptors exert two main functions: they can mediate antigen uptake or trigger downstream signalling or both. Binding of an individual PAMP to its corresponding PRR triggers a unique intracellular signalling cascade leading to DC maturation, which mediates the innate and adaptive immune response [Akira *et al.*, 2006]. Despite their individual nature there is a certain amount of cross-over in recognition of PAMPs (i.e. TLR7, TLR8 and RIG-I all recognised ssRNA), as well as extensive cross talk and synergy between different PRRs [Lee & Kim, 2007] [Kawai & Akira, 2011]. As a consequence, the overall outcome of pathogen recognition is often balanced by cross talk between different PRRs and is therefore dependent on the PAMP signature carried by the pathogen.

The first molecular component of a PRR was identified in *Drosophila* in 1996 and called Toll [Lemaitre *et al.*, 1996] and just one year later the human homologue, Toll-like receptor 4 (TLR4), was cloned [Medzhitov *et al.*, 1997]. Since then various classes of PRRs have been identified including additional members of the TLR family, nucleotide binding and oligomerisation domain (NOD)-like receptors (NLRs), retinoid acid-inducible gene I (RIG-I)-like helicases (RLHs), C-type lectin receptors (CLRs) and cytosolic DNA sensors [Heine, 2011] (Figure 1.3). The exact repertoire and expression level varies among cell types depending on their specific role in the immune response.

1.1.5 Toll-like receptors

Human toll-like receptors (TLRs) are type I membrane proteins that are homologous to the *drosophila* toll gene [Medzhitov *et al.*, 1997]. This group consists of several members that together recognize a wide range of PAMPs, including lipid, protein carbohydrate and nucleic acid signatures from a variety of different pathogens [Kawai & Akira, 2010]. Structurally all

Table 1.1: Pathogen recognition receptors and their ligands. Adapted from [Netea & van der Meer, 2011].

TLRs			
PRR	Recognised PAMP	Microorganism in Which PAMP Is Found	Signalling Molecule
TLR2-1	Triacyl lipopeptides	Bacteria	MyD88-TIRAP
TLR2-2	Peptidoglycan	Bacteria	MyD88-TIRAP
	Lipoarabinomannan	Mycobacteria	
	Phospholipomannan	Candida	
	Glycosylphosphatidylinositol	Trypanosoma	
TLR2-6	Diacyl lipopeptides Lipoteichoic acid Zymosan	Mycoplasma Streptococcus Saccharomyces	MyD88-TIRAP
TLR3	ssRNA virus dsRNA virus	West Nile virus Reovirus	TRIF
TLR4	Lipopolysaccharide Fungal mannans Envelope proteins	Gram-negative bacteria Candida Respiratory syncytial virus	MyD88-TIRAP, TRIF-TRAM
TLR5	Flagellin	Flagellated bacteria	MyD88
TLR7 and TLR8	ssRNA viruses	Influenza virus, vesicular stomatitis virus	MyD88
TLR9	dsDNA viruses CpG motifs	Herpes simplex virus Bacterial and fungal DNA	MyD88
CLRs			
PRR	Recognised PAMP	Microorganism in Which PAMP Is Found	Signalling Molecule
DC-SIGN	Mannans	HIV-1, Dengue, M. tuberculosis	Unknown
Mannose receptor	Fungal mannans	Candida	
Dectin-1	Beta-1,3-glucans	Fungi	SYK-CARD9, RAF1
Dectin-2FcR	Mannans	Candida hyphae	SYK-CARD9
MINCLEFcR	Mannans	Candida	SYK
Mannose-binding lectin	Mycobacterial cord factor	Mycobacteria	Soluble receptor
	Repetitive oligosaccharides	Bacteria and fungi	
NLRs			
PRR	Recognised PAMP	Microorganism in Which PAMP Is Found	Signalling Molecule
NOD1	Muramyl tripeptide peptidoglycans	Gram-negative bacteria	RIP2
NOD2	Muramyl dipeptide peptidoglycans	Gram-positive bacteria	RIP2
NLRP1	Anthrax toxin	Bacillus anthracis	ASC-caspase-1
NLRP3	Peptidoglycans Bacterial toxins	Bacteria Listeria, staphylococcus	ASC-NAIP5, caspase-1 ASC-caspase-1
NLRC4	Flagellin	Shigella, salmonella, legionella	
AIM2	dsDNA	Francisella tularensis	
RLHs			
PRR	Recognised PAMP	Microorganism in Which PAMP Is Found	Signalling Molecule
RIG-I	Short dsRNA 5' Triphosphate ssRNA	Paramyxoviruses, orthomyxoviruses, rhabdoviruses, flaviviruses	IPS1
MDA5	Long dsRNA	Paramyxoviruses, orthomyxoviruses, rhabdoviruses, flaviviruses Picornaviruses, reoviruses, flaviviruses	IPS1

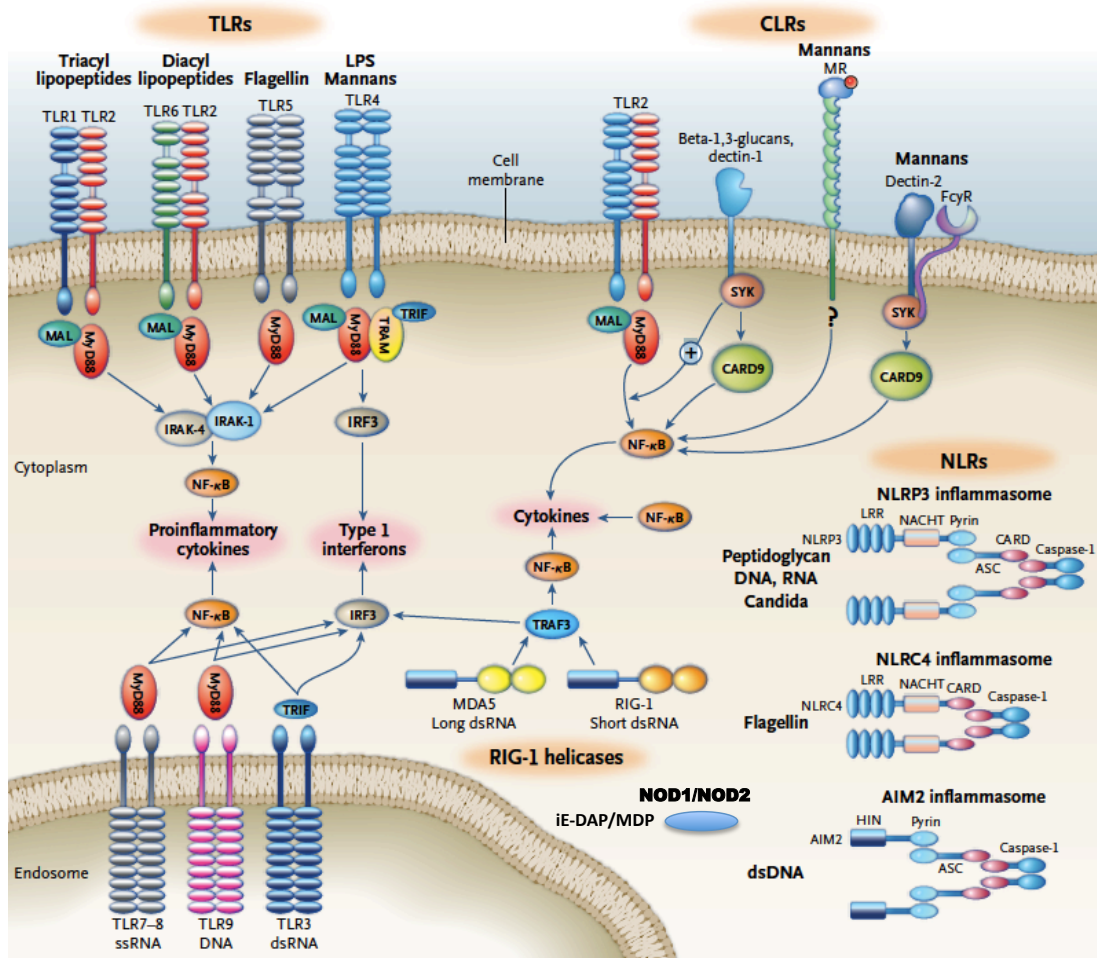


Figure 1.3: Pathogen recognition receptors. The figure shows the complex interplay between different PRR signalling pathways triggered in response to recognition of various PAMPs. PRR from the five different PRR families, TLRs, NLRs, CLRs, RLHs and cytosolic DNA sensors, are shown. PRRs are expressed on the cell surface, within the endosome or in the cytoplasm and recognize a variety of different pathogenic components from various sources. Ligand binding triggers various intracellular signalling pathways which lead to the production of pro-inflammatory cytokines and type I IFNs. In addition, a group of PRRs has been shown to activate the inflammasome resulting in the secretion of IL-1 β and IL-18. Figure adapted from [Netea & van der Meer, 2011].

TLRs are comprised of an extracellular ectodomain containing leucine-rich repeats (LRRs) that mediate PAMP recognition, a transmembrane domain and an intracellular Toll/IL-1 receptor (TIR) domain mediating downstream signal transduction [Jin & Lee, 2008]. So far 10 different TLRs (TLR1-10) have been discovered in humans with each TLR having a distinct function in terms of PAMP recognition (the only exception are TLR7 and TLR8 which have both been shown to respond to the same ssRNA ligands [Heil *et al.*, 2004]) and subsequent immune activation [Akira *et al.*, 2006]. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed

on the cell surface whereas TLR3, TLR7, TLR8 and TLR9 are located within endosomal compartments [Nishiya & DeFranco, 2004]. Cellular localisation of TLRs is thought to be important for their function as it affects ligand accessibility and signal transduction.

The first receptor to be identified was TLR4, the receptor for lipopolysaccharide (LPS), which is responsible for the induction of an inflammatory response to Gram-negative bacteria [Hoshino *et al.*, 1999]. LPS associates with the cell surface receptor CD14 and together with MD-2 triggers TLR4 in an indirect manner. In contrast, gram-positive bacteria which contain lipoteichoic acid (LTA) instead of LPS activate TLR2 in combination with either TLR1 or TLR6 [Ozinsky *et al.*, 2000]. Other bacterial components, for example flagelin (via TLR5), are also strong inducers of TLR signalling [Hayashi *et al.*, 2001].

Viruses are mainly recognised by their RNA or DNA genomes, which encode for structural proteins and are essential for their propagation. TLR3 recognizes dsRNA intermediates that are generated during viral replication [Tabeta *et al.*, 2004] and is also triggered by the synthetic ligand Poly(I:C) [Alexopoulou *et al.*, 2001]. TLR7 and TLR8 are both located on endosomal membranes. They bind to uridine-rich ssRNA and are also induced by synthetic components like R848 or imiquimod [Jurk *et al.*, 2002] [Hemmi *et al.*, 2002]. Similarly, the activator of TLR9 are dsDNA nucleotides bearing unmethylated CpG base pairs from viruses including herpes simplex virus 1 and 2 (HSV-1, HSV-2) [Bauer *et al.*, 2001]. The endosomal location of these receptors is essential for their function as many viruses traffic through endosomal compartments and the proteolytic environment promotes digestion of the viral particle and release of the genomic material [Ahmad-Nejad *et al.*, 2002]. Besides viral RNA and DNA, some viral envelope proteins can also be recognised. For example, the fusion protein of respiratory syncytial virus (RSV) or the envelope protein of mouse mammary tumor virus (MMTV) both activate TLR4 [Burzyn *et al.*, 2004] [Kurt-Jones *et al.*, 2000].

Extensive cross-talk between different TLRs is possible due to extensive similarities between the different signalling pathways [Kawai & Akira, 2007]. In general, PAMP binding induces TLR cross-linking which, in turn, leads to the recruitment of a variety of TIR adaptor molecules including MyD88, TIRAP (Mal), TRIF (TICAM1) or TRAM [Medzhitov *et al.*, 1998] [Yamamoto *et al.*, 2003b] [Yamamoto *et al.*, 2002]. The MyD88-dependent pathway is common to all TLRs except TLR3 [Adachi *et al.*, 1998].

MyD88 recruitment to the TIR domain initiates sequential binding and phosphorylation of IL-1 receptor-associated kinase 1 (IRAK1) [Wesche *et al.*, 1997]. TNF receptor-associated factor 6 (TRAF6) also temporarily associates with the receptor complex before dissociating to form a multiprotein complex with TAK1 (TGF-Beta-Activated Kinase-1), TAB1 (TAK1-Binding Protein-1) and TAB2 (TAK1-Binding Protein-2). TRAF6 functions as ubiquitin

E3 ligase which activates the protein kinase TAK1 [Cao *et al.*, 1996]. TAK1 is necessary for the activation of the MAPK signalling cascade leading to the phosphorylation of AP-1. Simultaneously, TAK1 phosphorylates $\text{I}\kappa\text{B}$ kinases promoting phosphorylation and degradation of $\text{I}\kappa\text{B}$ and subsequent translocation of $\text{NF-}\kappa\text{B}$ into the nucleus [Wang *et al.*, 2001]. Together, the combined activation of these transcription factors leads to the induction of pro-inflammatory cytokines such as $\text{TNF-}\alpha$, IL-1 and IL-12. TRAF6 also activates TANK, which interacts with TBK1 and $\text{IKK-}\varepsilon$ to activate IRF-3 and IRF7 [Konno *et al.*, 2009]. Activation of this signalling pathway is required for the induction of type I interferons and thus the establishment of an antiviral state. The stimulation of TLR3 occurs independently of MyD88. Instead TLR3 activates IRF3 and $\text{NF-}\kappa\text{B}$ via the adaptor molecule TRIF [Yamamoto *et al.*, 2003a]. In contrast, TLR4 is able to activate both MyD88- and TRIF-dependent signalling pathways by engaging the two adaptor proteins sequentially at the cell surface and in the endosomes, respectively [Kagan *et al.*, 2008]. Hence, its localisation control the access to distinct pools of signalling proteins.

1.1.6 RIG-I-like helicases

The introduction of pathogen-derived nucleic acids into the cytoplasm is a sign of infection and initiates a strong innate response. Recent studies have identified a group of receptors shown to be important for recognition of viral RNA in the cytoplasm of infected cells. These sensors belong to the RIG-I-like helicases include the retinoic acid-inducible gene (RIG-I), melanoma-differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology-2 (LGP2) [Yoneyama & Fujita, 2007] [Loo & Gale, 2011]. RIG-1 and MDA5 both contain a DExD/H box RNA helicase domain for ligand recognition and two caspase-recruitment (CARD) domains required for downstream signalling [Yoneyama *et al.*, 2004] [Kang *et al.*, 2004]. This occurs via the common adaptor protein IPS-1 (VISA or MAVS), which is localised at the outer mitochondrial membrane, and leads to the activation of $\text{NF-}\kappa\text{B}$ as well as IRF-3 and -7 [Kawai *et al.*, 2005]. In contrast, LGP2 lacks the caspase recruitment domain (CARD) and over expression in cell culture inhibits virus-induced activation of type I IFNs, suggesting a role as a negative regulator. However, its role *in vivo* is not established [Rothenfusser *et al.*, 2005] [Saito *et al.*, 2007].

It has been reported that RIG-I and MDA5 recognise distinct types of viral RNA. MDA5 is triggered by high molecular weight dsRNA fragments [Gitlin *et al.*, 2006] and is involved in the antiviral response against picornaviruses such as encephalomyocarditis virus (EMCV) and Theiler's virus, as shown in MDA5 knockout mice [Kato *et al.*, 2006]

[Xagorari & Chlichlia, 2008]. RIG-I responds to a wide variety of different ssRNA viruses including VSV, Hepatitis C and Sendai virus. It preferentially associates with shorter RNA containing a 5'-triphosphate moiety [Hornung *et al.*, 2006] [Pichlmair *et al.*, 2006] [Baum *et al.*, 2010]. The requirement of 5'-triphosphates for recognition is important for the strict differentiation between viral and host RNA. Cellular mRNAs are capped and processed and therefore lack this important recognition motif. However, also short synthetic dsRNA fragments, which do not bear any 5'-triphosphates, activate RIG-I and induce the secretion of type I IFNs [Kato *et al.*, 2008].

1.1.7 Cytosolic dsDNA sensors

While the recognition of extracellular DNA mainly involves TLR9, cytosolic DNA appears to require a novel group of intracellular sensors [Barber, 2011]. The first identified cytosolic DNA sensor, named DNA-dependent activator of IFN-regulatory factors (DAI) binds AT-rich B-form dsDNA [Takaoka *et al.*, 2007] and induces a strong type I IFN response. Surprisingly, a recent report implicates the RNA binding protein RIG-I in recognition of B-form DNA after transfection into the cytoplasm [Ablasser *et al.*, 2009]. This is thought to occur via an indirect mechanism by which cytoplasmic DNA is transcribed into dsRNA intermediates with 5'-triphosphate moiety by RNA polymerase III [Chiu *et al.*, 2009]. Both DAI and RIG-I induce the production of type I IFNs through STING, an ER-resident adaptor protein [Ishikawa & Barber, 2008], and the TBK1/IRF3 dependent pathway. Recently a third DNA sensor has been identified. This sensor, LLRFIP1 recognizes AT-rich B-form DNA as well as GD-rich Z-form DNA and triggers the production of IFN- β in a β -catenin and IRF3 dependent manner [Yang *et al.*, 2010a].

In addition to type I IFNs, cytosolic DNA also triggers the production of IL-1 β and IL-18. This occurs via the DNA sensor AIM2, a member of the IFN-inducible HIN200 family, which promotes the assembly of an inflammasome in an ASC-dependent manner [Hornung *et al.*, 2009] [Fernandes-Alnemri *et al.*, 2009] [Bürckstümmer *et al.*, 2009].

1.1.8 NOD-like receptors

Nod-like receptors (NLRs) belong to the group of intracellular PRRs and were first identified in plants where they play a central role in the resistance against microbial pathogens [Dangl & McDowell, 2006]. In general, NLRs are composed of a C-terminal LRR motif, responsible for pathogen sensing, a centrally located nucleotide-binding oligomerisation domain (NOD), which plays an essential role in activation, and a variable N-terminal effector domain

like CARDs, baculovirus inhibitor repeats (BIRs) or pyrin domains (PYD) [Ye & Ting, 2008].

More than 20 members have been identified, however, most of them have as yet unknown functions. NOD1 and NOD2 both recognize parts of peptidoglycan (GN), an essential component of bacterial cell walls: while NOD1 recognizes diaminopimelic (iE-DAP) derived from most Gram-positive and few Gram-negative bacteria [Chamaillard *et al.*, 2003], NOD2 is activated by muramyl dipeptide (MDP), which is a ubiquitous PGN component found in almost all bacteria [Girardin *et al.*, 2003]. Interestingly, a recent study suggests that NOD2 also binds viral ssRNA [Sabbah *et al.*, 2009]. However the role of NOD2 in the antiviral response requires further investigation. Both NOD1 and NOD2 contain N-terminal CARD domains and initiate downstream signalling via RIP2 and MAP kinases, thereby activating NF- κ B and promoting the secretion of pro-inflammatory cytokines. Dysregulation of NOD1 and NOD2 signalling is involved in a variety of human diseases [Elinav *et al.*, 2011]. Genetic variation in NOD1 has been associated with development of atopic eczema and asthma [Hysi *et al.*, 2005], whereas polymorphisms in NOD2 are linked to Crohn's disease, one of the major forms of inflammatory bowel disease [Ogura *et al.*, 2001].

The NALP superfamily is characterised by the presence of pyrin effector domains and plays a crucial role in regulating caspase-1 activity by forming a multiprotein complex known as the inflammasome [Gross *et al.*, 2011]. Caspase-1 is responsible for the processing and subsequent release of pro-inflammatory cytokines IL-1b and IL-18. In addition to a wide variety of PAMPs including flagellin and viral RNA [Miao *et al.*, 2006] [Allen *et al.*, 2009], the inflammasome is also triggered by a variety of non-microbial danger signals like asbestos, extracellular ATP or uric acid crystals which are collectively referred to as damage-associated molecular patterns (DAMPs) [Piccini *et al.*, 2008] [Schorn *et al.*, 2011].

1.1.9 C-type lectin receptors

C-type lectin receptors are broadly expressed on DCs and perform a variety of essential functions [van Kooyk, 2008]. Their extracellular carbohydrate recognition domain binds to simple monosaccharides and complex N- or O-linked sugars on glycosylated self or foreign antigens. Antigen binding promotes receptor oligomerisation and internalisation of bound antigen through receptor-mediated endocytosis. In addition CLRs are often associated with immunostimulatory or immunosuppressive function through the use of cytoplasmic ITIM or ITAM motifs, respectively [Kanazawa *et al.*, 2004].

Conventional DCs express a variety of CLRs including the mannose receptor, Dectin-1 and Langerin, whereas plasmacytoid DCs are characterised by the expression of BDCA-2

[Valladeau *et al.*, 2000] [Dzionek *et al.*, 2001] [Yokota *et al.*, 2001] [Romani *et al.*, 2010]. Due to their ability to recognize self-carbohydrates, CLRs are often thought to play an important role in homeostatic control and antigen capture in the absence of stimulation often leads to tolerance. They may also play a role in recognizing aberrant glycosylation patterns associated with tumors. This function is exploited by many pathogens, which have evolved to bind to CLRs to evade efficient recognition by the immune system [van Kooyk *et al.*, 2004]. Another notable function of C-type lectin function is the recognition of necrotic cells and their role in cross-presentation of dead-cell-associated antigens [Sancho *et al.*, 2009].

1.1.10 Genetic polymorphisms in PRRs and disease associations

The crucial role of PRR signalling in the innate immune response is highlighted by the observation that genetic polymorphisms in a variety of these receptors and their downstream signalling pathways can affect susceptibility to disease and cause immunodeficiencies [Netea & van der Meer, 2011].

Within the TLR-4 gene two co-segregating SNPs, Aps299Gly and Thr399Ile have been described, which lead to decreased cytokine secretion by epithelial cells *in vitro* and have been shown to be associated with hypo-responsiveness to inhaled LPS, septic shock and a variety of diseases including Legionnaire's as well as periodontal disease [Schröder & Schumann, 2005] [Schröder *et al.*, 2005].

Further studies have identified germ-line mutations in *IRAK-4* and *MyD88* [Picard *et al.*, 2003], which represent central nodes of PRR signalling adaption. These mutations lead to increased susceptibility to infection by pyrogenic bacteria [Chapel *et al.*, 2005] [von Bernuth *et al.*, 2008], a phenomenon which occurs during childhood but is lost in adulthood. This suggests that these defects could be, in part, compensated for by the development of protective B and T cell responses [Bousfiha *et al.*, 2010]. Similarly, children with a polymorphism in TLR3, which recognises viral nucleic acids, are susceptible to reoccurring encephalitis caused by infection with Herpes Simplex virus - 1 [Zhang *et al.*, 2007].

Genetic variation is not limited to TLRs as other PRRs have also been shown to be important for human disease. NOD2, for example, constitutes a major risk factor for developing Crohn's disease, an inflammatory condition of the bowel [Hugot *et al.*, 2001]. Mutations in the leucine-rich repeat domain are thought to lead to a loss-of-function mutation [Ogura *et al.*, 2001] resulting in decreased production of antibacterial defensins in the gut, an unregulated cytokine response, and defects in autophagy [Kobayashi *et al.*, 2005]

[Watanabe *et al.*, 2004] [Cooney *et al.*, 2010]. Together these abnormalities are thought to impair the defense against invading microorganisms in the gut. This leads to the hypothesis that Crohn's disease is a primary immunodeficiency syndrome of gut mucosal immunity [Marks *et al.*, 2010].

These findings highlight the importance of PRRs in the immune response against a variety of pathogens. Defects can lead to a variety of different disease states, whereby the exact strength and clinical manifestation depend on the nature of the PRR carrying the mutation. In general, it seems that the clinical severity decreases with age, possibly due to partial compensation by adaptive responses.

1.1.11 Distribution of innate receptor expression among human DC subsets

In humans, DCs are a heterogeneous group of cells that are segregated into a variety of subsets according to localisation, phenotype and immune function [Ju *et al.*, 2010]. Distinct subsets display significant variation in the type of PRRs expressed and consequently variation in the type of pathogen sensed and the immune responses initiated. Whereas some PRRs like RIG-I seem to be expressed ubiquitously across all DC subsets [Kramer *et al.*, 2010], the expression of others, for example TLRs, follow a distinct distribution among different DC subtypes [Kokkinopoulos *et al.*, 2005]. In addition, PRR expression has also been shown to vary with the DC maturation stage adding to the complexity [Kramer *et al.*, 2010].

In general human DCs can be divided into two main populations: CD11c⁺ myeloid DCs (mDCs) and CD11c⁻ CD123⁺ plasmacytoid DCs (pDCs) [Ju *et al.*, 2010].

Myeloid DCs are widely distributed within the human body and comprise multiple unique subsets [Ueno *et al.*, 2010]. Even though a comprehensive profiling study is difficult to conduct due to technical limitations [Schreibelt *et al.*, 2010], it is thought that mDCs express all extracellular TLRs (TLR1, TLR2, TLR4, TLR5 and TLR6) as well as the endosomal receptors TLR3 [Muzio *et al.*, 2000] and TLR8. However, it is important to note that small variations even within different mDC subtypes have been observed [Piccioli *et al.*, 2007]. In addition, mDCs express a wide variety of C-type lectins that characterise their tissue localisation [MacDonald *et al.*, 2002].

Plasmacytoid DCs are primarily located in the blood or secondary lymph nodes and are identified by the expression of the lectin receptor BDCA-2 [Dzionek *et al.*, 2001]. Functionally, pDCs are characterised by their capacity to produce high amounts of type I IFNs in response to viral infection and are therefore considered the first line in anti-viral de-

fense [McKenna *et al.*, 2005] [Reizis *et al.*, 2011]. Accordingly, pDC largely lack expression of cell surface TLRs but have the capability of recognizing viral antigen using TLR7 and TLR9 [Kadowaki *et al.*, 2001] [Jarrossay *et al.*, 2001] [Krug *et al.*, 2001]. Interestingly, however, pDCs do not express TLR3, which is responsible for the detection of viral dsRNA [Matsumoto *et al.*, 2003]. pDCs also express TLR1 but lack the heterodimeric binding partner TLR2 and hence fail to respond to their corresponding ligands [Kadowaki *et al.*, 2001].

1.1.12 Monocyte-derived dendritic cells generated *in vitro*

As circulating mDCs exist at low frequency and are difficult to isolate from tissue, many studies are based on *in vitro* generated DCs. In humans, DCs can be derived from bone marrow-derived CD34⁺ or from blood monocytes. In the latter systems, monocytes are isolated from whole blood and cultured in GM-CSF and IL-4 *in vitro* [Sallusto & Lanzavecchia, 1994]. Monocyte-derived DCs (MDDCs) have been shown to share many phenotypic and functional characteristics with mucosal mDCs, including low expression of MHC and co-stimulatory molecules and high levels of DC-SIGN, and are readily matured by exposure to a variety of inflammatory stimuli [Cella *et al.*, 1997]. The PRR expression profile of MDDCs includes the ubiquitous cytosolic RNA and DNA sensors as well as NOD2 [Cooney *et al.*, 2010]. Furthermore, they express all extracellular TLRs (TLR1, TLR2, TLR4, TLR5 and TLR6) as well as the endosomal TLR3 and TLR8 [Kramer *et al.*, 2010]. In contrast, MDDCs show no expression of TLR7 or TLR10 [Hasan *et al.*, 2005]. Similarly, an early report suggests that MDDCs lack expression of and TLR9 [Jarrossay *et al.*, 2001]. However, more recent studies contradict this finding [Hoene *et al.*, 2006]. Therefore, the expression of TLR9 by DCs remains controversial.

1.2 An introduction to HIV-1/AIDS

In 1981, the New York Times reported increasing incidence of a rare lung infection, Pneumocystis carinii pneumonia (PCP), and an aggressive form of Kaposi's sarcoma (KS) amongst young gay men in New York and California [Gottlieb *et al.*, 1981]. This condition was termed acquired immunodeficiency syndrome (AIDS) by the CDC in 1982. Only two years later, Franoise Barr-Sinoussi and Luc Montagnier published a seminal paper in Science that described the isolation of a retrovirus from lymph nodes of patients in the early phase of AIDS [Barré-Sinoussi *et al.*, 1983]. In 1984, Robert Gallo and colleagues demonstrated that this retrovirus, then known as HTLV-III and now termed human immunodeficiency virus 1 (HIV-

1), was the causative agent of AIDS [Gallo *et al.*, 1984].

HIV-1/AIDS is now a global pandemic affecting all parts of society. Among the worst affected are women and children in Sub-Saharan Africa and Asia. In 2009 the World Health Organisation (WHO) published devastating statistics according to which 33.3 million people are living with HIV-1 and 1.8 million die of AIDS every year (<http://www.who.int/hiv/data/en/>). A stark reflection of the fact that this pandemic is far from being under control, is that a further 2.6 million new infections occur every year. As a result of statistics like these, HIV-1/AIDS has captured the attention of world leaders, the media and the medical and public health communities perhaps more than any other disease affecting the developing world.

Extensive scientific efforts led to the elucidation of the genetic and structural organisation and the important factors that control the replication and life-cycle of HIV. This information facilitated the rapid development of a specific diagnostic assay for HIV-1 antibodies and, importantly, the first antiretroviral drug azidothymidine (AZT) in 1987 [Nakashima *et al.*, 1986]. Currently Highly Activate Antiretroviral Therapy (HAART) consists of a variety of different drugs, each targeting a different stage of the viral life-cycle. When used in combination these drugs have proven very efficient in temporarily educing viral replication. It extends the duration and quality of life for people living with HIV-1 as well as reduces the frequency of transmission, especially from mother to child. Importantly, their use in combination can prevent, or at least greatly slow down, the appearance of drug resistant strains.

However, HAART is life-prolonging but unable to eradicate the virus from infected individuals due to the existence of latent viral reservoirs in quiescent CD4⁺ T cells and macrophages.

In an effort to develop alternative treatment options and, ideally, an effective vaccine, the scientific community has since been focussing on gaining further insights into the viral life-cycle, the virus-host interactions and the immune response to the virus.

1.2.1 The origin and evolution of HIV

HIV arose as a result of zoonotic transmission from immunodeficiency virus (SIV) infecting non-human primates to human hunters in West Africa [Gao *et al.*, 1999] [Sharp & Hahn, 2008]. Two types of HIV, HIV-1 and HIV-2, have been isolated to date [Hirsch *et al.*, 1989], with HIV-1 being the cause of the current world-wide pandemic.

Phylogenetic analyses of HIV-1 suggest that three independent cross-species transmission events gave rise to independent HIV-1 groups: major (M), non-major (N) and outlier (O). Groups M and N were introduced into the human population from chimpanzees

through blood borne transmissions, whereas group O may have originated from gorillas [Van Heuverswyn *et al.*, 2006]. Group M is the predominant circulating HIV-1 group and can currently be divided into at least nine genetically distinct subtypes, also referred to as clades, which are typically associated with certain geographical regions [Robertson *et al.*, 2000].

HIV-1 evolves rapidly [Wolinsky *et al.*, 1996] and diversification is driven by host immune pressures and reaches a delicate survival balance that allows the virus to avoid elimination without sacrificing too much replicative fitness induced by potentially detrimental mutations [Korber *et al.*, 2001]. The virus has several intrinsic mechanisms that enable rapid evolution within the host: a fast generation time with 10^{10} - 10^{12} newly produced virions per day, an error-prone reverse transcriptase enzyme that lacks proofreading activity leading to the introduction of 0.22 mutations per genome per cycle [Drake, 1993] and, lastly, a high level of recombination with 3 recombination events per genome per replication cycle [Wolinsky *et al.*, 1996]. This extensive viral diversity, both at the global level and at that of the infected individual, has important implications for host control of the virus as well as antiretroviral treatment and vaccine development.

1.2.2 Genetic and structural organisation of HIV-1

The mature HIV-1 virion is composed of a central cone-shaped core formed by structural proteins and is surrounded by a lipid envelope derived from the infected cell (Figure 1.4). The viral lipid envelope contains important glycoproteins necessary for attachment to target cells. In addition to the structural proteins, each virion also contains the virally-encoded protease (PR), which is essential for the proteolytic cleavage of viral proteins, reverse transcriptase (RT) enzyme, that transcribes the RNA genome into dsDNA, integrase (IN), which finally integrates the DNA into the host genome, and p6, a small protein essential for the last steps in viral budding and release of virions from the cell.

Each HIV-1 virion carries two identical copies of the positive ssRNA genome which are encapsidated within the central capsid structure. The genome of HIV-1 is approximately 9.2 kb in length and contains a packaging signal termed the Psi element [Clever & Parslow, 1997]. It is flanked by two long terminal repeat (LTR) sequences that contain promoters, enhancers and other sequences required for binding of viral and cellular transcription factors, such as NF- κ B and AP-1, involved in viral transcription [Pereira *et al.*, 2000]. The viral genome encodes for 15 proteins in 9 overlapping reading frames, three of which produce the major Gag, Pol and Env polyproteins. These are synthesised first and then cleaved by host or viral proteases to form the mature proteins: matrix (MA), capsid (CA), nucleocapsid (NC) and

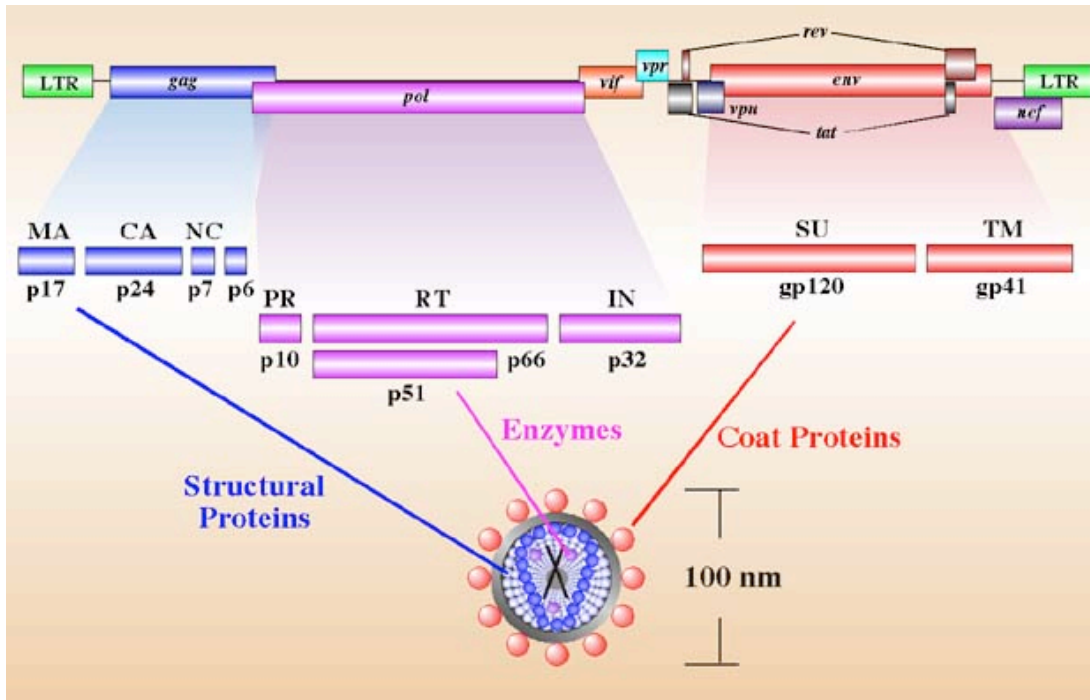


Figure 1.4: Genetic and structural organisation of HIV-1. Genomic organisation of the HIV-1 genome with 9 ORFs encoding for the main Gag, Pol and Env polyproteins as well as the accessory genes. This figure also contains a simplified diagram of the mature HIV-1 virion. Trimeric gp120/gp14 is embedded in a lipid bilayer lined by MA, which surrounds a cone shaped core structure. Two copies of the viral ssRNA are located within the viral capsid structure, which also contains the viral IN, RT and PR as well as the accessory and various host proteins. IN = Integrase, RT = Reverse transcriptase, MA = Matrix, NC = Nucleocapsid, PR = Protease, CA = Capsid. Adapted from www.stanford.edu/group/virus/retro/2005gongishmail/RetroviridaeHomePage.html.

p6 released from Gag, protease (PR), reverse transcriptase (RT) and integrase (IN) produced from Pol and gp120 and gp41 derived from the 160 kDa Env precursor [Pettit *et al.*, 2004]. In addition, HIV-1 genome contains two other genes, *tat* and *rev*, essential for replication, as well as four other accessory proteins *nef*, *vpu*, *vpr* and *vif* which are not essential for HIV-1 replication *in vitro* [Anderson & Hope, 2004].

1.2.3 The HIV-1 life cycle

HIV-1 infections can occur by sexual exposure through the genital tract or rectal mucosa, intravenous drug use, contaminated blood products or through vertical transmission from mother to child, either during birth or through breast milk. HIV-1 infects a variety of cells comprising the innate and adaptive immune system including T cells, monocytes, macrophages, dendritic and microglial cells of the CNS [Lifson *et al.*, 1986] [Vazeux *et al.*, 1987].

As illustrated in Figure 1.5, the viral life cycle is initiated by sequential binding of gp120 to its primary receptor CD4 [Dalglish *et al.*, 1984] and one of the two co-receptors, the chemokine receptors CXCR4 or CCR5 [Lifson *et al.*, 1986] [Berger *et al.*, 1999]. Co-receptor usage not only facilitates the interaction of the virion and the cell surface, thereby increasing the probability of fusion, but also directs the cellular tropism of HIV-1. The transmitted virus is predominantly macrophage-tropic (R5 type) as it primarily infects mucosal CCR5 expressing cells such as macrophages and memory T cells. A genetic variant involving a 32 base pair deletion in the CCR5 gene (CCR5- Δ 32) results in a truncated C-terminus. This leads to down regulation of CCR5 and, to a lesser extent, CXCR4 expression on the cell surface. In the homozygous state this mutation confers almost complete resistance to HIV-1 infection, and in the heterozygous state it provides partial resistance with slower disease progression [Samson *et al.*, 1996]. In contrast, T cell tropic isolates (X4 type) utilize CXCR4 which is expressed on a variety of cells including naive and resting CD4 T cells, macrophages and thymic precursors. X4 viruses usually emerge later in the course of chronic infection and are associated with disease progression [Connor *et al.*, 1997].

Whereas gp120 mediates the interaction between the virus and its binding sites, gp41 mediates fusion of viral and cellular membranes. Unlike other viruses that enter via receptor-mediated endocytosis, HIV-1 virions fuse directly with the plasma membrane leading to release of the viral core into the cytoplasm. Entry of subviral particles into the cytoplasm triggers viral uncoating and the formation of the reverse transcription complex [Goff, 2001] containing the viral RNA genome as well as RT, MA, NC, IN and Vpr [Nermut & Fassati, 2003]. The viral reverse transcriptase catalyses the reverse transcription of the HIV-1 RNA genome, producing proviral DNA which translocates into the nucleus as nucleoprotein-preintegration complex (PIC) [Sherman & Greene, 2002]. Nuclear import can occur across the intact nuclear membrane of non-dividing cells [Lewis & Emerman, 1994] and requires a multitude of host proteins, but the exact mechanism is still unclear. The full-length viral transcript is integrated into the host genome by the IN enzyme [Vanegas *et al.*, 2005] and serves as template for transcription of viral ORFs by RNA Pol II [Kingsman & Kingsman, 1996]. Transcription is initiated from the LTRs which contain several enhancer sites that allow binding of the Tat transactivator and the transcription factors NF- κ B and AP-1. Unspliced and partially spliced viral RNAs are exported from the nucleus into the cytoplasm and serve as template for viral protein synthesis. Finally HIV-1 uses the parts of the host ESCORT pathway to assemble into mature viral particles and bud from the plasma membrane [Bukrinskaya, 2004] [Usami *et al.*, 2009].

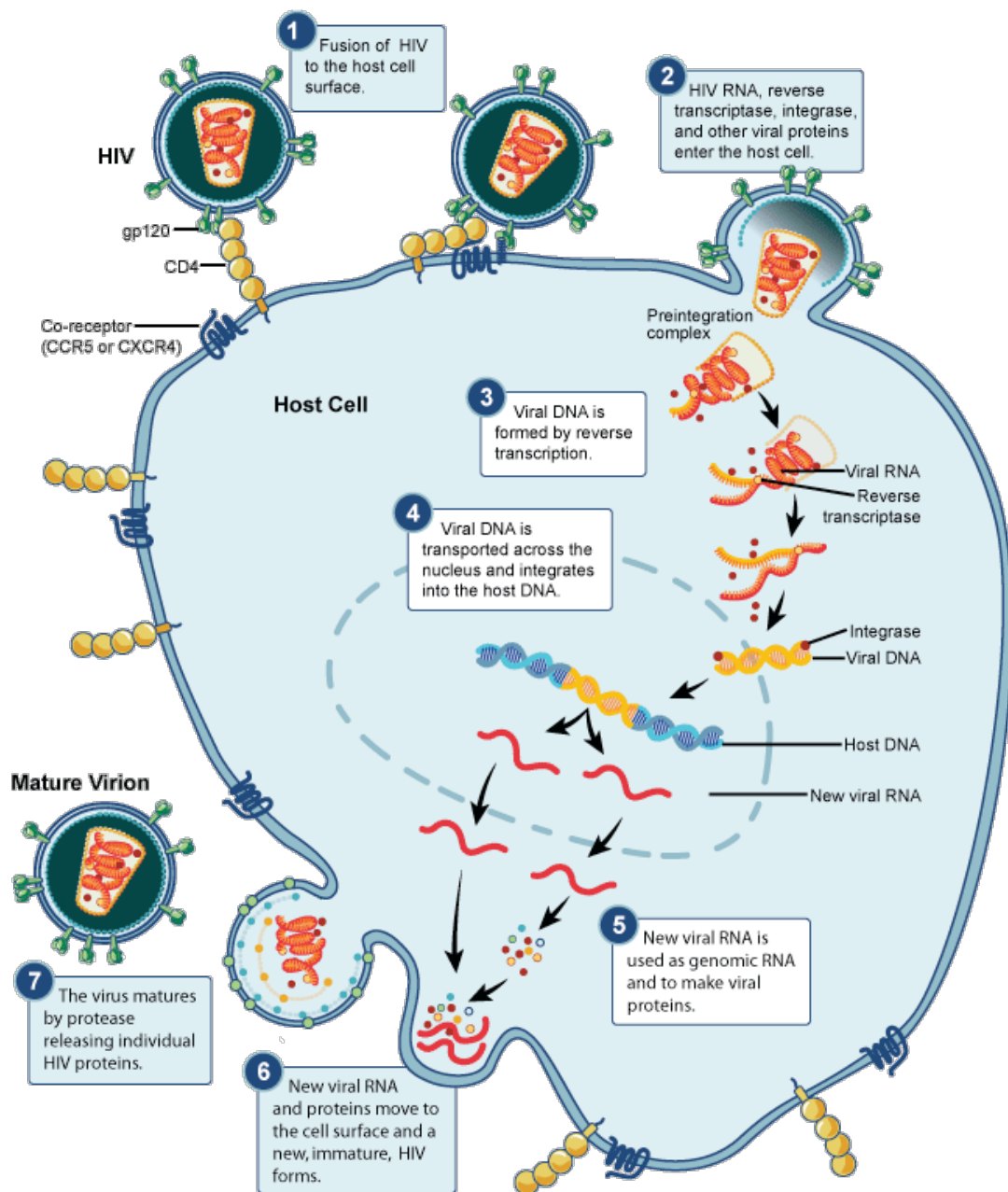


Figure 1.5: The HIV-1 replication cycle. The virus enters the cells by binding to its receptor CD4 and a co-receptor which promotes membrane fusion. Once in the cytoplasm, it uncoats and the single-stranded RNA genome is reverse transcribed into DNA by the viral RT enzyme. The pre-integration complex translocates into the nucleus where the provirus integrates into the host genome using a range of different host factors. Viral RNAs are produced and translated into polypeptides which are cleaved into individual proteins by viral and host proteases. Full-length viral RNA is used as genomic RNA for progeny virus which assembles and buds off the plasma membrane. Figure adapted from NIAID.

1.2.4 HIV-1 accessory proteins

HIV-1 also encodes for accessory proteins that are expressed at different stages during the viral life cycle to increase infectivity.

Tat is a virally-encoded potent transcriptional transactivator which enhances viral mRNA synthesis by approximately one hundred fold [Peruzzi, 2006]. It binds to the transactivation response element (TAR element) located at the 5' end of viral transcripts, and has multiple functions. Tat recruits factors with chromatin remodeling activity, thereby facilitating access to transcriptional start sites. It also acts together with CDK9/cyclinT1 to phosphorylate RNA pol II C-terminal domain (CTD) and therefore promotes transcription initiation [Puglisi *et al.*, 1992] [Aboul-ela *et al.*, 1995]. Following transcription initiation, Tat also recruits PCAF, facilitating elongation of the viral transcripts [Zhou & Sharp, 1996] [Yang *et al.*, 1996]. In addition to its direct role in promoting viral transcription, Tat may also impair the RNA silencing machinery by inhibiting Dicer [Triboulet *et al.*, 2007].

Rev coordinates the splicing of viral RNAs [Pollard & Malim, 1998]. It binds to unspliced and partially spliced viral transcripts in the nucleus and promotes their export out of the nucleus into the cytoplasm. Rev therefore allows full-length RNA transcripts to escape splicing by the host machinery and be packaged into newly synthesised viral particles [Hope, 1997].

Vif is synthesised during the late phase of the HIV-1 life-cycle [Rose *et al.*, 2004] and its primary function is to prevent the incorporation of APOBEC3G into newly synthesised virions by targeting it for degradation [Sheehy *et al.*, 2003]. APOBEC3G is an antiretroviral protein that, in the absence of vif, is incorporated into the intact HIV-1 virion and, when released into newly infected cells, catalyses the deamination of cytidine residues in single stranded DNA. This results in the creation of G-A hypermutations in newly synthesised viral DNA and the subsequent loss of replicative ability of the virus [Harris *et al.*, 2003].

Vpr forms part of the preintegration complex [Bachand *et al.*, 1999]. First identified as an enhancer of gene expression, it has also been shown to increase the fidelity of RT, which lacks proofreading ability [Mansky, 1996]. Vpr also targets PIC to the nucleus [Agostini *et al.*, 2002].

Vpu is unique to HIV-1, as HIV-2 produces Vpx. It has been shown to increase the release of mature virions by decreasing the degradation of its receptor CD4 through interaction with its cytoplasmic tail [Tiganos *et al.*, 1997].

Nef is produced early during infection and has several important functions [Cullen, 1991]. In infected cells, Nef decreases the expression of various cell surface receptors, including CD4 as well as HLA-A and B, to enhance viral release and protect the cells from CTL recognition and killing, respectively [Garcia & Miller, 1991] [Ross *et al.*, 1999] [Chen *et al.*, 2006]. Nef also modulates T cell signalling to increase viral replication [Simmons *et al.*, 2005]. In addition, it regulates cholesterol trafficking and supplies newly synthesised cholesterol to the site of viral budding [Zheng *et al.*, 2003].

1.2.5 The course of HIV-1 infection

The course of HIV-1 infection can be divided into the following five distinct phases from the time of transmission to the development of AIDS (Figure 1.6): the initial eclipse phase which constitutes the time between transmission and detection of viral RNA in the blood, the burst of viral replication in the acute phase, the reduction in viral load and the establishment of viral set point, the chronic phase and, lastly, the progression to AIDS [McMichael *et al.*, 2010].

Eclipse phase The time between infection and the detection of viral RNA is referred to as the eclipse phase. Single genome amplification studies show that about 80% of mucosally established HIV-1 infections occur by a single CCR5 tropic founder virus [Keele *et al.*, 2008]. This suggests that the genital barrier provides a robust physical barrier to HIV-1 infection. Most studies investigating the early events of HIV-1 transmission are based on *in vitro* experiments using mucosal explants and *in vivo* models of macaques infected with SIV either intravaginally or intrarectally. Studies of the SIV vaginal challenge model [Miller *et al.*, 2005] indicate that HIV-1 follows a local expansion and stage dissemination model [Haase, 2011]. The successful founder virus crosses the epithelia by transcytosis [Bomsel, 1997] and infects 'resting' CD4 T cells in the underlying submucosa. Subsequently, local infection is established which is supported by early innate immune responses leading to the recruitment of additional target cells to the site of infection [Li *et al.*, 2009a]. During this phase HIV-1 is thought to contact mucosal DCs through the C-type binding lectin DC-SIGN which binds to the heavily glycosylated Env protein. As will be discussed in greater detail later, the interaction between mucosal DCs and HIV-1 is central in the dissemination of the virus throughout the secondary lymphoid tissue where activated CD4⁺ CCR5⁺ target T cells are present at high levels.

Acute phase of HIV-1 infection As HIV-1 reaches the draining lymph nodes, the availability of target cells increases and the virus can replicate freely leading to an exponential increase in plasma virus levels [Celum *et al.*, 2001]. During this acute phase, patients

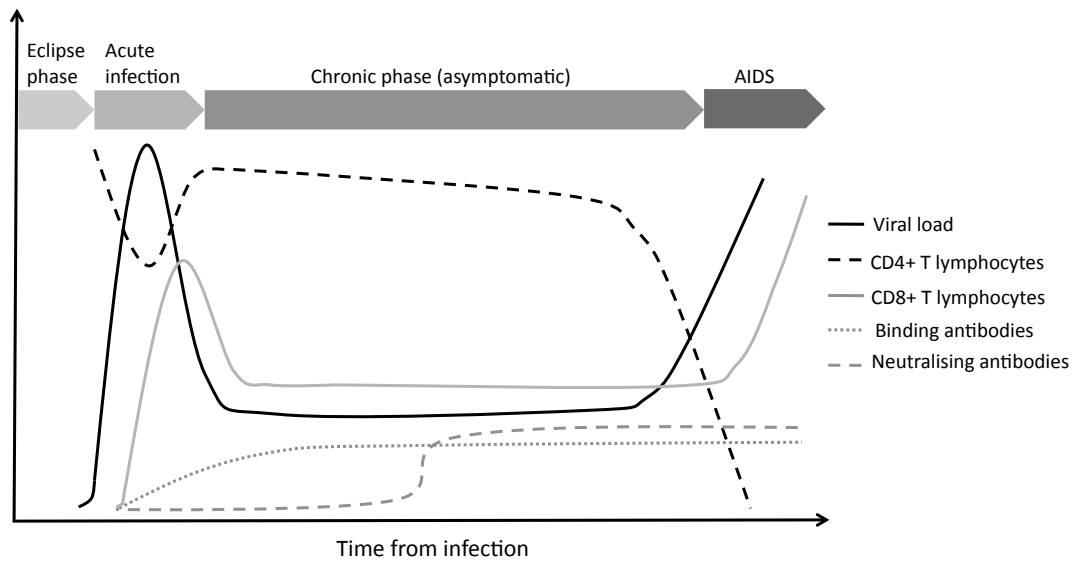


Figure 1.6: A typical time course of untreated HIV-1 infection from transmission to the progression to AIDS. Following transmission, HIV-1 disseminates to secondary lymphoid organs leading to a rapid increase in viraemia in the early acute phase which declines to a setpoint. This early phase of viral replication is accompanied by a rapid depletion of CD4⁺ lymphocytes especially in the gut-associated lymphoid tissue (GALT). The fall in viraemia coincides with the first appearance of HIV-specific CD8⁺ T cell responses which play an important role in temporarily controlling viral replication and rescuing CD4 counts. HIV-1 specific antibodies become detectable after the reduction in viral load, however, neutralising antibodies only begin to appear during the chronic phase of infection. The chronic phase is characterised by a stable viral load and a slow decline in CD4 counts. Continued viral replication and immune evasion finally exhausts the immune system leading to opportunistic infections and AIDS. Adapted from www.wikipedia.org/.

test positive for viral p24 antigen and anti-HIV-1 antibodies. Similarly to SIV infection in rhesus macaques, HIV-1 replication is most prolific in the gut-associated lymphoid tissue (GALT), which is predominantly populated by CCR5-expressing effector memory CD4⁺ T cells [Veazey *et al.*, 1998]. High levels of viral replication in the GALT can rapidly deplete up to 80% of CD4 T cells within the first 3 weeks of infection [Guadalupe *et al.*, 2003] [Brenchley *et al.*, 2004]. T cell death is not restricted to infected cells but also affects uninfected bystander T cells [Perfettini *et al.*, 2005]. Although the exact mechanisms causing this immunopathology are not fully understood, they are thought to be the consequence of target-cell infection and virus-induced Fas-mediated apoptosis [Douek *et al.*, 2003]. This profound depletion is associated with the destruction of the intestinal lamina propria and submucosa, allowing translocation of apoptotic microparticles and microbial products derived from commensal bacteria into the blood [Brenchley *et al.*, 2006]. The breakdown of

integrity of the epithelial barrier in the gastrointestinal tract has been proposed as one of the main driving forces of systemic immune activation [Brenchley & Douek, 2008] which is characteristic for the chronic phase of HIV-1 infection in humans and SIV infection of rhesus macaques. In contrast, SIV-infected sooty mangabeys, which are a natural host for SIV infection and commonly used as a non-pathogenic primate model for HIV-infection, have minimal levels of immune activation, preserved mucosal CD4⁺ T cells and no evidence of microbial translocation despite chronic high levels of virus replication [Silvestri *et al.*, 2003]. The exact mechanism by which they limit immune activation, despite the presence of high amounts of virus, remains unknown. Interestingly, it has been shown that pDCs from sooty mangabeys produce less IFN- α in response to TLR7 and TLR9 ligands *ex vivo*, possibly due to genetic polymorphisms affecting the signalling cascade [Mandl *et al.*, 2008]. This suggests that innate immune signalling may be important in the pathogenic nature of HIV-1 and is therefore worth investigating.

Establishment of viral set point and the chronic phase Following the burst of viral replication and a sharp decrease in CD4⁺ target cells, HIV viral load reaches its plateau 21-28 days post-infection. Thereafter it decreases to a much lower steady state level referred to as viral set point. The magnitude of the initial peak viraemia and set point reflect the balance between viral turnover and the immune response attempting to control the virus. The fall in viraemia coincides with the first appearance of HIV CD8⁺ T-cells, suggesting an important role for CTLs in the control of viral replication. This is supported by data from the monkey model showing that artificial depletion of CD8⁺ T cells in SIV-infected rhesus macaques results in a massive burst of viraemia which, in turn, leads to a rapid progression to AIDS [Schmitz *et al.*, 1999]. Later studies showed that depletion of CD8⁺ T cells leads to a massive burst of activated CD4⁺/CCR5⁺ cells, suggesting that increased virus replication may be a result of increased levels of target cells instead of a lack of CD8⁺ T cells [Zhang *et al.*, 2004b]. However, inhibition of CD4⁺ T cell expansion by IL-15 did not abrogate the rapidly progressive infection in these monkeys. This suggests that CD8⁺ T cells have a more complex effect on the outcome of SIV infection than just regulating the availability of target cells [Okoye *et al.*, 2009].

An additional piece of compelling evidence for the importance of CD8⁺ T-cells comes from several recent large-scale genome-wide association studies (GWAS), which found that polymorphisms in the human leukocyte antigen (HLA) loci are the only SNPs significantly associated with control of HIV-1 [Limou *et al.*, 2009] [International HIV Controllers Study *et al.*, 2010]. Nevertheless, the huge mutational capacity of HIV allows for the generation of viral variants

that escape recognition by HIV-1 specific CTLs and, in some cases, this can lead to a loss of control and progression to AIDS [Goulder *et al.*, 1997]. Indeed, it may be argued that the existence of CTL escape mutations demonstrates that HIV-1-specific CTLs must be controlling viral replication to some extent in order for there to be sufficient evolutionary pressure to select for these mutations [Leslie *et al.*, 2004]. The constant struggle between the host and the virus is one of the most striking features of HIV-1 infection. The set point viral load reached is predictive of the time to an AIDS defining illness and is likely to be determined by host genetic factors, the quality and quantity of the host HIV-specific immune response as well as the fitness of the transmitted founder virus.

1.2.6 The role of mucosal dendritic cells in early HIV-1 infection

During HIV-1 transmission, the cells initially targeted by the virus are mainly immature DCs, Langerhans cells, CD4⁺ CCR5⁺ T cells and macrophages that reside in the subepithelium of mucosal surfaces [Lederman *et al.*, 2004]. Hu and colleagues used SIV and a rhesus macaque intravaginal transmission model to demonstrate that the virus can rapidly penetrate the vaginal mucosa and infect or associate with intraepithelial DCs [Spira *et al.*, 1996] [Hu *et al.*, 2000]. Therefore, immature DCs are thought to be the principal viral targets and seem to play an important role in viral transmission and dissemination to the draining lymph nodes [Reece *et al.*, 1998] [Shattock & Moore, 2003]. Indeed, in human cervical explants exposed to HIV-1, the migratory DC population residing in the tissue accounts for 90% of HIV-1 dissemination [Hu *et al.*, 2004].

1.2.7 HIV-1 infection of DCs has several outcomes

Accumulating evidence suggests that HIV-1 exploits dendritic cells to enhance systemic viral dissemination and impair host-mediated control of infection [Altfeld *et al.*, 2011]

Human mucosal DCs express high levels of the C-type lectin receptor DC-SIGN which binds to the heavily glycosylated HIV-1 envelope protein (Env). *In vitro* studies using monocyte-derived dendritic cells as a model system demonstrate that C-type lectin receptor Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) acts as an adhesion receptor sequestering virus particles into a specialised endosomal compartment [Jameson *et al.*, 2002] [Soilleux *et al.*, 2001]. The encounter between HIV-1 and immature DCs can have three distinct outcomes (Figure 1.7) [Piguet & Steinman, 2007].

Firstly, HIV-1 viral particles can be endocytosed and destroyed in the late endosome, potentially leading to antigen presentation. Secondly, a significant 5-10% of internalised virions evade degradation and are protected for up to two days in a non-lysosomal compartment [Kwon *et al.*, 2002] which is rich in tetraspanins such as CD82 and CD9 [Garcia *et al.*, 2008]. Virus-bearing DCs migrate to the draining lymph nodes including the GALT, where T cells are present at high levels. DC and T cell conjugates are formed to allow direct cell-to-cell transfer of sequestered virus particles from virus-bearing DCs to target cells through so-called virological synapses. This mode of transmission is more effective than infection by cell free virus as it delivers the virus directly to its entry receptor [Wiley & Gummuluru, 2006].

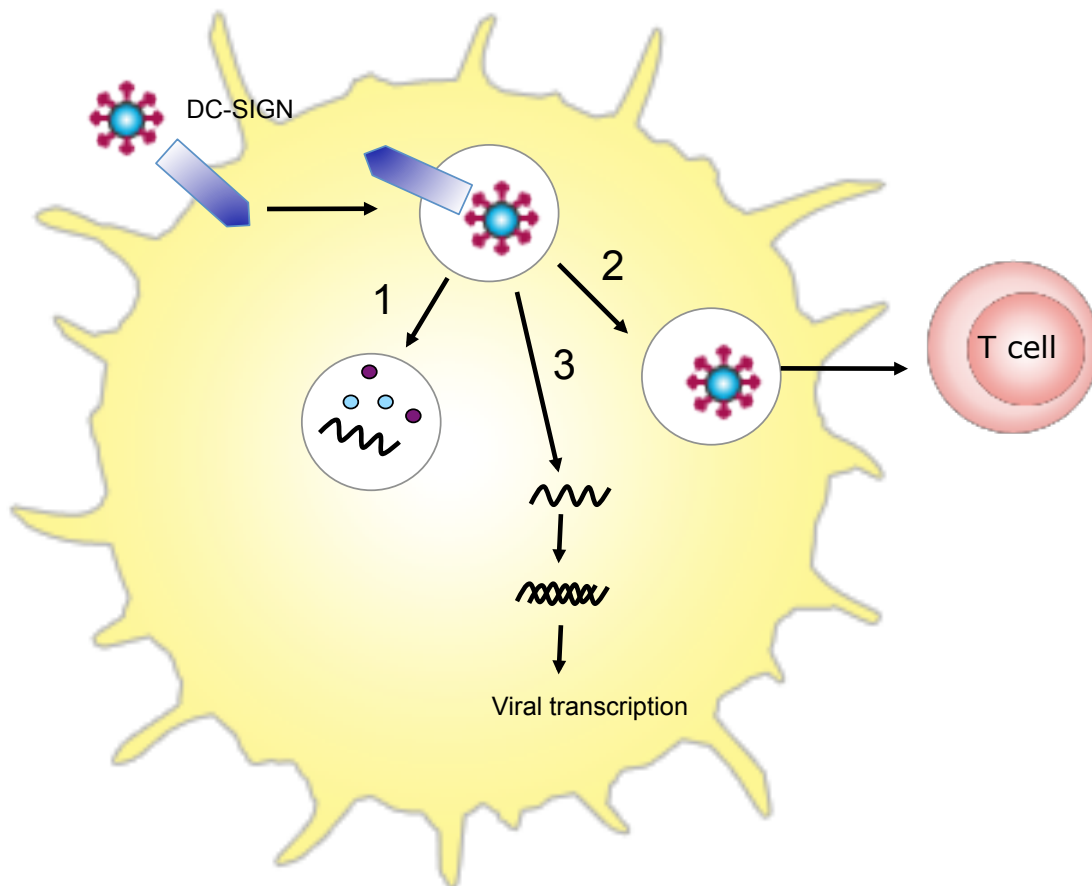


Figure 1.7: HIV-1 infection has several different outcomes. DCs bind HIV-1 mainly through DC-SIGN. This leads to uptake of the virus into an endosome-like compartment. From there it is either 1) degraded or 2) retained intact and is recycled back to the cell surface from where it can be directly passed on to $CD4^+$ T cells in trans. 3) Additionally, DCs can be productively infected, however, replication is low.

Thirdly, HIV-1 has been shown to productively infect DCs [Gringhuis *et al.*, 2010], although this remains controversial as some groups claim that HIV-1 does not infect DCs [Manel *et al.*, 2010]. It is generally well-accepted that HIV-1 replication in DCs is inefficient.

This may partially result from low CD4 and co-receptor expression and the presence of viral restriction factors such as APOBEC3G, Tetherin, Trim22 and Trim5a. However, the presence of early viral transcripts, 6 hrs post-infection, suggests that the production of mature viral particles is inhibited post-integration [Gringhuis *et al.*, 2010]. Additional studies are required to elucidate the exact life-cycle of HIV-1 in DCs.

1.2.8 Innate immune recognition of HIV-1 by PRRs

The HIV-1 virion carries several components that have the potential to trigger DC maturation and activate the innate immune system. In addition, further PAMPs could be generated during different phases of the viral life cycle. The hypothetical possibilities for PAMP recognition on HIV-1 infection are described in Figure 1.8. A GU-rich ssRNA fragment, derived from the HIV-1 LTR, is a potent activator of TLR7 and stimulates DCs to secrete type I interferons and pro-inflammatory cytokines [Heil *et al.*, 2004]. TLR3 may also be involved in sensing of HIV-1-derived dsRNA replication intermediates which enter the endosome by viral endocytosis or autophagy of viral material in the cytoplasm [Gougeon & Piacentini, 2009]. Once in the cytoplasm, the genomic material, including ssRNA or RNA:DNA duplex and dsDNA arising from reverse transcription, is well shielded by protein complexes but could in theory trigger nucleic acid sensors. Similarly, newly synthesised viral transcripts could be recognised by MDA-5 or RIG-I, provided they are uncapped and clearly recognizable as foreign.

Despite these potential mechanisms by which HIV-1 could trigger the activation of innate signalling pathways, monocyte-derived DCs are not activated by HIV-1. *In vitro* studies examining a wide variety of maturation markers show that HIV-1 infection of DCs fails to induce dendrite formation [Hodges *et al.*, 2007], complete up-regulation of co-stimulatory molecules [Harman *et al.*, 2011] and pro-inflammatory cytokine IL-12p70 [Fantuzzi *et al.*, 2004], resulting in an impaired T cell proliferative response [Donaghy *et al.*, 2003]. Furthermore, exposure of DCs to HIV-1 impairs their maturation in response to other stimulation, for example, to triggering of TLR4 by LPS, and promotes production of IL-10 and induction of a regulatory (Treg) cell response. [Granelli-Piperno *et al.*, 2004] [Smed-Sørensen *et al.*, 2005]. However, HIV-1 is able to promote DC migration to lymph nodes where HIV-1-infected DCs attract and contact T cells essential for viral transmission in trans [Felts *et al.*, 2010]. Some of these studies have provided conflicting results which may in part be explained by different experimental conditions such as length and dose of virus exposure and stimuli to induce DC maturation. In summary, HIV-1 infection of DCs during early infection induces a DC phe-

notype characterised by partial maturation, which is beneficial for the virus as it promotes propagation while preventing an effective immune response.

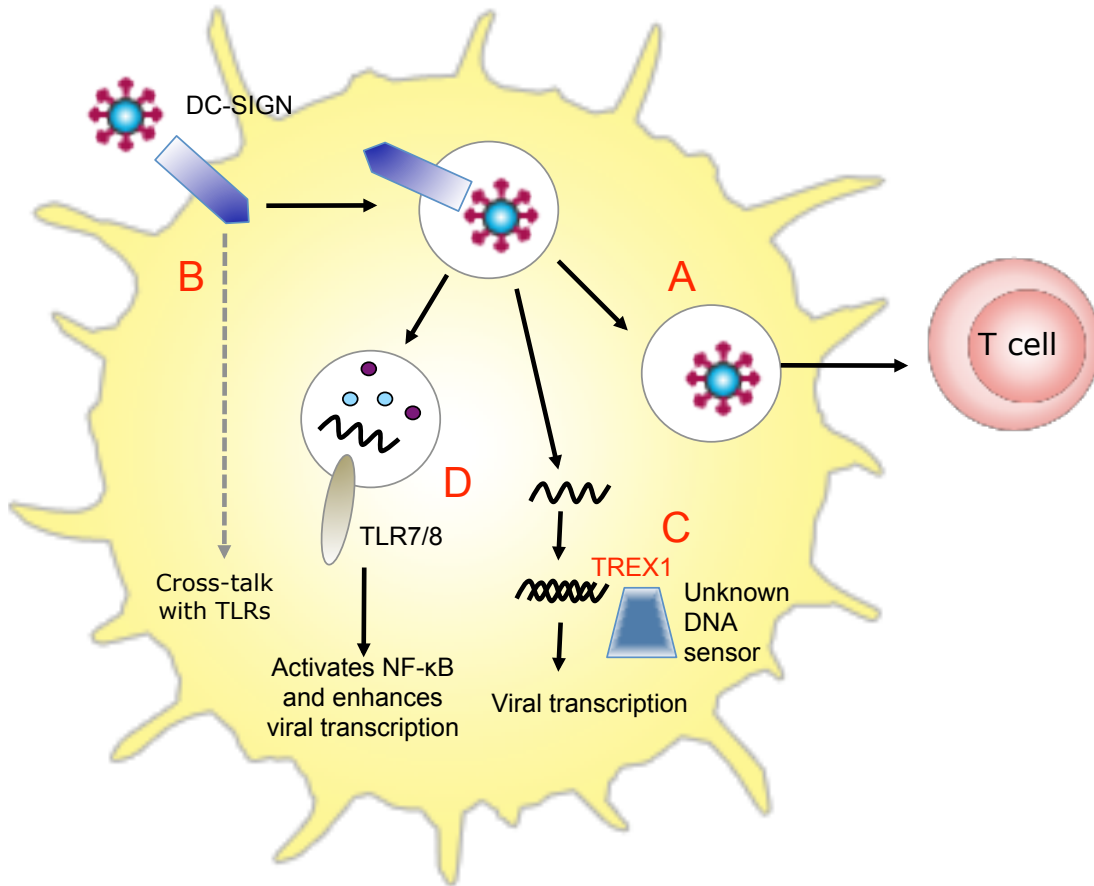


Figure 1.8: Subversion of PRR signalling by HIV-1. (A) HIV-1 has been shown to efficiently 'hide' in an endosome-like compartment where it remains intact. This may prevent the access of endosomal TLR8 to the viral nucleic acid. (B) Binding to DC-SIGN does not only lead to viral uptake but also triggers an intracellular signalling cascade which has been shown to modulate the innate immune response. (C) Reverse transcribed viral DNA can be sensed via an as yet unknown cytoplasmic sensor. However, the exonuclease TREX1 digests excess viral DNA making it unavailable for detection. (D) HIV-1 infection has been shown to lead to TLR7/8 dependent NF-κB activation which promotes viral transcription.

1.2.9 HIV-1 subverts innate immune signalling in DCs

Accumulating evidence suggests that HIV-1 actively avoids recognition by PRRs, either by hiding in a specialised compartment and thus shielding its PAMPs, or by interfering with the downstream signalling pathways (Figure 1.8). Even more strikingly, HIV-1 seems to exploit PRR recognition to dampen the antiviral response, enhance its transmission to T cells, and

increase viral replication.

DC-SIGN and DCIR bind to the glycosylated HIV-1 Env In addition to functioning as an adhesion receptor, DC-SIGN seems to be critically involved in the innate immune evasion of HIV-1. It is believed that DC-SIGN has evolved to bind self sugars on ICAM-2 and ICAM-3, thereby mediating DC transendothelial migration and the initial contact between DCs and resting T cells [Geijtenbeek *et al.*, 2003], respectively. However, several pathogens have evolved the ability to bind to this receptor, for example Hepatitis C virus, Dengue virus, Mycobacterium tuberculosis and also HIV-1 [Lozach *et al.*, 2004] [Tassaneetrithep *et al.*, 2003] [Geijtenbeek *et al.*, 2003] [Geijtenbeek *et al.*, 2000]. Interestingly, the majority of pathogens recognised by DC-SIGN tend to cause chronic infections, where the TH1 versus TH2 balance is crucial to their persistence [Engering *et al.*, 2002].

Binding of HIV-1 Env to DC-SIGN triggers a signalling cascade which shapes both innate as well as adaptive immune responses and promotes HIV-1 infection and transmission. Intracellular signalling is thought to occur via the recruitment of LARG to an incomplete ITIM motif on the cytoplasmic tail of DC-SIGN [den Dunnen *et al.*, 2009]. This activates a range of different kinases including Rho, which has been shown to enhance viral synapse formation via the activation of downstream Cdc42 [Hodges *et al.*, 2007], ERK, which is associated with a Th2 type response [Caparrós *et al.*, 2006] and Raf, which modulates TLR signalling and leads to increased IL-10 transcription [Gringhuis *et al.*, 2007]. Therefore, DC-SIGN stimulation results in a transcriptional profile distinct from that obtained following TLR stimulation, and is characterised by increased expression of immunosuppressive genes and down regulation of TLR pathway components [Hodges *et al.*, 2007]. In conclusion, the current data suggest that signalling through DC-SIGN intersects with TLR signalling, modulating or even abrogating the immune response to disable effective immune recognition of HIV-1 and enable viral escape.

In addition to DC-SIGN, the novel DC immunoreceptor DCIR has also been shown to bind the HIV-1 Env protein [Lambert *et al.*, 2008]. Similar to DC-SIGN, DCIR contains an intracellular ITIM motif, and signalling leads to the activation of Src homology 2-containing tyrosine phosphatase 1 (SHP-1) and SHP-2, thereby facilitating transmission of HIV-1 from DCs to T cells and enhancing infection [Lambert *et al.*, 2011].

HIV-1 exploits TLR8 signalling to enhance its replicative capacity A short ssRNA fragment derived from the HIV-1 LTR has been shown to be an effective TLR8 agonist [Heil *et al.*, 2004] [Alter *et al.*, 2007]. Indeed, exposure of MDDCs to HIV-1 triggers TLR8

signalling and NF- κ B p65 activation which leads to the recruitment of cyclin-dependent kinase 7 (CDK7) to viral LTRs [Gringhuis *et al.*, 2010]. CDK7 phosphorylates the RNAPII CTD, thus promoting transcriptional initiation of integrated provirus. However, increased initiation of transcription leads to the accumulation of abortive viral transcripts. Signalling via DC-SIGN is required for efficient elongation as it activates Raf-1 which leads to phosphorylation of NF- κ B p65 on ser 276, which in turn induces the recruitment of positive elongation factor b (p-TEFb) to the transcription site [Gringhuis *et al.*, 2010]. Despite this evidence which suggests that HIV-1 actively triggers TLR8 signalling and downstream NF- κ B activation, it does not fully mature DCs, nor does it lead to the production of type I IFNs. Indeed, a study by Harman *et al.* showed that, despite using high concentrations of virus, HIV-1 fails to induce IRF3 activation and therefore an antiviral IFN response [Harman *et al.*, 2011]. Together these data suggest that HIV-1 is able to selectively exploit individual components of the TLR8 signalling cascade to enhance its replication while remaining undetected.

Sensing of the HIV-1 capsid Once in the cytoplasm, the HIV-1 capsid is recognised by the host restriction factor TRIM5 α which accelerates uncoating of the core and decreases HIV-1 infectivity [Stremlau *et al.*, 2006]. It has recently been shown that binding of the capsid to TRIM5 α also triggers innate immune signalling by activating TAK-1, leading to the transcription of NF- κ B and AP-1 as well as enhanced IRF-3-mediated IFN- β transcription [Pertel *et al.*, 2011]. This detection mechanism occurs at an early stage of the HIV-1 life cycle prior to integration. Following integration, *de novo* synthesised capsid can be detected by binding to cyclophilin A, leading to DC maturation characterised by a strong type I IFN response and the induction of HIV-1 specific CD4⁺ and CD8⁺ cells. However, this particular signalling pathway is only activated during co-infection with SIV particles expressing Vpx and serves to overcome the low replication of HIV-1 in DCs [Manel *et al.*, 2010].

HIV-1 escapes recognition by an unknown cytoplasmic DNA sensor A recent report provides evidence that reverse transcribed viral DNA produced within the cytoplasm is capable of triggering the expression of type I IFNs via a pathway dependent on STING, TBK-1 and IRF3 [Yan *et al.*, 2010]. The exact DNA sensor remains to be identified. However, excess viral DNA in the cytoplasm is digested by the cytosolic exonuclease TREX1, allowing the escape of HIV-1 from this nucleic DNA sensor [Geijtenbeek, 2010].

1.2.10 Innate immune responses in HIV-1 infection - protective or detrimental?

The findings highlighted above suggest that HIV-1 may exploit a range of different PRRs in order to enhance amplification, systemic dissemination and subvert the immune response to remain undetected. Despite a complex interaction between HIV-1 and mucosal DCs, it is clear that DCs fail to efficiently detect incoming HIV-1 virions and are therefore unable to prevent or limit HIV-1 infection. This implies that it may be beneficial to enhance recognition of HIV-1 by priming the innate immune response. However, activation of the innate immune response can have detrimental consequences. In the chronic phase extensive activation of the innate immune response causes chronic immune activation which leads to depletion of cells in the GALT and therefore irreversible damage to the host immune system.

As a consequence it seems that HIV-1 faces the challenge of striking a balance between activating the innate immune system in a way to allow replication but at the same time reducing immune control to prevent recognition. The mechanisms by which HIV-1 achieves this are still unknown. One intriguing mechanism to subtly fine-tune cellular functions comes from the recently established field of microRNAs.

1.3 MicroRNAs

1.3.1 MicroRNAs are post-transcriptional regulators of gene expression

One of the most intriguing findings of the last decade has been the identification of small RNA species that inhibit gene expression at a post-transcriptional level, a process referred to as RNA interference (RNAi). One special class of small RNAs, which have attracted attention as modulators of gene expression in plants and eukaryotes, are the ~18 - 22 nucleotide long non-coding microRNAs (miRNAs) [Lagos-Quintana *et al.*, 2001]. It has become very clear that miRNAs modulate a range of cellular processes including development, hematopoiesis, immunity and oncogenesis. Dysregulation of miRNA activity is associated with a variety of diseases.

Currently there are 1,048 entities listed in the human miRNA database (microrna.sanger.ac.uk, version 13.0) and *in silico* predictions estimate that 30-50% of the human transcriptome are likely to be under miRNA regulation [Lewis *et al.*, 2005] [Friedman *et al.*, 2009].

1.3.2 The biogenesis of microRNAs

MiRNAs are often mapped to intergenic regions, but can also be encoded within introns or exons of protein-coding genes. Intergenic miRNAs exist as independent transcriptional units or in polycistronic clusters containing several miRNAs that show similar expression profiles [Kim & Nam, 2006] [Krol *et al.*, 2010]. Almost all miRNAs are initially transcribed by RNA polymerase II, as part of a long primary miRNA (pri-miRNA) precursor consisting of multiple stem loop structures [Lee *et al.*, 2004] (Figure 1.9). Some primary transcripts produce a single miRNA, whilst others are grouped in clusters and expressed together on a single polycistronic transcript. Like other Pol II transcripts, pri-miRNAs are capped and polyadenylated. Primary miRNA transcripts are subsequently cleaved by the nuclear RNase III type enzyme Drosha [Lee *et al.*, 2003] which, together with the double-stranded RNA-binding protein DGR8, forms the Microprocessor complex [Denli *et al.*, 2004] [Han *et al.*, 2004]. The cropping process releases individual hairpin precursors (pre-miRNA) which are subsequently transported into the cytoplasm by exportin-5 [Lund *et al.*, 2004a]. On reaching the cytoplasm, further processing is carried out by the RNase II type enzyme Dicer which removes the terminal loop region to generate a 18 - 22 nucleotide long mature miRNA duplex [Bernstein *et al.*, 2001] [Hutvagner *et al.*, 2001]. The duplex is unwound and one strand is usually incorporated into the RNA-induced silencing complex (RISC) where it acts as a guide strand to direct the RISC to the complementary mRNA target [Khvorova *et al.*, 2003] [Schwarz *et al.*, 2003]. The RISC then silences gene expression either via degrading or destabilising the target mRNA or through interfering with translation.

1.3.3 The function of eukaryotic miRNAs

Eukaryotic miRNAs classically bind to the 3' untranslated region (3'UTR) of target mRNAs in a sequence specific manner, leading to inhibition of translation and/or decrease in mRNA stability [Zhao & Liu, 2009]. Unlike plant miRNAs, which require near-perfect sequence complementarity to the mRNA transcript targeted for cleavage, eukaryotic miRNAs pair imperfectly with sites in the 3'UTR of their target mRNAs [Brodersen & Voinnet, 2009]. Intriguingly, perfect base pairing is only required between nucleotides 2-8 at the 5' end of the miRNA - the so called seed region. This increases the number of potential target sites, making target prediction a difficult and time-consuming computational challenge.

Binding of eukaryotic miRNAs to their complementary sequence in the 3'UTR can mediate target gene repression by a variety of different mechanisms [Zhao & Liu, 2009]: (1) miRNA binding can promote deadenylation and destabilisation of the mRNA target [Fabian *et al.*, 2011],

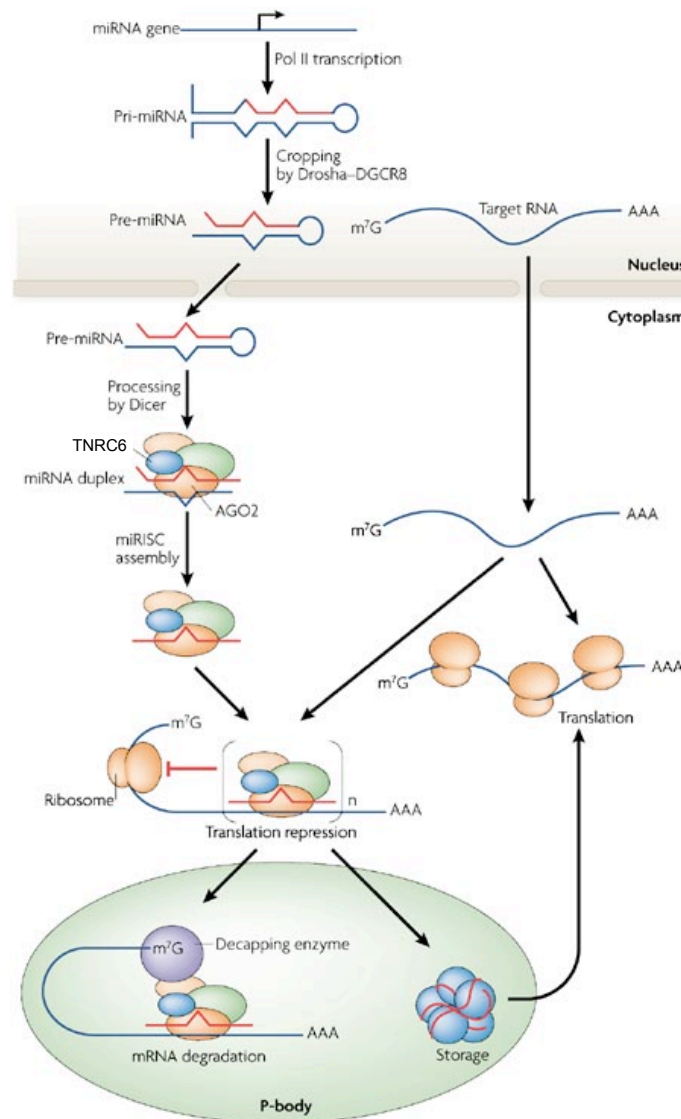


Figure 1.9: The biogenesis and function of mammalian miRNAs. MicroRNAs are transcribed by Pol II from introns and intergenic regions into long (60 - 100 nt) primary transcripts (pri-miRNA) with a stem-loop structure. The stem-loop structure is cropped by Drosha into precursor miRNAs (pre-miRNAs) and transported out of the nucleus by exportin-5. In the cytoplasm pre-miRNAs are processed by Dicer into 20-22 nt mature miRNA duplexes. The duplex is unwound and loaded into the RISC, a multi-protein silencing complex with an argonaute protein at its core [Hammond *et al.*, 2001] and a variety of other cofactors such as the TNRC6 family [Meister *et al.*, 2005]. The miRNA guides the RISC to the matching binding site within the 3'UTR of target mRNAs. Mammalian miRNAs can bind their target with imperfect sequence complementarity. The characteristic bulge in the middle prevents target cleavage by RISC. Instead, miRNA binding leads to translational repression by interfering with the ribosomal machinery. Furthermore, bound mRNA can be sequestered into P-bodies and either stored there or enter the mRNA decay pathway. Figure taken from [Rana, 2007].

(2) miRNA:mRNA pairs can be sequestered to specific foci, referred to as processing bodies (P-bodies), for storage or degradation [Nathans *et al.*, 2009] or (3) miRNA binding can interfere with translation at the post-initiation phase by disrupting elongation and thus affect target expression at the protein level [Mathonnet *et al.*, 2007].

In addition, a range of non-classical miRNA binding modes have been observed. Some miRNAs, for example miR-16, function together with the RNA binding protein tristetraprolin (TTP) to bind to AU-rich elements (AREs) within the 3'UTR of target mRNAs to accelerate mRNA decay [Jing *et al.*, 2005]. Complicating matters further, the classical function of miRNAs as negative regulators of gene expression has recently been challenged by the observation that selected miRNAs bind to sites in the 5'UTR of their target genes and enhance gene expression [Ørom *et al.*, 2008]. The mechanism of miRNA-mediated regulation from these sites has not as yet been defined.

Prediction and validation of miRNA targets is currently a major challenge [Thomas *et al.*, 2010]. Putative miRNA targets can be predicted using various computational tools which incorporate multiple factors including perfect seed matching, thermodynamic stability of the miRNA:mRNA duplex as well as the position of the miRNA binding site within the 3'UTR [Witkos *et al.*, 2011]. As bioinformatic target prediction can yield hundreds or even thousands of possible targets for a single miRNA, experimental validation on the transcriptional as well as protein level is essential. Several high-throughput methods including gene expression microarray analysis and proteomic approaches are being developed to investigate the complex effects of single miRNAs on the cellular proteome [Ørom & Lund, 2010]; this will be discussed in Chapters 5 and 6.

1.3.4 The role of miRNAs in the innate immune response

Similar to transcription factors, miRNAs are dynamic regulators in innate immune cells and play a pivotal role in their development, maturation and function [Taganov *et al.*, 2007]. Given their role in innate immune responses, aberrant miRNA expression can give rise to impaired innate immune function and is even sufficient to cause disease. Dysregulation of selected miRNAs has been observed in patients with, for example, Crohn's disease [Wu *et al.*, 2010a], rheumatoid arthritis [Pauley *et al.*, 2008] [Alsaleh *et al.*, 2009] or multiple sclerosis [Otaegui *et al.*, 2009] [Du *et al.*, 2009].

The importance of miRNAs in the regulation of innate immune responses has been underlined by an increasing number of studies demonstrating that miRNA expression is modulated under a range of inflammatory conditions. The first expression profiling study carried out by

Taganov et al showed that stimulation of human monocytic THP-1 cells with LPS induces the expression of miR-146, miR-155 and miR-132 [Taganov *et al.*, 2006]. Since then further studies using a range of different cell types, stimuli, time points and, most importantly, profiling technologies have lead to the identification of additional miRNAs that are regulated in response to immune activation. As can be seen from Table 1.2 most data obtained so far focuses on TLR-inducible miRNAs and less is known about the effect of other PRRs, such as RIG-I or NOD2, on cellular miRNA expression.

Similar to inflammatory regulated genes, miRNA regulation is controlled in a time-dependent manner, leading to the definition of early and late response miRNAs. The regulation of miRNA expression can occur at three different levels: transcription, processing and subcellular localisation [Davis-Dusenbery & Hata, 2010]. Selective induction of miRNA expression is initially controlled via transcriptional increase of pri-miRNAs [Ruggiero *et al.*, 2009]. For example, the expression of miR-146 and miR-155 is induced via activation of the central transcription factor NF- κ B [Taganov *et al.*, 2006]. However, inflammation or other types of cellular stress have also been shown to affect various stages of miRNA processing. The abundance of RNAi components themselves can be influenced during the immune response. For example, type I interferons have been shown to inhibit Dicer expression, decreasing the processing of miRNAs on a global level [Wiesen & Tomasi, 2009]. Other factors, like the SMAD proteins, which regulate miR-21 expression, control miRNA processing in a ligand-dependent manner [Davis *et al.*, 2008]. Lastly, miRNAs have been shown to associate with stress granules following the onset of cellular stress, although the role of subcellular localisation in the control of miRNA function remains unknown [Leung *et al.*, 2006].

1.3.5 A central role of miR-155 in the innate immune response

A consensus is emerging which suggests that miR-155, miR-146, miR-132, miR-21 and miR-9 form a small group of miRNAs that are induced fairly ubiquitously across multiple cell types and upon stimulation with a range of different bacterial and viral stimuli [O’Connell *et al.*, 2007]. First identified as the tumor promoting oncomiR termed BIC [Tam *et al.*, 1997], miR-155 has now emerged as a master regulator of the innate as well as the adaptive immune response. Mice deficient for miR-155 show clear defects in a range of immunological processes affecting DC as well as B and T lymphocyte function, suggesting an important role for miR-155 in the homeostasis and function of the immune system [Rodriguez *et al.*, 2007] [Thai *et al.*, 2007]. In the search for targets, several large-scale approaches have been used to gain insight into the gene networks affected by miR-155.

Table 1.2: MicroRNAs regulated downstream of PRR signalling. Adapted from [O'Neill *et al.*, 2011].

Upregulated				
miRNA	TLRs	Signalling molecules	Target	References
miR-155	TLR2, TLR3, TLR4, TLR9	MYD88, TRIF, NF- κ B, KSRP	MYD88, TAB2, IKK ϵ , C/EBP β , TNF, SHIP1, SOCS1	[Taganov <i>et al.</i> , 2006] [O'Connell <i>et al.</i> , 2007] [Thai <i>et al.</i> , 2007] [Tang <i>et al.</i> , 2010] [Ceppi <i>et al.</i> , 2009] [Tili <i>et al.</i> , 2007] [Ruggiero <i>et al.</i> , 2009] [Androulidaki <i>et al.</i> , 2009]
miR-146	TLR2, TLR3, TLR4, TLR5	MYD88, NF- κ B	IRAK1, IRAK2, TRAF6	[Ceppi <i>et al.</i> , 2009] [Landgraf <i>et al.</i> , 2007] [Hou <i>et al.</i> , 2009] [Bazzoni <i>et al.</i> , 2009] [Taganov <i>et al.</i> , 2006]
miR-132	TLR4, TLR9		p300, ACHE	[Ruggiero <i>et al.</i> , 2009] [Bazzoni <i>et al.</i> , 2009] [Lagos <i>et al.</i> , 2010] [Shaked <i>et al.</i> , 2009]
miR-21	TLR4	MYD88, TRIF, NF- κ B	IL-12p35, PDCD4	[Sheedy <i>et al.</i> , 2010] [Landgraf <i>et al.</i> , 2007] [Lu <i>et al.</i> , 2009b]
miR-223	TLR4		TLR4, TLR3, IKK	[Ceppi <i>et al.</i> , 2009] [Li <i>et al.</i> , 2010a] [Liu <i>et al.</i> , 2009]
miR-147	TLR2, TLR3, TLR4	MYD88, TRIF, NF- κ B, IRF3		
miR-9	TLR2, TLR4, TLR7, TLR8	MYD88, NF- κ B	NF- κ B1	[Bazzoni <i>et al.</i> , 2009]
miR-125b	TLR4	NF- κ B	TNF	[Alsaleh <i>et al.</i> , 2009] [Tili <i>et al.</i> , 2007]
let-7e	TLR4	AKT1	TLR4	[Androulidaki <i>et al.</i> , 2009]
miR-27b	TLR4	NF- κ B	PPAR γ	[Jennewein <i>et al.</i> , 2010]
Downregulated				
miRNA	TLRs	Signalling molecules	Target	References
miR-125b	TLR4	NF- κ B, AKT1	TNF	
let-7i	TLR4	NF- κ B, C/EBP β	TLR4	[Iliopoulos <i>et al.</i> , 2009] [Chen <i>et al.</i> , 2007] [O'Hara <i>et al.</i> , 2010]
miR-98	TLR4			[Hu <i>et al.</i> , 2010]

They provide evidence that miR-155 plays a dual role in the innate immune response. Its role early in infection is to enhance the pro-inflammatory response, by repressing a variety of different negative regulators including SH-2-domain-containing inositol-5-phosphatase 1 (SHIP-1) [Yamanaka *et al.*, 2009] and the suppressor of cytokine signalling 1 (SOCS1) [Yasukawa *et al.*, 2000], both of which are negative regulators of TLR signalling. Simultaneously, miR-155 possesses anti-inflammatory potential and provides a negative feedback loop that prevents excessive immune activation during later time points. For example, knockdown of miR-155 in human MDDCs increased the expression of the pro-inflammatory cytokine IL-1 β [Ceppi *et al.*, 2009]. Similarly, over expression of miR-155 represses TAB2, an essential component of the TLR signalling network [Ceppi *et al.*, 2009].

1.3.6 PRR-responsive miRNAs modulate innate immune responses

The function of other PRR-responsive miRNAs is less well defined, mainly due to the problem of target identification and the complexity of the immune system. Knockout mouse models that can provide a bigger picture are still missing. Hence, only a limited number of individual targets exist which, together, can only provide a small snapshot of a miRNA's overall function. Mounting evidence suggests that PRR-responsive miRNAs work together to balance the innate response to invading pathogens by modulate strength and timing. In addition, they may provide an important switch between the strong initial pro-inflammatory response and the resolution phase of the inflammatory process [O'Neill *et al.*, 2011].

miRNAs as negative regulators of PRR signalling Several miRNAs have been shown to regulate PRR signalling in a post-transcriptional manner by targeting essential pathway components. Modulation could occur on the level of receptor expression and bioinformatic analysis indicates that miRNAs could directly regulate the expression of different PRRs [Heikham & Shankar, 2010]. This could function as a negative feedback loop to completely shut down or limit signalling following stimulation and avoid excessive pro-inflammatory responses. Stimulation of TLR4 in mouse macrophages, for example, leads to up regulation of let-7e which, in turn, binds to a site in the TLR4 mRNA 3'UTR, resulting in decreased TLR4 expression and thus decreased cytokine responsiveness to LPS [Chen *et al.*, 2007] [Androulidaki *et al.*, 2009]. Albeit it is tempting to assume that miRNAs may play a role in shaping the PRR repertoire of specific cells, no supporting evidence has so far been provided.

Pathogens may carry a variety of PAMPs and therefore trigger multiple PRRs. Thus, targeting more central components further downstream of receptor engagement would allow simultaneous toning down of multiple signalling pathways. A study with *H. pylori* demonstrates that miR-155 decreases the expression of the universal TLR adaptor MyD88 [Ceppi *et al.*, 2009]. In addition, miR-155 simultaneously represses TAB2, which functions further downstream of TRAF6, and activates MAPK kinases [Tang *et al.*, 2010]. Similarly, miR-146, which is up regulated in a MyD88-dependent manner, represses the expression of IRAK1, IRAK2 and TRAF6, which in turn function as crucial adaptor proteins downstream of MyD88 [Taganov *et al.*, 2006]. The adaptor MAL that links TLR2 and TLR4 signalling [Verstak *et al.*, 2009], undergoes proteosomal degradation after signalling and is also targeted by miR-145 [Mansell *et al.*, 2006] [Starczynowski *et al.*, 2010]. However, it is unknown if PRR signalling alters the expression of miR-145.

In addition, miRNAs may control the expression of downstream pro-inflammatory transcription factors. In fact, transcription factors and miRNAs often modulate one another and form regulatory loops. For example, TLR4-mediated maturation of monocytes and neutrophils induces the expression of miR-9 in an NF- κ B-dependent manner. Up regulation of miR-9 in turn decreases the activation of NF- κ B by repressing NF- κ B1 expression, an important transactivator of the NF- κ B p65 subunit. It therefore not only tones down TLR signalling but also limits its own expression [Bazzoni *et al.*, 2009].

MicroRNAs modulate cytokine output Computational analysis indicates that the 3'UTRs of chemokines, cytokines and their receptors generally only contain few miRNA binding sites compared to the genome as a whole [Asirvatham *et al.*, 2008] [Asirvatham *et al.*, 2009]. However, as already mentioned, miRNAs may control cytokine secretion via indirect mechanisms by modulating TLR signalling, targeting transcription factors or repressing global regulators of cytokine expression like SOCS1 [Lu *et al.*, 2009a] [Wang *et al.*, 2010]. Similarly, LPS stimulation induces miR-21 which down regulates IL-10 indirectly by targeting the protein programmed cell death 4 (PDCD4) [Sheedy *et al.*, 2010]. There is accumulating evidence suggesting that miRNAs may also be able to control cytokine expression by non-classical mechanisms. The TLR4-responsive miR-221, in cooperation with the RNA binding protein tristetraprolin (TTP), binds to AREs in the TNF mRNA transcript and accelerates its decay [Tili *et al.*, 2007]. Conversely, binding of miRNAs to AREs can also protect RNA transcripts from decay as shown for the interaction between miR-4661 and IL-10 mRNA [Ma *et al.*, 2010].

1.3.7 The role of miRNAs in virus-host interaction

There is a growing amount of evidence that miRNAs play a critical role in intricate host-pathogen interaction networks. Due to early studies in plants and invertebrates, the RNAi pathway was initially thought to have evolved as a defense mechanism against viral infection. Indeed several viruses encode for RNAi suppressors that directly interfere with discrete steps in the cellular RNA silencing machinery. Examples of these suppressors include primate foamy virus Tas [Lecellier *et al.*, 2005], Vaccinia virus E3L, Influenza A virus NS1 [Li *et al.*, 2004], Ebola virus VP35 [Haasnoot *et al.*, 2007] and the HIV-1 Tat protein [Bennasser *et al.*, 2005]. However, recent reports strongly suggest a more complex role of miRNA mediated gene silencing in the intricate interaction between viruses and their host.

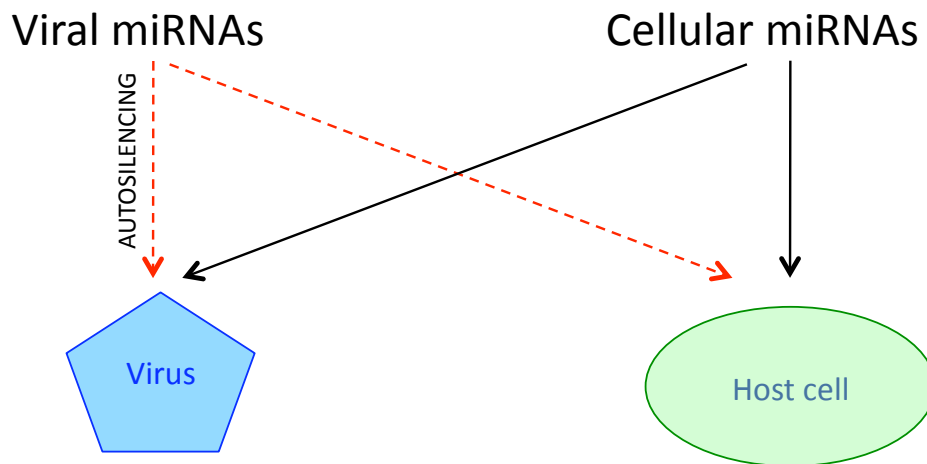


Figure 1.10: MicroRNAs in host-virus interaction. Upon viral infection cellular miRNAs have been shown to be differentially expressed. In addition viruses encode for their own short RNAs which can enter the cellular RNAi pathway and function as miRNAs. Both cellular and viral miRNAs have been shown to have effects on the host and viral replication. If viral miRNAs target back to their site of origin in the viral genome, this is termed autosilencing.

1.3.8 Cellular miRNAs involved in viral infection

As described previously, viral infection can modulate expression of cellular miRNAs; and cellular miRNAs are not only involved in antiviral host defense but can also modulate the cellular environment thereby indirectly impact on viral pathogenesis (Figure 1.10). For example, human papillomavirus encodes for two oncogenic proteins, E6 and E7, which inhibit p53 and RB pathways. E6 expression down regulates the expression of p53-associated miRNAs

miR-34a and miR-218 [Wang *et al.*, 2009a] [Martinez *et al.*, 2008] promoting cell growth and enhancing tumorigenicity.

Several cellular miRNAs exhibit significant homology to selected regions of viral transcripts. For example, miR-32 restricts the accumulation of the retrovirus primate foamy virus type 1 (PFV-1) in human cells by directly targeting the viral transcript [Lecellier *et al.*, 2005]. Similarly, miR-24 and miR-93 have been shown to target VSV transcripts in infected mice [Otsuka *et al.*, 2007]. However, the significance of these miRNAs in a physiologically relevant setting needs to be established. Several antiviral miRNAs were shown to contribute to establishing HIV-1 latency in resting CD4⁺ T cells by directly targeting the viral transcript [Huang *et al.*, 2007b]. HCV infection induces a strong type I IFN response which rapidly induces the expression of miR-196, miR-296, miR-351, miR-431, and miR-448, all of which may directly target the HCV genome and attenuate viral replication [Pedersen *et al.*, 2007]. An exception is miR-122 which is highly expressed in the liver and binds to the 5' UTR of the HCV genome, thereby significantly enhancing viral replication [Jopling *et al.*, 2005] [Jopling *et al.*, 2008]. This miRNA may play a key role in directing the tissue tropism as well as increasing the pathogenicity of HCV.

1.3.9 Virus-encoded miRNAs

In 2004, Pfeffer *et al.* cloned a miRNA of viral origin from B cells latently infected with Epstein Barr Virus (EBV) and thus were the first to identify a virally encoded miRNA. Since then many human viruses including HIV-1, HSV-1 and HSV-2, have been shown to encode for functional microRNAs that can be processed by the cellular RNAi machinery and may perturb the host cell's overall miRNA milieu [Nair & Zavolan, 2006] [Jurak *et al.*, 2010] [Voinnet, 2005].

Due to their inherent complementarity to the site of origin within the viral genome, viral miRNAs (vmiRNAs) could potentially also target back to the virus and down-regulate viral replication, a process often referred to as 'autosilencing' (Figure 1.10). However, the role of this mechanism in viral infection remains controversial. While it seems unnecessary and could have antiviral potential, it may also be exploited by the virus as a means of regulating expression levels and establishing latency. For example, the SV40 miRNA miR-S1, which accumulates during late infection, represses the viral T-antigen and may help to prevent killing of infected cells by T-antigen-specific CTLs [Sullivan *et al.*, 2005]. In addition, HSV-1 and HSV2 express viral miRNAs to repress the expression of two early viral proteins, ICP0 and ICP34.5, and are thought to stabilize the latent state [Umbach *et al.*, 2008]. However,

the relevance of these mechanisms for viral infection *in vivo* remain controversial.

Viral miRNAs also have the potential to share sequence homology with specific sites in cellular mRNAs and therefore be involved in directly regulating the host environment. Stable expression of multiple Kaposis' sarcoma-associated herpes virus (KSHV) miRNAs in cell lines represses thrombospondin 1 (THBS1) expression. Down regulation of THBS1 expression may be desirable for viral propagation as it has been shown to target TGF- β to inhibit cell survival and proliferation [Samols *et al.*, 2007]. One particular KSHV miRNA (miR-K12-11) is an orthologue of miR-155 and acts as an oncomiR supporting tumor formation, especially in immunocompromised patients [Gottwein *et al.*, 2007] [Skalsky *et al.*, 2007]. Finally, KSHV miR-K7, among other viral miRNAs, targets MICB which is expressed as a response to cellular stress and viral infection and is a ligand for NK cells. Therefore, down regulation of MICB by viral miRNAs may contribute to the immune evasion strategies of selected viruses [Nachmani *et al.*, 2009].

1.3.10 The complex role of miRNAs in the host-pathogen interaction

The role of miRNAs in viral infection is clearly complex. It can be manipulated by both the host and the pathogens and can have a profound effect on both. It is likely that viral pathogenesis as well as the establishment of an antiviral immune response is subject to control by miRNAs and understanding this complex interaction represents a novel basis for the development of treatment strategies. The ability to harness miRNAs for therapeutic strategies is underlined by the development of a miR-122-specific antisense oligonucleotide that has already been shown to be effective in the treatment of HCV *in vivo* [Branch & Rice, 2010] and is progressing into clinical trials.

1.4 Aims

Despite many years of intensive HIV/AIDS research, the mechanisms by which the virus evades the innate immune response remain poorly understood. The interaction between DCs and HIV-1 has been under particularly thorough investigation, but the entire complexity of this interaction has not yet been fully defined.

Given the emerging role of miRNAs in initiating and controlling innate immune responses and their central role in host-virus cross talk, the aim of this work is to explore the significance of cellular miRNAs during HIV-1 infection of human monocyte-derived dendritic cells *in vitro*.

We use genome-wide profiling based on microarray technology to characterise differential miRNA expression in MDDCs induced by exposure to HIV-1. Given that HIV-1 actively subverts the innate immune response and fails to fully activate MDDCs, we plan to compare the observed changes in miRNA expression with those observed during DC maturation. We hope that this might provide clues as to whether these miRNAs are involved in the antiviral response or exploited by HIV-1 as a means of immune escape.

Secondly, a comprehensive target search combining bioinformatic as well as a variety of experimental validation strategies is used to determine targets for selected miRNAs. After gaining insight into which gene networks are subject to regulation of this particular miRNA, we will attempt to explore the biological function of the chosen miRNA on DC phenotype and function as well as viral pathogenesis using a variety of molecular biology techniques. Therefore, the aim of this work is to identify novel miRNAs involved in the innate antiviral immune response of DCs in the context of HIV-1 infection.

Chapter 2

Materials and Methods

2.1 Solutions and biochemical reagents

D10 DMEM (Sigma) supplemented with 10% (v/v) heat-inactivated (56°C for 20 min) FCS (Seralabs), 2 mM L-glutamine, 1 U/ml penicillin and 50 μ g/ml streptomycin.

R10 RPMI (Sigma) supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 1 U/ml penicillin and 50 μ g/ml streptomycin.

MACS buffer Phosphate-buffered saline (PBS), 2% (v/v) FCS, 4ml 0.5M EDTA.

PBA PBS, 0.5% BSA, 0.01 g NaN₃.

FACS fix PBS, 1% (v/v) formaldehyde.

50× TAE buffer 242 g of Tris base, 57.1 ml acetic acid, 100 ml of 0.5 M EDTA pH8.

Lysis buffer 0.5% NP-40, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA.

LB (Luria Bertani) medium 10 g/l bactotryptone (BD Biosciences), 5 g/l yeast extract (Meford) and 10 g/l NaCl (Sigma) in distilled water. The solution was autoclaved at 15 psi for 20 min and cooled before use.

Ampicillin A ready made 100 mg/ml stock solution was obtained from Sigma and stored at -20°C.

Agar plates LB agar (as for LB medium but with the addition of 15 g/l bactoagar (BC Biosciences) was autoclaved at 15 psi for 20 min and cooled to below 50°C before the addition of ampicillin to a final concentration of 100 μ g/ml. Agar containing the

antibiotic were then poured into 9 cm petri dishes and allowed to cool. Once set, plates were stored at 4°C.

2.2 Antibodies

All antibodies used are listed in Table 2.1.

Table 2.1: *List of antibodies used.*

	Species	FACS	WB	Confocal	Company
DC-SIGN	mouse	1:100			eBiosciences
CD86	mouse	1:100			eBiosciences
CD80	mouse	1:50			eBiosciences
CD83	mouse	1:20			eBiosciences
HLA-DR	mouse	1:50			BD Biosciences
α -LC3B	rabbit			1:200	Cell signalling
α -MxA	mouse		1:2500	1:200	Abnova
α -MxA	rabbit		1:2500	1:200	Abnova
α -HA	mouse	1:1000			Kindly provided by Prof. Allan Townsend
α -mouse HRP	goat		1:10000		Sigma
α -rabbit HRP	goat		1:10000		Sigma
α -mouse 800	goat		1:10000		LICOR
α -rabbit 680	donkey		1:10000		LICOR
α -mouse AF488	goat	1:500		1:200	Invitrogen
α -rabbit AF488	goat			1:200	Invitrogen
α -mouse AF568	goat			1:100	Invitrogen

2.3 Molecular biology

2.3.1 Plasmids and constructs

The pmiR-GLO plasmid was obtained from Promega and 3'UTRs were inserted into the multiple cloning site using SalI and NheI restriction endonucleases (see Figure 2.1).

The HIV-1_{NL4.3-BaL} plasmid was generated using a T tropic HIV-1_{NL4.3} vector as a backbone and replacing the envelope sequence with that of the M tropic HIV-1_{BaL} strain [Fackler *et al.*, 2001].

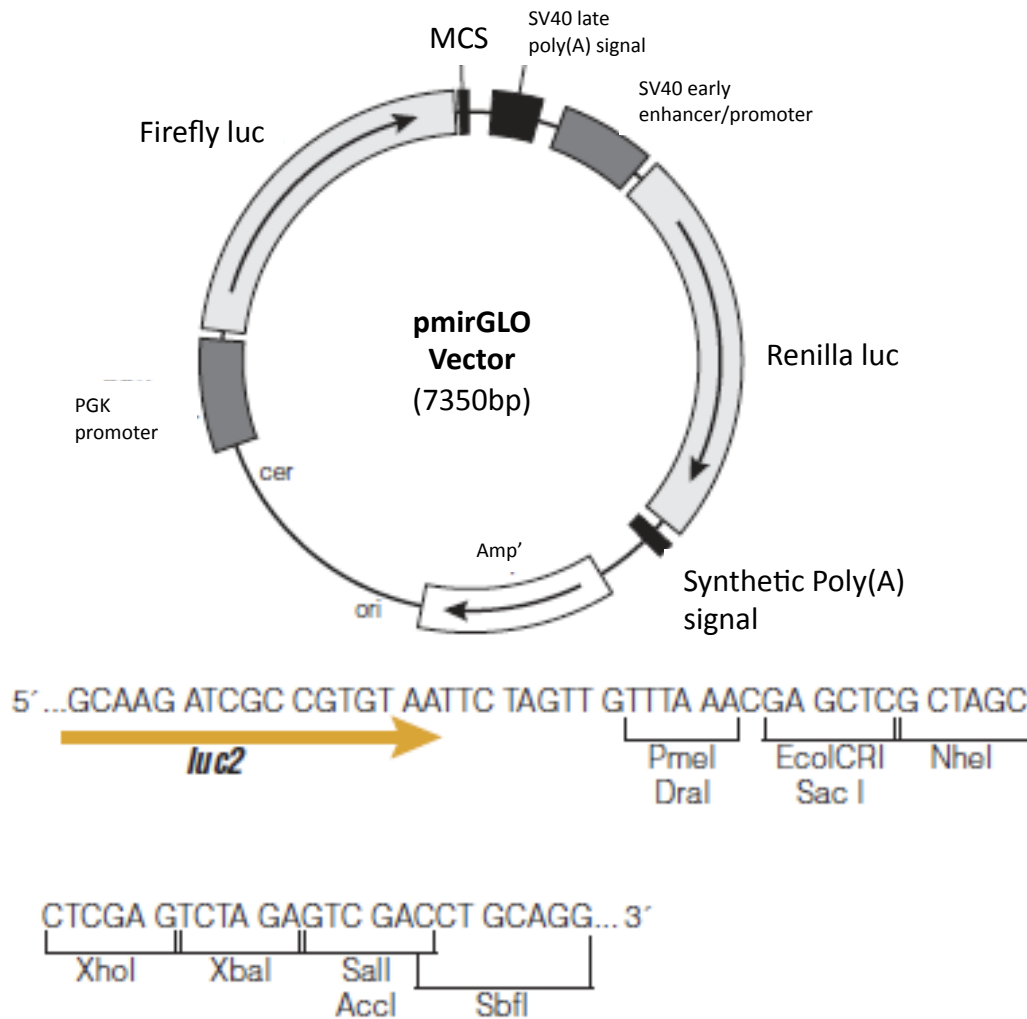


Figure 2.1: pmir-GLO luciferase reporter vector (Promega).

2.3.2 Isolation of genomic DNA

Genomic DNA was extracted from 5×10^6 freshly isolated PBMCs or 2 - 4 ml whole blood by isopropanol precipitation using the FlexiGene DNA purification kit (Qiagen) according to manufacturer's instructions. DNA concentration was assessed by UV spectroscopy using a NanoDrop ND-800 spectrophotometer. DNA was stored at -80°C until required.

2.3.3 Primer design

All primers used for 3'UTR PCR amplification were designed using the sequences published at Pubmed (<http://www.ncbi.nlm.nih.gov/>). Primers were designed to amplify full-length

3'UTR sequences and to insert a SalI and NheI restriction endonuclease site at each end of the PCR product. This allowed cleavage of the PCR product and vector with the same enzymes and subsequent oriented ligation of the PCR product into the vector. It was also ensured that the 3'UTR sequences remained in frame when cloned into the pmiR-GLO vector.

The melting temperature of a pair of primers is dependent upon the G/C content and was calculated using:

$$T_m = 64.9^\circ\text{C} + 41^\circ\text{C} \times \frac{G+C-16.4}{n}$$

where n is the total number of base pairs in the primer.

For PCR amplification, it is important that the melting/annealing temperatures of the two primers do not differ by more than 5°C. However the nature of the 3'UTR sequence, in which the G/C content often decreases further away from the stop codon, made primer design challenging. The primers used for amplification of the 3'UTRs are listed in Table 2.2.

2.3.4 Polymerase chain reaction (PCR)

3'UTR sequences were amplified from genomic DNA by PCR using the high-fidelity Phusion DNA polymerase (NEB). PCR setup and cycling conditions are shown in Table 2.3 and 2.4. For optimisation of large PCR products, the following parameters were varied: elongation time, buffer (HF vs. GC), DMSO concentration and MgCl₂ concentration (1.5 mM to 2 mM).

2.3.5 Agarose gel electrophoresis

DNA samples were resolved on 1% Agarose gels made by melting 1 g agarose (Sigma) in 100 ml TAE buffer. Liquid was cooled before 1 ng/ml ethidium bromide was added and the gel was poured. Loading buffer was added to the PCR product before it was loaded into the wells of the gel and run at 70 V. A 1 kb ladder (NEB) was used to assess the product size. DNA bands were visualised using a UV transilluminator and photographed using a UVP digital camera system. Low wavelength UV was used to visualise gels from which DNA fragments were to be excised.

2.3.6 Purification of PCR products

Gel fragments containing the DNA fragment of interest were excised from agarose gels using a clean microblade. DNA was recovered using the QIAquick Gel Extraction kit (Qiagen) according to manufacturer's instructions. Finally, DNA was eluted by applying 45 µl EB buffer onto the spin column, incubating for 1 min at RT and spinning at 13,000 rpm for 1

Table 2.2: Primers for 3' UTR amplification. Inserted restriction enzyme sites written in capitals.

Primer name	Sequence
CXCL10 FWD	TCGCTAGCaccagaggggagcaaaatcgatgc
CXCL10 REV	AGGTCGACtagcttttcttaattttccaggagagtacctc
IFIT2 FWD	TCGCTAGCgaatagagatgtggtgccactagg
IFIT2 REV	AGGTCGACtggaatcagtgacttttatttctcataacagagg
GCH1 FWD	TCGCTAGCcttcattcagtggtgtctgcagaacc
GCH1 REV	AGGTCGACaataggaccaacttcaggaaacatacactc
ING4 FWD	TCGCTAGCtaagggccttgattccaacacagtctc
ING4 REV	AGGTCGACgaatgctcagcatgtttactaaaacagtgctc
MAP1LC3C FWD	TCGCTAGCccatgtcgggaaggatgtgtgctc
MAP1LC3C REV	AGGTCGACggacacctgcccatgcagg
SOCS3 FWD	TCGCTAGCgggttaaaggcgcaaggggc
SOCS3 REV	AGGTCGACgtgctctttattataaattactgaaatgtttctttctg
CD209 FWD	TCGCTAGCagaacttcaccccttttaagctacagtcc
CD209 REV	AGGTCGACtgcaaggcagccaagcaaggacac
SIRP1B FWD	TCGCTAGCtgaccctcagtcctgtgctgctc
SIRP1B REV	AGGTCGACtaatgaagtgcacttgagcagagactgaagg
ARHGAP31 FWD	TCGCTAGCccaagaatggaagctgtgaggagac
ARHGAP31 REV	AGGTCGACcggagagacaaaacaagaactagagtttaaatg
IRF3 FWD	TCGCTAGCccctcgctcctcatggtgtgc
IRF3 REV	AGGTCGACgatcatagcaggaaaccagtttattggttgag
RAB1B FWD	TCGCTAGCaggggacacatggagtgggacag
RAB1B REV	AGGTCGACaaaccctcagctcaccggagg
NOD1 FWD	TCGCTAGCaggatgctttctgttcatgggggttttgc
NOD1 REV	AGGTCGACaagagtgttttcagtattttatacaaatgagctggc
AP2B1 FWD	TCGCTAGCaagactgggtccagtaccctcaacc
AP2B1 REV	AGGTCGACgtaaatgtgagtttattgacgaagacacaagg
ATG12 FWD	TCGCTAGCccacaaagaaaatcaacttgctactacatgaaatgg
ATG12 REV	AGGTCGACgtgttattccaatatttaggacatagtaggcactc
CHORDC1 FWD	TCGCTAGCtgggagatggaaggaaggctattac
CHORDC1 REV	AGGTCGACttgacaaaccgtagttaaaccatagctacc
DAGLA FWD	TCGCTAGCaccccagttgcgtggccagc
DAGLA REV	AGGTCGACgtgcttctacatccagtttatacacagctc
MLLT10 FWD	TCGCTAGCaagacttagtgataaaactgggcctgtagc
MLLT10 REV	AGGTCGACacattttgtacatttttaatacatgtggaattttacatctagg
STX12 FWD	TCGCTAGCtgctccgatcgttctccgc
STX12 REV	AGGTCGACtctgtaataggtttattaagaccacctaggag
FKBP1A FWD	TCGCTAGCaggaaatggcctcctcccttagctc
FKBP1A REV	AGGTCGACctgagaaaagccgcataaagcacttttattgc
PTPN7 FWD	TCGCTAGCccctgccaccctccgtg
PTPN7 REV	AGGTCGACgagtcagtcacccaagaagtctttattttccac
GIN1 FWD	TCGCTAGCtattcattaaaaacgttgatagaagaagtatcttgac
GIN1 REV	AGGTCGACggctgaacaataagaattttattgtctcacag
MFN1 FWD	TCGCTAGCaatagagattgctttggtgacctgataggagg
MFN1 REV	AGGTCGACgaaactggtgaggcatttccaccagc
S100B FWD	TCGCTAGCattagaaagcagccaacctttcctgtaacag
S100B REV	AGGTCGACctttttattgaacgaggccctcgcg
PCSK7 FWD	TCGCTAGCctcagggcctgacagtgtggg
PCSK7 REV	AGGTCGACttggaagggttttattttggaaaagctgtgcag
PPP2R1B FWD	TCGCTAGCgaggagcaggagggaag gcc
PPP2R1B REV	AGGTCGACgaactcatatcagtttaagagaacaactctggag
RARB FWD	TCGCTAGCacattttctagctacttcaaacattccccag
RARB REV	AGGTCGACtatattttattctttccaggagactccatgg
DDX17 FWD	TCGCTAGCaccactcaagtggtagtgactccagc
DDX17 REV	AGGTCGACactaagaatagtaacatagctttcagcatcctgtg
TMED2 FWD	TCGCTAGCaagcctctctctgat gatccaaact cag
TMED2 REV	AGGTCGACttaccgaacctgggcatattagatacaacc
DHX15 FWD	TCGCTAGCttcagtgcttaga actgaagtattgaggagac
DHX15 REV	AGGTCGACggccaaaattagataaaatagactttattgaagtcagtg
TGM4 FWD	TCGCTAGCcttgctgatgctgtggagccttagttg
TGM4 REV	AGGTCGACagtttaatttaaagcttattgatcatttgagcaaatctctgg
MXA FWD	TCGCTAGCccacactctgtccagccccgta
MXA REV	AGGTCGACtgtctgctagaatgagtttatt

Table 2.3: *Phusion PCR reaction setup.*

Component	Volume
gDNA (200 ng/ μ l)	1.2 μ l
10 $\times$ buffer	10 μ l
DMSO	1.5 μ l
Primer FWD (10 μ M)	2.5 μ l
Primer REV (10 μ M)	2.5 μ l
Pol enzyme	0.5 μ l
Water	30.8 μ l
Total	50 μ l

Table 2.4: *PCR cycling conditions.*

Temperature	Time	No. of cycles
98°	30 sec	
94°C	10 sec	
65°C	20 sec	5
72°C	90 sec	
94°C	10 sec	
60°C	20 sec	10
72°C	90 sec	
94°C	10 sec	
58°C	20 sec	20
72°C	90 sec	
72°C	10 min	
4°C	hold	

min.

2.3.7 Digestion and DNA with restriction endonucleases

Purified PCR products were digested for 1 hr at 37°C using SalI and NheI according to the reaction setup shown in Table 2.5. Digested PCR products were purified using the PCR Purification kit (Qiagen) according to manufacturer's instructions and eluted in 30 μ l EB buffer for subsequent ligation. For maximum recovery the elution step was performed twice by reapplying the eluate onto the same column again. If the amount of PCR product was very low, the MinElute PCR Purification kit (Qiagen) was used and PCR products were eluted using 10 μ l EB buffer.

Table 2.5: *Restriction enzyme digest.*

Component	Volume
PCR product	42.5 μ l
10 $\times$ buffer H	5 μ l
BSA	0.5 μ l
NheI	1 μ l
SalI	1 μ l
Total	60 μ l

2.3.8 Annealing of two oligonucleotides

For cloning of the perfect miR-650 binding site, two complementary oligonucleotides (shown in Table 2.6) were designed that contained the miR-650 target region and when annealed produced overhangs complementary to those created by SalI and NheI digest of the pmiR-GLO vector. Furthermore, an internal NotI restriction enzyme site was inserted for confirmation. Both oligonucleotides were diluted to a final concentration of 1 μ g/ μ l in nuclease-free water. Then 2 μ l of each oligonucleotide were combined with 46 μ l of Oligo Annealing Buffer supplied with the pmiR-GLO vector (Promega) and heated at 90°C for 3 min. In order to allow annealing, the mixture was transferred to a 37°C water bath for 15 min. For ligation, annealed oligonucleotides were diluted 1:10 with nuclease-free water to a final concentration of 4 ng/ μ l.

Table 2.6: *Complementary oligonucleotides for cloning of miR-650 binding site.*

Oligo name	Sequence
miR-650 FWD	CTAGCTAGCGGCCGCTAGTgtcctgagagcgtgcctcctG
miR-650 REV	TCGACaggaggcagcgtctcaggacACTAGCGGCCGCTAbG

2.3.9 Preparation of vector for cloning

2 μ g pmiR-GLO vector were digested using the same restriction endonucleases, i.e. SalI and NheI, as for the PCR product and the reaction setup is shown in Table 2.7. After 60 min incubation at 37°C the reaction was stopped by heating the mixture to 80°C for 15 min. In order to prevent self-ligation digested vector was dephosphorylated using the Antarctic Phosphatase (NEB) (See Table 2.7). The mixture was incubated at 37°C for 30 min and the reaction was inactivated by incubation at 65°C for 15 min. The vector was run on an agarose gel to separate uncut from cut vector and subsequently purified using the Gel Extraction kit (Qiagen) according to manufacturer's instructions.

Table 2.7: Preparation of *pmiR-GLO* vector for cloning.

Component	Volume
Plasmid	2 μ g
10 $\times$ buffer H	5 μ l
BSA	0.5 μ l
NheI	1 μ l
SalI	1 μ l
Water	up to 50 μ l
Total	50 μ l
	60 min at 37°
	15 min at 80°
Phosphatase buffer 10 $\times$	6 μ l
Phosphatase	1 μ l
Water	3 μ l
Total	60 μ l
	30 min at 37°
	15 min at 65°

2.3.10 Ligation

Digested and purified PCR product was cloned into the digested, dephosphorylated and purified *pmiR-GLO* vector using standard T4 ligase (NEB). This was achieved by mixing 100 ng of digested, dephosphorylated and purified vector with PCR product at a molar ratio of 1:3 (or alternatively 2 μ l of annealed oligonucleotides) and adding 1 μ l buffer, 0.5 μ l T4 ligase and in a final volume of 10 μ l. The reaction mixture was incubated at 16°C for 3 hrs before being transformed into competent *E.coli*.

2.4 Bacterial culture

2.4.1 Bacterial strains

Different *E.coli* strains used for transformation:

- Library efficiency DH5 α (Invitrogen):

Genotype: F- φ 80lacZ Δ M15 Δ (lacZYA-argF)U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ -.

- XL-10 Gold (Stratagene):

Genotype: Tetr Δ (mcrA)183 Δ (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacIqZ Δ M15 Tn10 (Tetr) Amy Camr].

2.4.2 Transformation of competent *E.coli*

Competent cells were thawed on ice. For XL-10 Gold bacteria 2 μ l β -mercaptoethanol was added to 50 μ l competent cells for 10 min with gentle shaking every 2 min. Then 1 μ g of plasmid or 2 μ l of ligation mixture were added to 50 μ l bacteria and gently mixed. The mixture was incubated on ice for 30 min, heat shocked at 42°C for 45 sec and immediately transferred onto ice for another 2 min. The bacteria were then added to 1 ml of SOC medium without antibiotics that had been pre-warmed to 37°C. After allowing the bacteria to grow at 37°C for 1 hr with shaking, 50 μ l were plated on LB agar plates containing 100 μ g/ml ampicillin and incubated at 37°C overnight.

2.4.3 Starter culture preparation

After allowing colonies to grow, plates were stored at 4°C for up to one month. Single bacterial colonies were selected and used to inoculate 10 ml of LB media containing 100 μ g/ml selective antibiotic and cultured overnight shaking at 225 rpm at 37°C.

2.4.4 Extraction of bacterial plasmids

Bacterial cultures were centrifuged at 2,000 rpm for 5 min to pellet bacterial cells. The bacterial plasmid were extracted from *E.coli* using the QIAprep Spin Miniprep kit (Qiagen) according the manufacturer's instructions. Finally, 50 μ l EB buffer were added to the spin column and allowed to incubate for 1 min at RT before being centrifuged at 13,000 rpm for 1 min to elute the DNA. The quantity and quality of the eluted DNA was assessed using the Nanodrop 2100. For greater quantities of DNA, the starter culture was transferred to 2.5 l LB media, grown overnight and the DNA was extracted using the Plasmid Mega kit (Qiagen).

2.4.5 Confirmation of insert

Successful ligation was firstly confirmed by excising the insert using the same restriction enzymes, SalI and NheI, used for ligation. Furthermore, inserts were sequenced by the WIMM core sequencing facility (University of Oxford) using an ABI sequencer.

In order to confirm successful amplification of the HIV-1_{NL4.3-BaL} plasmid a restriction digest, using BamHI and SalI (NEB), was performed. Digestion gave rise to two different size fragments which were visualised by gel electrophoresis.

2.5 Cell culture

2.5.1 Cell lines and culture conditions

All cells were cultured in humidified incubators with 5% CO₂. HEK293T cells were obtained from CRUK cell bank and grown in D10. MDDCs and PBMCs isolated from human blood were cultured in R10. Cytokines and phytohaemagglutinin (PHA) were added as described. Cultured cell lines were routinely tested for mycoplasma infection.

2.5.2 Frozen storage of cells

For long-term storage, cells were pelleted, resuspended in freezing medium (90% FCS and 10% DMSO) and transferred into cryovials which were placed into isopropanol freezing pots and stored at -80°C. In order to recover frozen cells, they were rapidly thawed at 37°C resuspended in FCS and spun at 1,500 rpm for 5 min. Finally, cells were resuspended in the appropriate medium and cultured as described.

2.5.3 Isolation of human primary blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were extracted from leukocyte-enriched human blood supplied by the National Blood Service in the form of buffy coats. Donor blood was combined with warm RPMI at a ratio of 1:6, underlayered with 15 ml of Lymphoprep and centrifuged for 25 min at 2,000 rpm without brake. The mononuclear cell layer was harvested from the interface between the higher density Lymphoprep and the lower density blood-RPMI mixture using Pasteur pipettes. The PBMCs were subjected to three 10 min washes with ice-cold RPMI at decreasing speeds (1,800 rpm, 1,500 rpm, 1,100 rpm) and used for the generation of monocyte-derived dendritic cells or CD4⁺ T cell enrichment.

2.5.4 Generation of human monocyte-derived dendritic cells (MDDCs)

CD14⁺ cells were positively selected from PBMCs by magnetic bead separation. 10⁹ PBMCs were resuspended in 2.75 ml ice-cold MACS buffer and 250 µl CD14 MicroBeads (Miltenyi Biotec) were added. After a 20 min incubation at 4°C the cells were washed with 30 ml cold MACS buffer (1,200 rpm for 10 min at 4°C) to remove unbound beads and resuspended in 3 ml of MACS buffer. An LS MACS column with a pre-filter column was placed in the magnetic field of a MACS separator, and rinsed with 3 ml MACS buffer. The cell suspension

was added onto the column and unlabelled cells were collected in the effluent. The column was washed three times with 3 ml MACS buffer to remove all unbound cells. Then the column was removed from the magnetic field, 5 ml of MACS buffer was added to the column and labelled cells were flushed out immediately by depression of a plunger. Cells were washed with 30 ml cold RPMI, plated out in R10 at a concentration of 10^6 cells/ml and cultured for 5 days in the presence of 40 ng/ml recombinant IL-4 and 40 ng/ml recombinant GM-CSF (Preprotech). At day 3, 5 ml of fresh R10, 40 ng/ml GM-CSF and 40 ng/ml IL-4 were added to each plate. Immature MDDCs were harvested at day 5.

2.5.5 CD4⁺ T cell enrichment

PBMCs were blasted by adding 10 μ g/ml phytohemagglutinin (PHA) and CD4⁺ T cells were used 3 days post-stimulation. Enrichment was performed by negative selection using the CD4⁺ T Cell Isolation kit II (Miltenyi). Briefly, 10^7 PHA-blasted PBMCs were resuspended in 40 μ l MACS buffer and 10 μ l of Biotin-Antibody Cocktail were added. Cells were incubated for 10 min at 4°C before addition of 30 μ l Anti-biotin MicroBeads. After incubating the mixture for an additional 15 min at 4°C, cells were washed with 30 ml MACS buffer and spinning at 12,000 rpm for 10 min. In the meantime, an LC MACS column was placed in the magnetic field and prepared by rinsing with 3 ml of MACS buffer. The cell pellet was resuspended in 500 μ l MACS buffer and the suspension was applied onto the MACS column. The column was washed three times by adding 3 ml of MACS buffer and allowing the entire column to empty completely each time. The entire effluent was collected as this fraction represents the enriched CD4⁺ T cells. The collected CD4⁺ T cells were washed with RPMI (12,000 rpm for 10 min at 4°C) and cultured in large tissue culture flasks at a concentration of 10^6 cells/ml R10.

2.5.6 Stimulation of human monocyte-derived dendritic cells

For DC stimulation with PRR agonists, 2×10^6 cell DCs were resuspended in 1 ml R10 and seeded into 12-well plates. Cells were stimulated with various PRR ligands (Invivogen); concentrations listed in Table 2.8. Generally DCs were stimulated for 24 hrs unless stated otherwise.

Table 2.8: *Concentration of PRR agonists used for MDDC stimulation.*

TLR	Ligand	Concentration
TLR1/2	Pam ₃ CSK ₄	1 μ g/ml
TLR2	HKLM	10 ⁸ cells
TLR3	Poly(I:C)	25 μ g/ml
TLR4	LPS	1 μ g/ml
TLR5	Flagellin	1 μ g/ml
TLR6/2	FSL-1	1 μ g/ml
TLR7	Imiquimod	10 μ g/ml
TLR8	ssRNA40/LyoVec	10 μ g/ml
TLR9	ODN2006	3 μ M
RIG-I	Poly(I:C)/LyoVec	10 μ g/ml
NOD2	MDP	10 μ g/ml
Control	ssRNA41/LyoVec	10 μ g/ml
Control	LyoVec	10 μ g/ml

2.5.7 Small molecule inhibitors

Chloroquine, meanserin hydrochloride, or paranthenolide were obtained from Sigma. Where it was used, DCs were pre-incubated for 30 min with 50 nM chloroquine, 30 μ g/ml mianserin hydrochloride, or 10 μ M paranthenolide 30 min prior to stimulation with ssRNA40.

2.5.8 Dendritic cell nucleofection

Transient transfection of DCs was performed using the human DC Nucleofector kit (Amaxa). 3×10^6 DCs were resuspended in 100 μ l human dendritic cell transfection solution and mixed with 5 μ l miRNA inhibitor or mimic (50 μ M stock solution) (Applied Biosystems). The mixture was transferred to a cuvette and electroporated using program U-02. Warm R10 was added to the cuvette immediate after nucleofection and cells were transferred to a 6-well plate containing 3 ml of R10 giving final concentration of miRNA inhibitor or mimic of 82.5 nM. Cells were incubated for 24 hrs to allow for target gene expression or knockdown before experiments were performed.

2.5.9 Surface staining of cells for flow cytometry

The required amount of cells were washed in cold PBS. Dead cells were stained using the Violet LIVE/DEAD Cell Stain (Invitrogen) according to the manufacturer's instructions. Briefly, cells were resuspended in 1 ml PBS + 1 μ l LIVE/DEAD Cell Stain and incubated for 15 min at RT. Cells were washed with PBA and resuspended in a minimal amount of PBA.

Cell surface staining was performed at 4°C for 30 min with directly conjugated antibodies against various surface markers (eBioscience or BD Biosciences). Unbound antibody was removed by washing with cold PBA and cells either underwent intracellular staining or were fixed in 200 μ l FACS fix solution. For intracellular staining, cells were permeabilised in 100 μ l Fix/Perm solution (BD Biosciences) at 4°C for 20 min and washed with 1 ml 1 $\times$ Perm/Wash buffer. Then cells were stained with antibody for 20 min at 4°C washed with 1 ml 1 $\times$ Perm/Wash buffer and fixed in 200 μ l 1% PFA. Samples were run on a Cyan flow cytometer (Dako) and data was analysed using FlowJo.

2.5.10 Luminex cytokine assay

On day 5, 3×10^6 DCs were transfected with miRNA mimic and cultured for 24 hrs. Then DCs were harvested, resuspended in 1.5 ml R10 and stimulated with ssRNA40 (10 μ g/ml). Supernatants were collected 24 hrs post-stimulation and cytokine concentrations were measured. A 17-plex human cytometric (Luminex) bead immuno-assay was performed using a 17-plex human cytokine kit (Bio-Rad), according to the manufacturer's instructions. Data were extracted using a Bio-Plex 200 reader.

2.5.11 Transient transfection of HEK293T cells

pmiR-GLO vector and miRNA mimics were co-transfected in a 96-well plate using Lipofectamine 2000 (Invitrogen). A reverse transfection protocol was chosen. For each sample well, 20 ng pmiR-GLO vector and 3.75 pmol miRNA mimic (25 nM final concentration) were added to 25 μ l Opti-MEM (Invitrogen). In a separate tube 0.3 μ l Lipofectamine 2000 was added to 25 μ l Opti-MEM and incubated for 5 min at RT. After 5 min incubation, the two mixtures were combined, mixed and incubated for 30 min at RT. In the meantime, HEK293T cells, which were 70-80% confluent on the day of transfection, were harvested, counted and resuspended in D10 without antibiotics. After 30 min, 10^4 HEK293T cells in a volume of 100 μ l D10 were added to the transfection mixture and cultured for 48 hrs before measuring the luciferase expression.

2.6 RNA analysis

2.6.1 Extraction of mRNA

DCs were harvested, washed once with cold PBS and pelleted in an Eppendorf tube. For mRNA analysis, RNA was extracted using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Homogenisation of uninfected cells was performed with a needle (0.8 mm) whereas HIV-1 infected cells were lysed in the Cat.3 laboratory using QIAshredder columns (Qiagen). RNA was eluted in nuclease-free water and stored at -80°C until further use.

2.6.2 Extraction of total RNA

As the RNeasy kit is selective for longer RNA transcripts. Hence for miRNA analysis, total RNA was extracted using the miRNeasy kit (Qiagen) according to manufacturer's specifications with one modification: the chloroform purification step was performed twice to remove any residual phenol. Furthermore, as described above, uninfected cells were homogenised using a needle (0.8 mm) and HIV-1-infected cells were lysed in the Cat.3 laboratory using QIAshredder columns (Qiagen). Total RNA was eluted in nuclease-free water and RNA was stored at -80°C until further use.

2.6.3 RNA quality control

RNA concentration was assessed by UV spectrophotometry using the NanoDrop ND-800 spectrophotometer, using $1.2\mu\text{l}$ of sample. The purity was assessed by the $260\text{nm}/280\text{nm}$ absorbance ratio, which indicates protein contamination, and the $230\text{nm}/280\text{nm}$ absorbance ratio, which indicated salt and phenol contamination. In addition, samples for microarray analysis were subjected to microelectrophoretic analysis using the Agilent RNA 6000 Nano Gel kits on an Agilent Bioanalyzer 2100. This determines the RNA quality by calculating an RNA integrity number (RIN) number for each sample.

2.6.4 Gene expression microarray analysis

DCs were transfected with miRNA mimic and RNA was extracted 24 hrs post transfection using the RNeasy kit as described above. Following quality control only samples which satisfied the following criteria were taken further for microarray analysis: $260/280 \geq 1.8$, $230/280 \text{ ratio} \geq 1.5$ and RNA integrity number (RIN) ≥ 7

Total RNA was amplified and labelled using the GeneChip WT Terminal Labelling kit (Affymetrix) and hybridised to Affymetrix Human Gene 1.0 ST Arrays (Sample labelling I performed myself and hybridisation was done under the guidance of Dr. Dilair Baban at the Wellcome Trust Centre for Human Genetics (University of Oxford)).

A total of 2 μg RNA and the appropriate amount of GeneChip Eukaryotic Poly-A RNA Spike-in controls were reverse transcribed to double stranded cDNA in two separate steps (1st and 2nd strand cDNA synthesis). The transcripts were amplified by *in vitro* RNA synthesis. The cRNA yield and size distribution was confirmed by microelectrophoresis using the Bioanalyzer 2100. The cRNA was then purified and reverse transcribed back into cDNA, fragmented and labelled the by terminal deoxynucleotidyl transferase (TdT). Each chip was incubated with 10 μg fragmented cDNA for 16 hrs at 45°C in a hybridisation chamber with rotation at 60 rpm. Unhybridised probes were removed and fluorescent images were captured using the Gene Array Scanner 3000 (Affymetrix). It was confirmed that all quality controls met the Affymetrix quality assessment guidelines. In addition, on-chip normalization was performed to allow comparison between different arrays and the resulting raw data was analysed using Genespring GX software. Statistical analysis was performed using a two-way ANOVA test with Bonferroni adjustment.

2.6.5 miRNA profiling by microarray

DCs were infected with HIV-1 or left uninfected and RNA was extracted at 2 hrs or 24 hrs post-infection using the miRNAeasy kit as described above. Following quality control only samples which satisfied the following criteria were taken further for microarray analysis: $260/280 \geq 1.8$, $230/280 \text{ ratio} \geq 1.5$ and RNA integrity number (RIN) ≥ 8 . Microarray analysis was carried out in collaboration with Oxford Gene Technology, first using a custom designed array platform with a dual colour setup whereby RNA from infected and uninfected cells was labelled with different dyes. Repeat analysis was performed using the Agilent miRNA microarray with a modified dual colour setup; each sample was labelled with the same dye and the second dye was used for a 'spike-in' RNA normalisation control. On-chip normalisation was performed and the raw data was analysed using Genespring GX software. Statistical analysis was performed using a two-way ANOVA test with Bonferroni adjustment.

2.6.6 DNase treatment

For quantitative real-time PCR analysis, some RNA samples were treated with the DNA-free kit (ABI) to remove genomic DNA contamination. This was only necessary if primers

amplified sequences from genomic DNA. Briefly, 1.5 μg RNA were incubated with 1 μl DNase I enzyme and 10 $\times$ buffer in a final volume of 22.5 μl for 20 min at 37°C. DNase I was inactivated by addition of 2.5 μl inactivation reagent for 2 min with occasional mixing. The inactivation reagent was pelleted by centrifugation at 13,000 rpm for 2 min. The supernatant was taken off and 9 μl were used for reverse transcription.

2.6.7 Measuring mRNA expression by Taqman quantitative PCR

A total of 500 ng RNA were reverse transcribed in a total volume of 20 μl using the High Capacity RNA-to-cDNA kit (ABI) which contains random hexamer primers and MultiScribe reverse transcriptase following the manufacturer's instructions. Taqman PCR primers were obtained from ABI and where possible primers were used which were shown not to amplify genomic DNA. Real-time quantitative PCR was performed in triplicates on the fast ABI 7500 system using the standard cycling conditions for 40 cycles. Each reaction contained 25 ng cDNA starting material, 10 μl Gene expression master mix (ABI) and 1 μl Taqman primer in a total volume of 20 μl . The efficiency of each Taqman primer set was assessed by performing serial dilution experiments. In each instance, gene expression was normalised using GAPDH as an endogenous control. Fold changes were calculated using the $\Delta\Delta C_t$ method:

$$\text{Relative expression ratio} = \frac{\{2^{-(C_t \text{GENE} - C_t \text{GAPDH})}\}_{\text{treated sample}}}{\{2^{-(C_t \text{GENE} - C_t \text{GAPDH})}\}_{\text{control sample}}}.$$

2.6.8 Measuring miRNA expression by Taqman quantitative PCR

Each miRNA was reverse transcribed from 10 ng total RNA in an individual reaction using a miRNA-specific RT primer and the miRNA Reverse Transcriptase kit (ABI). Taqman PCR was performed in triplicates as described above. In each instance, miRNA expression was normalized against the endogenous control RNU48 and relative miRNA expression was calculated using the $\Delta\Delta C_t$ method.

2.7 Protein biochemistry

2.7.1 Cell lysis

10^6 cells were lysed in 100 μ l 0.5% NP-40 lysis buffer supplemented with protease inhibitors (CompleteMini tablets, Roche) for 30 min on ice and cell debris was pelleted at 13,000 rpm for 10 min at 4°C. The protein concentration in the supernatant was determined by Bradford assay (Bio-Rad) and samples were stored at -80°C until further use.

2.7.2 Immunoblotting

Whole cell lysates containing 40 - 80 μ g protein were boiled for 10 min in 4 $\times$ NuPAGE LDS loading buffer (Invitrogen) and cooled on ice. 20 μ l sample was then loaded onto pre-cast 4-12% NuPAGE Bis-Tris gels and run at 160 V in NuPAGE MOPS running buffer. One well in each gel was loaded with 10 μ l of Full Rainbow Molecular Weight Marker (Amersham Biosciences). The proteins separated by SDS-page were electrophoretically transferred onto a PVDF membrane (Hybond-P, Amersham Biosciences) in NuPAGE transfer buffer at 20 V for 1 hr using a wet transfer XCell II Blot Module (Invitrogen). Membranes were blocked in 0.5% Tween/PBS supplemented with 5% milk powder for 1 hr at RT. Then membranes were incubated primary antibody, diluted in 5 ml 0.5% Tween/PBS according to manufacturer's recommended concentrations, overnight at 4°C. Membranes were washed three times for 10 min with 0.5% Tween/PBS before addition of the species-specific secondary antibody conjugated to horseradish peroxidase (HRP), diluted in 0.5% Tween/PBS supplemented with 5% milk powder, for 1 hr at RT. Finally, membranes were washed three times for 10 min with 0.5% Tween/PBS. All incubations were carried out with gentle agitation. Equal volumes of ECL Western Blotting Detection reagents (Amersham Biosciences) were mixed and applied to the membrane. Chemiluminescence was detected using photographic paper (Kodak) and a Xograph (Imaging system) developer.

For fluorescent detection the blocking step and all antibody incubations were performed in LI-COR blocking buffer. In addition, the last two wash steps before detections were performed with TBS without Tween. Membranes were allowed to dry and were detected using the Odyssey Western Blot Scanner (LI-COR).

2.8 Immunofluorescence and confocal microscopy

Treated DCs were transferred onto Poly-L lysine coated slides (BD Bioscience) at 200,000 cells/well and allowed to adhere for 1 hr. Cells were then fixed in 4% PFA for 10 min and permeabilised in 0.1% Triton for 7 min. Each well was washed three times with PBS + 2% BSA (PBA) before incubation with primary antibodies diluted in 100 μ l PBA overnight at 4°C. Finally, cells were washed three times with PBA and incubated with secondary antibody for 1 h at RT. Finally, cells were washed again with PBA and nuclei were stained by adding To-Pro far red (1:4,000 dilution) (Invitrogen) for 10 min. Slides were prepared with Vectashield without Dapi (Vector) and visualised under the confocal microscope (Bio-Rad).

2.9 Dual luciferase assay

HEK293T cells were co-transfected with pmir-GLO vector and miRNA mimic and luciferase activity was measured 48 hrs post-transfection by Dual-Luciferase Reporter Assay System (Promega). Culture supernatants were removed, cells were washed once with PBS and 20 μ l lysis buffer were added. After incubation for 15 min at RT with gentle agitation, lysates were frozen at -80°C to ensure complete lysis. After thawing, 15 μ l cell lysate were transferred to 96-well luminescence microplate (Falcon). In order to measure firefly luciferase activity, 50 μ l LARII substrate were added and luminescence was measured using the Fusion Instrument (Packard). After this measurement was completed, 50 μ l Stop and Glo reagent was added to each well. This quenched the firefly luciferase reaction and allowed quantification of renilla luciferase activity. Firefly luciferase activity was normalized against renilla activity. Reactions were carried out in quadruplicate and significance was determined by an unpaired t-Test.

2.10 Virology

2.10.1 Infection of monocyte-derived DCs with Influenza A virus

Influenza A/PR/8/34 stock (4,000 HAU/ml) was kindly provided by Prof. John McCauley (NIMR, London) and was stored at -80°C. 10^6 DCs were resuspended in 100 μ l RPMI supplemented with 2 μ g Trypsin (Sigma). Influenza A/PR/8/34 were added to cells and incubated together for 2 hrs at 37°C. After 2 hrs cells were washed twice with RPMI and resuspended in R10 at a concentration of 10^6 cells/ml for further culture.

2.10.2 Generation and of HIV-1 stock

HEK293T cells were grown until 70% confluent. 30 μ g HIV-1_{NL4.3-BaL} proviral plasmid was added to 1 ml of sterile 250 mM CaCl₂ solution and incubated at room temperature for 10 min. The mixture was added dropwise to 1 ml 2 $\times$ Hepes buffer pH 7.12 while vortexing constantly and incubated at RT for 20 min. The solution was then added dropwise to the tissue culture plate which was carefully moved from side to side to ensure thorough mixing of the transfection solution with the medium. Cells were incubated at 37°C for 6 hrs before the transfection medium was replaced with fresh D10. Virus was harvested 24 hrs and 48 hrs post transfection, filtered (0.45 μ m Milipore filter) and purified over a sucrose gradient by ultracentrifugation according to [Kutner *et al.*, 2009]. Virus was resuspended in PBS and stored in small aliquots at -80°C. Viral concentration was assessed by reverse transcriptase activity (QuanT-RT, GE Healthcare) and p24 Elisa. TCID₅₀ (50% Tissue Culture Infective Dose) and MOI (Multiplicity of Infection) were determined by titrating the virus using TZM-blue HeLa reporter cells as described in [Richards & Clapham, 2006].

2.10.3 Infection of monocyte-derived DCs with HIV-1

10⁶ DCs were resuspended in 100 μ l R10 and 50 ng HIV-1 were added. This corresponds to an MOI of 1. After 2 hrs incubation at 37°C cells were washed twice with RPMI and resuspended in R10 at a concentration of 10⁶ cells/ml for further culture. Supernatants were sampled for reverse transcriptase activity using the QuanT-RT kit (GE healthcare)

2.10.4 Setting up DC-T cell co-culture

10⁶ DCs were infected as described above for 2 hrs at 37°C. Cells were washed twice with R10 and 3 $\times$ 10⁶ PHA treated autologous CD4⁺ T cells were added. The cell mixture was cultured in 1 ml R10 in a 12-well plate and supernatants were sampled regularly for reverse transcriptase activity using the QuanT-RT kit (GE Healthcare)

2.10.5 Measuring RT activity in culture supernatants

Culture supernatants were collected and frozen at -80°C until further use. Samples were thawed and remaining cells were pelleted by spinning at 5,000 rpm for 5 min. 70 μ l supernatants were used for QuanT-RT assays (GE Healthcare) which were performed according to the manufacturer's specifications.

Table 2.9: *Primers for HIV-1 tat-rev PCR.*

Primer name	Sequence
Tat-rev FWD	ATGGCAGGAAGAAGCGGAG
Tat-rev REV	ATTCCTTCGGGCCTGTCG
GAPDH FWD	CCATGTTTCGTCATGGGTGTG
GAPDH REV	GGTGCTAAGCAGTTGGTGGTG

2.10.6 Measuring early viral gene transcription

RNA was extracted at 6 hrs and 24 hrs using the RNeasy kit (Qiagen), subjected to DNase digest and was reverse transcribed as described (all as described above). Early viral tat-rev transcripts and GAPDH were amplified by SYBR green quantitative PCR (SYBR green Master Mix from ABI) using primers published in [Gringhuis *et al.*, 2010] (Table 2.9). A melting curve was generated to ensure amplification of a single product. Finally, gene expression was normalised to GAPDH and relative quantification was performed using the $\Delta\Delta C_t$ method.

2.10.7 Statistical analysis

Technical replicated are shown as mean $\pm$ SD and biological replicates as mean $\pm$ SEM.

Statistical analysis was performed using a t-test (paired or unpaired) in GraphPad Prism. Statistical significance is shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ NS not significant.

Chapter 3

MicroRNA profiling in HIV-1-infected dendritic cells

3.1 Introduction

Gene silencing by miRNAs, as described earlier (see Section 1.3), is a post-translational regulatory mechanism that effects various cellular processes by fine-tuning gene expression. As HIV-1 relies heavily on the host cellular machinery for its survival and propagation, it is highly susceptible to changes in host gene expression. Although research is still in its infancy, evidence is emerging which indicates that cellular miRNAs may play an important role in HIV-1 pathogenesis [Sun & Rossi, 2011].

An early study by Triboulet *et al.* demonstrated that silencing of the cellular RNAi machinery, by knocking down Dicer and Drosha, increased the recovery of infectious viral particles from PBMCs isolated from HIV-1 positive patients [Triboulet *et al.*, 2007]. Nathan *et al.* later confirmed these findings by showing that disruption of P-body formation increases HIV-1 replication in single-round *in vitro* infection assays [Nathans *et al.*, 2009]. In accordance with the idea that the RNAi pathway has evolved as a cellular defence strategy against invading pathogens, HIV-1 was thought to inhibit this antiviral mechanism by encoding two global RNAi suppressors: the Tat protein which has been shown to inactivate Dicer *in vitro* [Bennasser *et al.*, 2005] and the TAR element which binds to TRBP making it unavailable for incorporation into the RISC loading complex [Bennasser *et al.*, 2006].

However, the capacity of HIV-1-encoded RNAi suppressors to hinder miRNA biogenesis has recently been challenged by a separate study which demonstrated that HIV-1 Tat is unable to globally inhibit miRNA production or induce the accumulation of pre-miRNAs as

would be predicted for inhibitors of Dicer [Lin & Cullen, 2007]. More importantly, HIV-1 infection of CD4⁺ T cells does not lead to a global decline in miRNA expression but results in differential regulation of miRNA expression [Triboulet *et al.*, 2007] [Yeung *et al.*, 2005]. Accumulating evidence suggests that alterations in the miRNA profile and the subsequent effects on gene expression may affect the balance of cofactors and restriction factors, thus impacting indirectly on several stages of the HIV-1 life cycle [Ouellet *et al.*, 2009]. In addition, selected miRNAs have been shown to target specific sites within HIV-1 transcripts, thus regulating HIV-1 replication in a direct manner. The complex role of cellular miRNAs in the life cycle of HIV-1 is only beginning to be explored [Sun & Rossi, 2011] and Tables 3.1 and 3.2 summarise the key findings so far.

Decreased miR-17 and miR-20a expression support HIV-1 replication in CD4⁺ T cells

As mentioned in Chapter 1.2, CD4⁺ T lymphocytes are the main targets for HIV-1 infection and readily support viral replication. A recent study shows that HIV-1 infection of Jurkat T cells decreases the expression of miR-17 and miR-20 by an as yet unknown mechanism [Triboulet *et al.*, 2007]. Both miRNAs are members of the miR-17/92 cluster and directly regulate the expression of the histone acetylase P300/CBP-associated factor (PCAF), an essential cofactor of the Tat transactivator. Over expression of miR-17 and miR-20 in PBMCs of HIV-1-infected donors inhibited the expression of PCAF, thus limiting viral replication. More importantly, restoration of PCAF expression using cDNA vectors lacking the 3'UTR sequence was sufficient to relieve miRNA-mediated suppression of HIV-1 replication [Triboulet *et al.*, 2007]. This study suggests that HIV-1 modulates miRNA expression in CD4⁺ T cells to create a favourable cellular environment, thus enhancing propagation.

Table 3.1: Cellular miRNAs targeting HIV-1 mRNAs.

miRNA	Target sites	Comments	Reference
miR-149 miR-324-5p miR-378	vpr vif vpu	- Target sites identified by <i>in silico</i> target prediction. - Not experimentally validated.	[Hariharan <i>et al.</i> , 2005]
miR-29a miR-29b	nef (3'LTR)	- Target site identified by <i>in silico</i> target prediction. - miR-29a and miR-29b inhibit Nef expression and HIV-1 replication in HEK293T and Jurkat cells. - miR-29a targets viral mRNA to P bodies; disruption of P bodies enhances HIV-1 replication. - miR-29a may provide a checkpoint in viral latency. - Group O and <i>nef</i> deleted HIV-1 contain non conserved nucleotides in seed region and cannot be targeted by miR-29a for transport to P bodies; this may be linked to lower infectivity.	[Hariharan <i>et al.</i> , 2005] [Ahluwalia <i>et al.</i> , 2008] [Nathans <i>et al.</i> , 2009]
miR-28 miR-125b miR-150 miR-223 miR-382	3' end of HIV-1	- More abundant in resting than activated CD4⁺ T cells. - Over expression of these miRNAs enhances viral recovery from HIV-1-infected CD4⁺ T cells from patients on HAART. - Contribute to latent state.	[Huang <i>et al.</i> , 2007b]
miR-28 miR-150 miR-223 miR-382	3' end of HIV-1	- Down regulated upon differentiation of monocytes into macrophages. - Knockdown of these miRNAs in monocytes increases susceptibility to HIV-1 infection. - Control HIV-1 tropism.	[Wang <i>et al.</i> , 2009b] [Swaminathan <i>et al.</i> , 2009]

Table 3.2: Cellular miRNAs targeting host factors involved in HIV-1 replication.

miRNA	Target site	Function and further comments	Reference
miR-17 miR-20a	PCAF	- miR-17 and miR-20a are down regulated in HIV-1 infected CD4⁺ T cells. - Inhibition of miR-17 and miR-20a in Jurkat cells enhances PCAF expression and HIV-1 replication.	[Triboulet <i>et al.</i> , 2007]
miR-198	Cyclin T1	- miR-198 is down regulated upon differentiation from monocytes to macrophages. - Down regulation of miR-198 is implicated in increased susceptibility of macrophages to HIV-1 infection.	[Sung & Rice, 2009]

Anti-HIV-1 miRNAs contribute to the establishment of viral latency

Cellular miRNAs have also been implicated in establishing HIV-1 latency in resting CD4⁺ T lymphocytes. A miRNA profiling study detected that a cluster of cellular miRNAs (mir-28, miR-125b, miR-150, miR-223 and miR-382) was enriched in resting CD4⁺ T cells as compared to activated CD4⁺ T cells [Huang *et al.*, 2007b]. Computational analysis identified putative binding sites for these miRNAs in the 3'UTR of HIV-1 mRNAs. Finally, combined inhibition of these miRNAs using antisense oligonucleotides increased the recovery of virus from resting CD4⁺ T cells isolated from HIV-1-infected individuals on suppressive antiretroviral treatment (HAART) [Huang *et al.*, 2007a].

Similarly, HIV-1 infection of CD4⁺ T cells has been shown to induce the expression of miR-29a, which targets a conserved site in the *nef* region. Over expression of miR-29a in Jurkat T cells suppresses Nef expression, decreases infectivity and reduces viral replication [Ahluwalia *et al.*, 2008]. A further study demonstrates that binding of miR-29a to its target site promotes the accumulation of HIV-1 transcripts in P bodies and leads to translational repression [Nathans *et al.*, 2009]. Storage of viral mRNAs within cellular P bodies may allow the virus to remain latent and to be reactivated under certain stimuli. Hence, the regulation of miR-29a may be an antiviral mechanism, but could also provide a subtle switch between latency and activation.

The role of cellular miRNAs that target sites in the HIV-1 genome and therefore inhibit viral replication is complex. Whereas it may seem logical that miRNA-mediated restriction of HIV-1 replication serves as an effective antiviral mechanism, the above-mentioned studies show that it may in fact be an integral part of viral pathogenesis. Who benefits from antiviral miRNAs *in vivo* - the virus or the host - remains to be elucidated.

The cellular miRNA repertoire dictates HIV-1 tropism

The miRNA repertoire varies greatly among different cell types and may contribute to the cellular tropism of HIV-1. For example, monocytes, which are refractory to HIV-1 infection, harbour higher levels of virus-targeting cellular miRNAs (mir-28, miR-150, miR-223 and miR-382) [Wang *et al.*, 2009b]. Differentiation of monocytes into macrophages is accompanied by a decline in the expression of these four miRNAs and a simultaneous increase in susceptibility to HIV-1 infection. However, it is worth noting that due to the interplay of multiple cellular factors, including the level of co-receptor expression, it remains controversial if these miRNAs alone are responsible for the increase in the cell's permissiveness to HIV-1 infection [Swaminathan *et al.*, 2009].

Differentiation of monocytes into macrophages is also accompanied by a decline in miR-198 expression [Sung & Rice, 2009]. This miRNA is not predicted to bind to viral transcripts. Instead, it regulates the expression of Cyclin T1, an essential cofactor for HIV-1 replication. Cyclin T1 is recruited to the TAR RNA structure by the Tat transactivator and, together with p-TEFb, enhances HIV-1 replication [Liou *et al.*, 2002]. Reduced miR-198 expression may therefore also contribute to the increase in infectivity observed upon differentiation from monocytes into macrophages.

MicroRNA profiling in HIV-1-infected primary human dendritic cells

As described in Chapter 1.2.6, the interaction between HIV-1 and DCs at the site of viral entry is central to viral dissemination and the establishment of persistent infection [Altfeld *et al.*, 2011]. However, the mechanisms by which HIV-1 subverts the function of DCs and the subsequent generation of an effective inflammatory response are not entirely clear.

It is widely accepted that HIV-1 escapes classical PRR-mediated innate immune responses in DCs through a variety of mechanisms [Mogensen *et al.*, 2010] [Yan & Lieberman, 2011]. Firstly, HIV-1 prevents the accumulation of PAMPs, such as cytosolic DNA, and therefore escapes recognition [Yan *et al.*, 2010]. Secondly, HIV-1 is capable of modulating PRR signalling. More specifically, HIV-1 has been shown to trigger TLR8 and DC-SIGN thereby inducing a complex signalling cascade in DCs [Hodges *et al.*, 2007] [Gringhuis *et al.*, 2010]. However, this leads to a gene expression profile different from that observed in classically matured DCs [Hodges *et al.*, 2007].

So far nothing is known about the expression pattern of cellular miRNAs in HIV-1-infected DCs and their potential role in shaping the innate responses against the virus. Given their potential role in antiviral defence as well as in viral escape and latency [Skalsky & Cullen, 2010], we hypothesise that miRNAs could be involved in the complex interaction between DCs and HIV-1.

I therefore sought to explore the impact of HIV-1 infection on the miRNAome of human dendritic cells. Due to the challenge of obtaining sufficient numbers of *ex vivo* isolated DCs from mucosal tissues, monocyte-derived dendritic cells (MDDCs) were chosen for this study. MDDCs have been widely adopted as *in vitro* model culture system to address some of the questions of DC function and HIV pathogenesis [Mallon *et al.*, 1999], as they resemble mucosal DCs in morphology as well as functional characteristics [Sallusto & Lanzavecchia, 1994] [Cella *et al.*, 1997]. The use of primary cells is of great importance for this study as the cellular miRNA repertoire varies significantly across different cell lineages [Petriv *et al.*, 2010].

The work shown in this chapter presents an attempt to detect changes in the miRNAome of monocyte-derived DCs in response to HIV-1 infection by using a microarray-based profiling method. Furthermore, it aims to distinguish between HIV-1-specific changes and those induced during DC maturation, by comparing the findings from the array study to miRNA expression in DCs stimulated with the TLR8 ligand ssRNA40, a short RNA fragment derived from the HIV-1 LTR, which has been shown to mature DCs [Heil *et al.*, 2004] [Alter *et al.*, 2007].

3.2 Aims

Aims of this chapter:

- To setup and characterise HIV-1 infection of DCs *in vitro*.
- To investigate whether exposure of DCs to HIV-1 alters the cellular miRNA expression profile using a microarray-based profiling method.
- To validate array findings using miRNA-specific quantitative PCR.
- To investigate differences between HIV-1-induced changes and miRNA expression in DC stimulated with the TLR8 ligand ssRNA40.

3.3 Results

3.3.1 Characterisation of *in vitro* HIV-1 infection of DCs

In preparation for miRNA profiling, I first sought to characterise the kinetics of HIV-1 replication in DCs by tracing the fate of captured virus over time. Initial experiments were performed using a variety of different techniques, including immunoblotting for viral p24 gag protein, qRT-PCR of early viral gene transcripts and quantification of reverse transcriptase activity in culture supernatants as a proxy for newly emerging virus (Figure 3.1).

As mucosal DCs encounter transmitted virus isolates, an HIV-1 strain (HIV-1_{NL4.3-BaL}) expressing M-tropic Env, capable of binding to the CCR5 co-receptor, was used. This viral clone was generated using the backbone of T-tropic HIV-1_{NL4.3} and replacing the envelope protein with that of the M-tropic HIV-1_{BaL} isolate [Fackler *et al.*, 2001]. At Day 5, immature DCs were harvested and 3×10^6 cells were resuspended in 300 μ l R10. DCs were challenged with HIV-1_{NL4.3-BaL} at a concentration of 50 ng p24 gag/ 10^6 DCs. This corresponds to

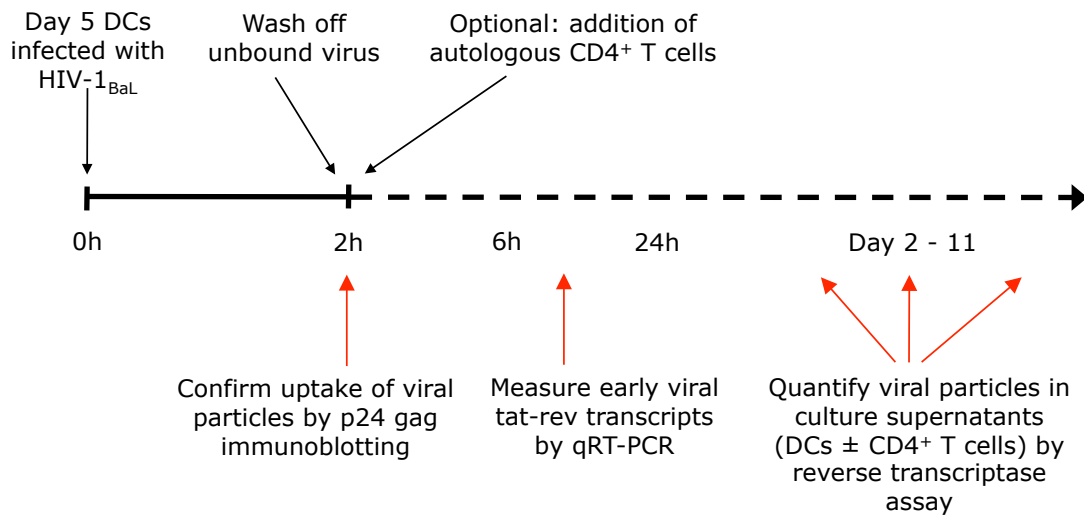


Figure 3.1: Time course of HIV-1 infection *in vitro*. 10^6 DCs were resuspended in $100\ \mu\text{l}$ R10 and infected with HIV-1_{NL4.3-BaL} (50 ng p24 gag) for 2 hrs. Unbound virus was washed off and DCs were resuspended in 3 ml R10. For co-culture experiments PHA-blasted CD4⁺ T cells were added after the wash step. Uptake of viral particles was confirmed by immunoblotting for HIV-1 p24 gag 2 hrs post-infection. Early viral tat-rev transcripts were measured by qRT-PCR at 6 hrs and 24 hrs post-infection. Newly produced viral particles were quantified in culture supernatants using an HIV-1-specific reverse transcriptase assay.

a multiplicity of infection (MOI) of 1 which is in accordance with the work published by other groups [Gringhuis *et al.*, 2010] [Blanchet *et al.*, 2010]. Any remaining unbound virus was washed off after 2 hrs and DCs were resuspended at 10^6 cells/ml R10 for further culture. Removal of the virus after an initial infection period is thought to mimic the transient exposure occurring *in vivo*.

3.3.2 Confirming the ability of DCs to capture and retain infectious HIV-1 particles

In order to confirm uptake of viral particles within the first 2 hrs of exposure, DCs were harvested 2 hrs post-infection, washed and lysed. Cell lysates were analysed for viral p24 gag by immunoblotting. Figure 3.2(A) shows that HIV-1 p24 gag can be detected in infected DCs at the time the virus is washed off. This confirms that DCs successfully capture HIV-1 virions during the initial exposure period.

Previous work has shown that viral particles captured by DCs can be retained for several days and transmitted to $CD4^+$ T cells in trans via formation of an infectious synapse [Pope *et al.*, 1994] [Jolly *et al.*, 2004]. In order to confirm whether the captured virus remains infectious and can be transmitted to $CD4^+$ T cells, an *in vitro* co-culture system was set up. Briefly, DCs were infected with HIV-1 as described above and stringently washed after 2 hrs. After removing unbound viral particles, autologous $CD4^+$ T cells were added at a ratio of 1:3. $CD4^+$ T cells were purified from PHA-activated PBMCs using negative magnetic bead isolation. Culture supernatants were tested for HIV-1 reverse transcriptase activity as a measure of newly emerging viral particles. This assay is based on the ability of HIV-1 reverse transcriptase to reverse transcribe an artificial ssRNA primer, thereby incorporating H^3 -labelled thymidine which provides a radioactive readout.

Figure 3.2(B) displays the replication kinetics of two different donors. For HIV-1-infected DCs, Figure 3.2(B) shows that an increase in reverse transcriptase activity can be expected around day 4. The addition of $CD4^+$ T cells to HIV-1-infected DCs significantly accelerated the appearance of viral particles in the supernatant to day 1-2, and resulted in higher viral titres compared to HIV-1-infected DCs alone. To demonstrate that this effect is due to trans infection, $CD4^+$ T cells were infected with HIV-1 directly. As expected, this was less efficient, showing similar kinetics as in the case of DCs alone, except that the rise in reverse transcriptase activity appears between days 2-4. This observation is consistent with other studies suggesting that infection of DCs is inefficient, and that captured HIV-1 particles can be retained in a specialised compartment from which they are recycled back to the cell surface

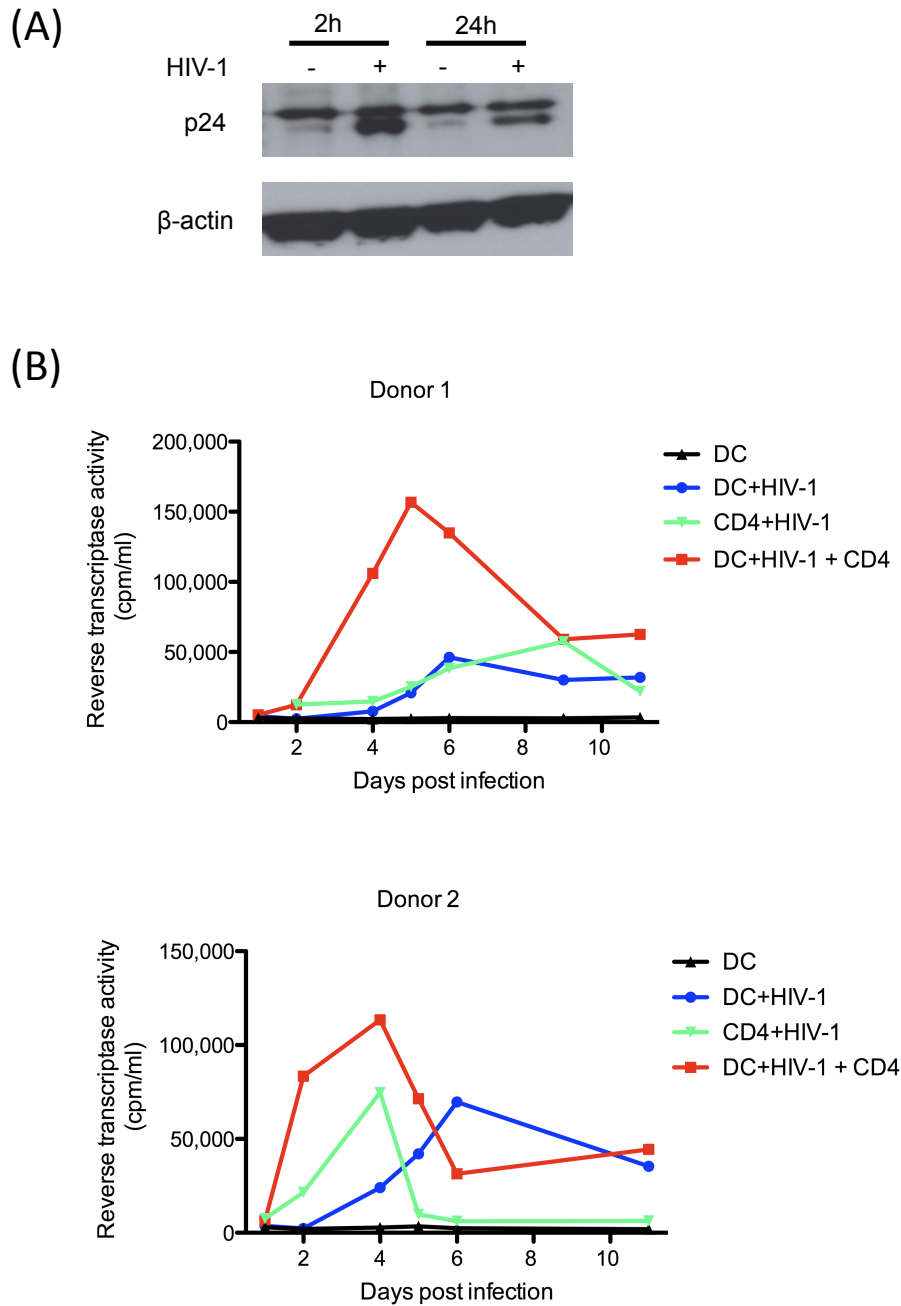


Figure 3.2: Investigation into the kinetics of HIV-1 infection of DCs *in vitro*. 10^6 DCs were infected with HIV-1 (50 ng p24 gag) or left uninfected. (A) Capture of viral particles was confirmed by immunoblotting for HIV-1 p24 gag antigen (top band) 2 hrs and 24 hrs post infection. The top band represents unspecific antibody binding. (B) 10^6 DCs were either left uninfected (DC) or infected with HIV-1 (DC+HIV-1) and cultured in 1 ml R10. Alternatively, 3×10^6 PHA activated autologous blasted $CD4^+$ T cells were added after the wash step (DC+HIV-1 + CD4). As an additional control, 3×10^6 $CD4^+$ T cells were directly infected with HIV-1 (50 ng p24 gag) and cultured alone (CD4 + HIV-1). Culture supernatants were probed for newly emerging viral particles using the Quanti RT kit (GE Healthcare). Addition of PHA-blasted autologous $CD4^+$ T cells to HIV-1 infected DCs accelerates and enhances the production of newly synthesised viral particles compared to DCs cultured alone or directly infected $CD4^+$ T cells (CD4+HIV-1). Data from (A) one or (B) two representative donors are shown.

and passed on to T cells in trans. Furthermore, it confirms that delivery of HIV-1 from DCs to CD4⁺ T cells enhances the infectivity of CD4⁺ T cells, underlining the importance for cell-to-cell transfer in viral dissemination.

3.3.3 Measuring early viral gene transcripts in HIV-1-infected DCs

The data shown above suggest that DCs only start releasing viral particles into the supernatants around day 4 post-infection. However, Gringhuis *et al.* have shown that viral transcription is active as early as 6 hrs post-transfection [Gringhuis *et al.*, 2010]. I therefore sought to determine whether HIV-1 infection results in successful integration and viral gene transcription at earlier time points. For this purpose, RNA was extracted from DCs infected with HIV-1 for 6 hrs and 24 hrs. Any remaining genomic DNA was removed by DNase digestion before the RNA was reverse transcribed. Finally, tat-rev transcripts were amplified using SYBR green-based quantitative PCR and tat-rev primers previously designed by Gringhuis *et al.* [Gringhuis *et al.*, 2010]. Figure 3.3(A) marks the location of the primers in relation to the HIV-1_{NL4.3} genome. The housekeeping gene GAPDH served as endogenous control and was used for normalisation. As seen in Figure 3.3(B) tat-rev transcripts were detected in HIV-1-infected DCs at 6 hrs and 24 hrs post-infection.

The work presented here confirms that the chosen infection conditions lead to a rapid uptake of HIV-1 particles by DCs. Furthermore, these particles remain infectious as HIV-1-bearing DCs efficiently enhance the infection of autologous CD4⁺ T cells. In agreement with the finding by Gringhuis *et al.*, the data also confirms that DCs are productively infected within the first 6 hours of infection, as indicated by the detection of early viral transcripts. However, newly synthesised viral particles only begin to emerge in supernatants approximately 4 days post-infection.

3.3.4 Assessing the antiviral state of HIV-1 infected DCs

As described in the Introduction, monocyte-derived DCs fail to fully mature in response to HIV-1 infection *in vitro* [Granelli-Piperno *et al.*, 2004]. In order to investigate whether the infection conditions described above induced DC maturation, I sought to investigate whether HIV-1-infected DCs mount an antiviral type I IFN response. MDDCs have been shown to secrete type I IFNs, predominantly IFN- β in response to infection with Influenza A and Sendai virus [Osterlund *et al.*, 2005]. Furthermore, IFN- α has been shown to induce a variety

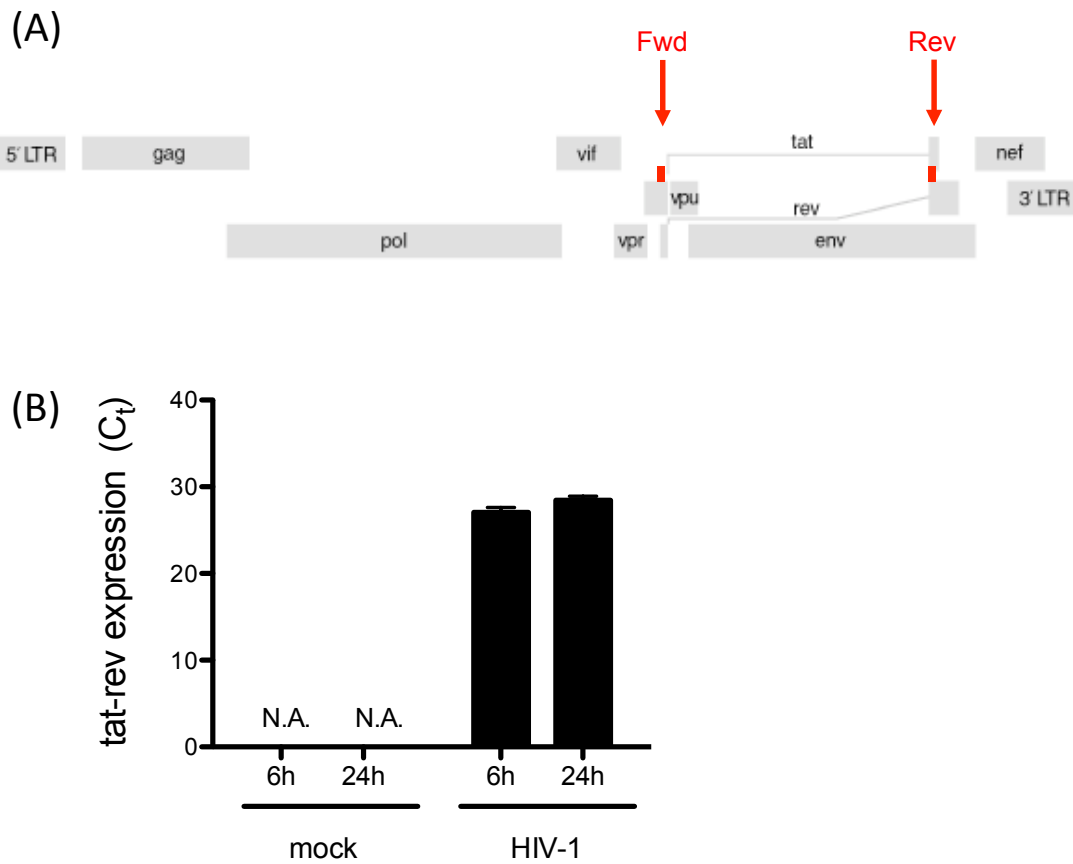


Figure 3.3: Detection of early viral gene transcripts in HIV-1-infected DCs.

(A) Alignment of tat-rev qRT-PCR primers to the genomic sequence of HIV-1_{NL4.3} (forward and reverse primers marked in red). **(B)** 10⁶ DCs were infected with HIV-1 (50 ng p24 gag) or left uninfected. Cells were harvested after 6 hrs and 24 hrs post-infection and RNA was extracted. After treatment with DNase I, RNA was reverse transcribed and tat-rev was amplified by SYBR green quantitative PCR (N.A = no amplification). Data from is from three different experiment shown as mean $\pm$ SD.

of HIV-1 restriction factors including APOBEC3G and Tetherin [Van Damme *et al.*, 2008] [Peng *et al.*, 2006]. As type I IFNs, especially IFN- α , exist in several different isoforms, I chose to monitor the expression of IFN-inducible protein myxovirus resistance protein A (MxA) as an indirect proxy for the antiviral state [Holzinger *et al.*, 2007]. DCs were infected with HIV-1 for 24 hrs or stimulated with the TLR8 ligand ssRNA40. DCs were harvested at various time points post-infection and MxA expression was monitored on an mRNA as well as protein level (Figure 3.4). The results collectively show that treatment of DCs with 10 $\mu\text{g/ml}$ ssRNA40 induces the expression of MxA, while infection with HIV-1 has no effect. This indicates that the DCs fail to induce an antiviral state in response to HIV-1 infection. Since then, several studies have revealed that HIV-1 has evolved several strategies to modulate the antiviral type I IFN response of monocyte-derived DCs [Manel *et al.*, 2010] [Yan *et al.*, 2010] [Harman *et al.*, 2011].

3.3.5 Setup of miRNA profiling experiment

Given the existence of multiple different phases of HIV-1 infection in DCs, it would have been preferable to carry out the profiling study at several different time points post-infection. However, due to constraints in terms of cost, a single time point was chosen for the initial screening study. I was keen to identify early changes in miRNA expression in order to minimise the detection of any secondary effects and negative feedback mechanisms, which may increase the complexity of the results. As a result, the profiling experiment was performed 2 hrs post-infection. Previous studies in the lab looking at signalling via DC-SIGN and NOD2 had shown significant changes in gene expression 2 hrs post-stimulation [Hodges *et al.*, 2007] [John Baker, unpublished data].

Despite the short nature of miRNAs, several tools have been developed for miRNA profiling including microarrays, stem-loop qRT-PCR arrays and deep sequencing. Methods vary greatly regarding cost, sample size, sample quantity, speed and requirement to identify new miRNAs [Kong *et al.*, 2009] [van Rooij, 2011]. Therefore, the choice depends on the individual requirements. In this study, a microarray platform is used to investigate changes in the miRNAome of DCs in response to HIV-1 infection. Microarray technology has emerged as a powerful high-throughput tool for studying the expression of thousands of small non-coding RNAs in response to a variety of different stimuli and disease states [Calin *et al.*, 2004] [Volinia *et al.*, 2006]. Microarray sample chips are manufactured by immobilizing antisense oligonucleotide probes onto a solid support platform. Appropriate probe design is limited by the short length of miRNAs and therefore rather challenging. Nevertheless, several platforms

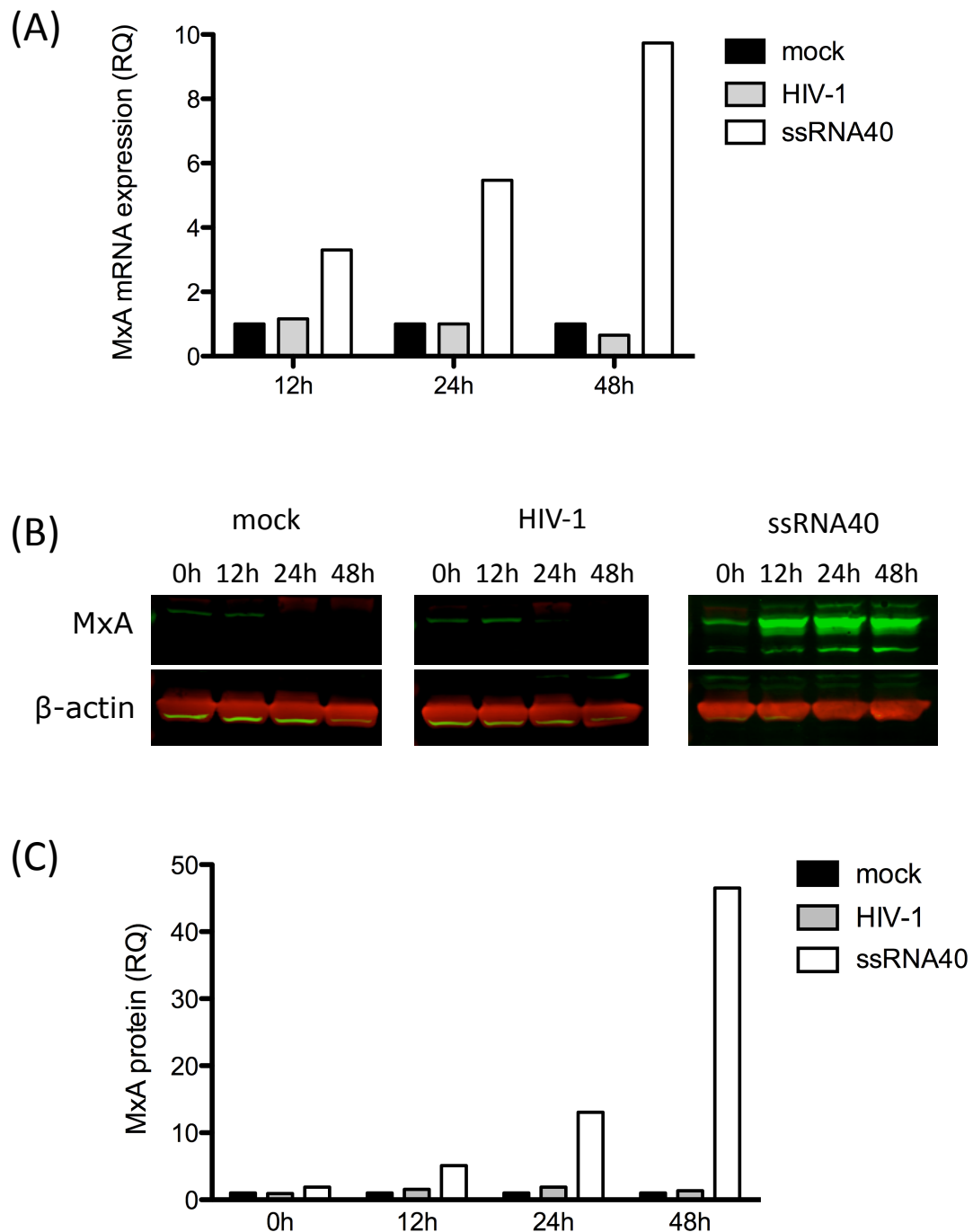


Figure 3.4: Monitoring the expression of IFN-inducible protein MxA following HIV-1 infection and ssRNA40 stimulation. 10^6 DCs were infected with HIV-1 (50 ng p24 gag), stimulated with ssRNA40 (10 μ g/ml) or left unstimulated. **(A)** RNA was isolated at various time points after stimulation and MxA mRNA was quantified by qRT-PCR. Gene expression was normalised against GAPDH. **(B)** Alternatively, DCs were lysed and MxA protein was detected by quantitative immunoblot (LI-COR) (green = MxA, red = β -actin). **(C)** Quantification of MxA protein expression from immunoblot. Protein expression is normalised against β -actin expression. Infection of DCs with HIV-1 fails to induce MxA expression in DCs compared to ssRNA40. Results are from one representative donor.

are currently available and provide an accessible and low cost tool for profiling miRNAs.

3.3.6 Sample generation for array analysis

Immature primary DCs derived from buffy coats were harvested on Day 5 and either infected with HIV-1 as described above, or left uninfected. After 2 hrs, cells were washed and total RNA was extracted using the miRNAeasy kit (Qiagen). As the efficiency of the downstream labelling reaction is inhibited by trizol, an additional chloroform extraction step was added to the RNA extraction protocol to avoid contamination. All samples were quality controlled using a Nanodrop US spectrophotometer and only samples with low salt ($260\text{nm}/230\text{nm} > 2$) and protein contamination ($260\text{nm}/280\text{nm} > 1.8$) were taken further. RNA is vulnerable to degradation by RNase enzymes and therefore relatively unstable. As miRNA microarrays are optimised to detect short RNA fragments, the use of degraded RNA may result in artefacts. All samples were therefore tested for RNA integrity by microelectrophoresis using the Agilent 3100 Bioanalyzer platform. Bioanalyzer traces of eight representative samples are displayed in Figure 3.5. For each sample, two dominant bands, which represent intact ribosomal RNA species, are visible. Significant RNA degradation is characterised by the appearance of multiple other bands. The relative size of the two main ribosomal RNA peaks is used to calculate an RNA integrity number (RIN). Only samples with a $\text{RIN} \geq 8$ (out of a maximum attainable score of 10) were used in these microarray studies.

3.3.7 Microarray analysis of HIV-1 infected DCs 2 hrs post-infection

The miRNA microarray analysis was carried out in collaboration with Oxford Gene Technology (OGT) on a dual colour miRNA microarray platform designed by the OGT bioinformatics group (Figure 3.6). The array was manufactured by Agilent using ink jet in-situ synthesis (IFISS) technology and covered all miRNA entities listed in miRBase Version 10. Briefly, RNA samples were labelled using a dual colour protocol whereby RNA from uninfected DCs was labelled with Cy5 (emission wavelength 670 nm) and RNA of HIV-1-infected DCs was labelled with Cy3 (emission wavelength 570 nm).

Three biological replicates were performed and, in each case, both conditions from the same individual were pooled and incubated with the array according to manufacturer's instructions. Unhybridised target was removed by washing, before the chip was scanned to detect the amount of fluorescence for each probe. Raw data containing the spot intensities

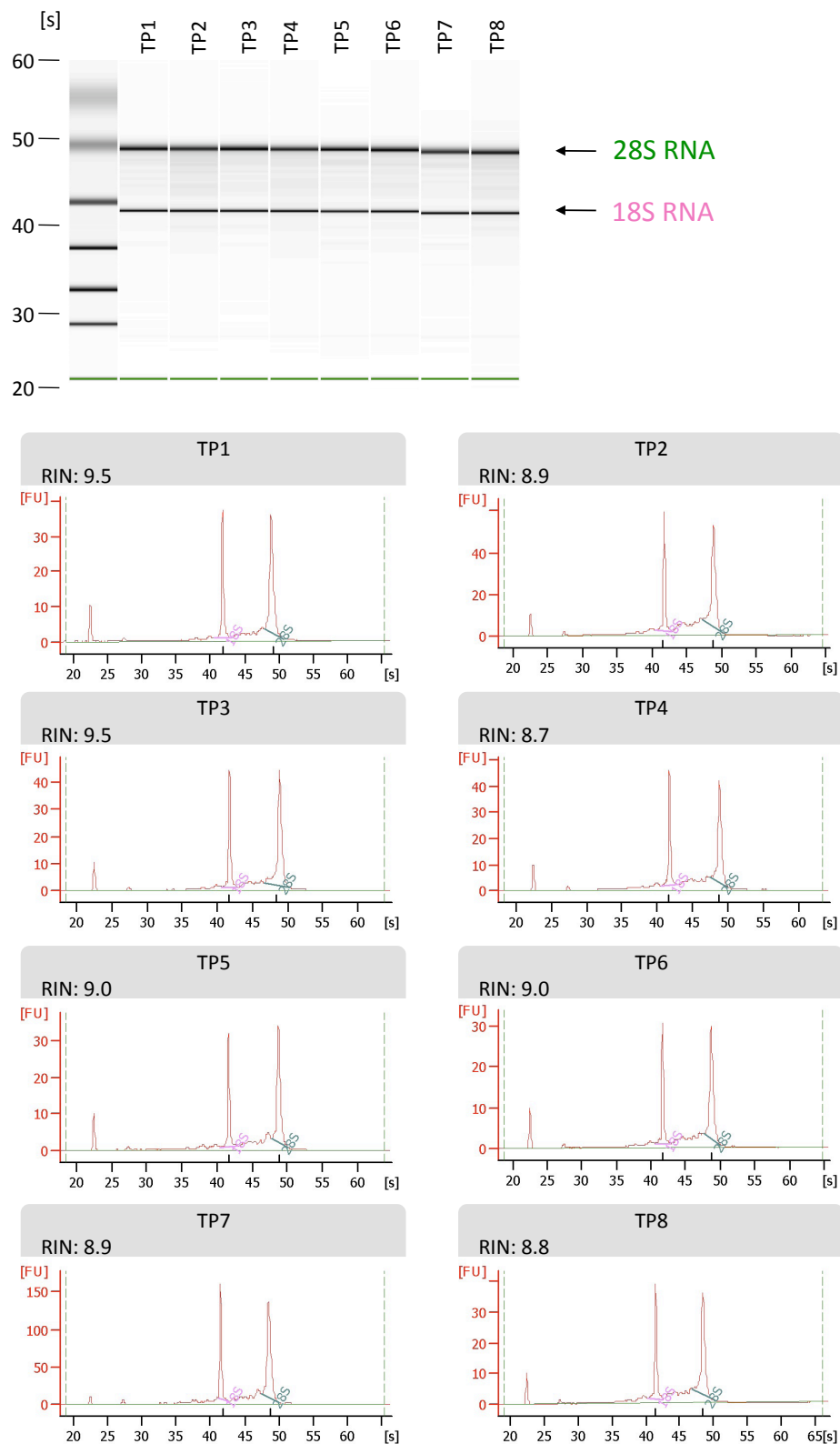


Figure 3.5: Validation of RNA integrity prior to array hybridisation. RNA samples were run on the Agilent Bioanalyzer 2100 platform using the Eukaryotic Total RNA Nano chip. One batch of representative samples are shown: samples **1,3,5,7**) uninfected, **2,4,6,8**) HIV-1-infected DCs. The two main bands represent 18S and 28S ribosomal RNAs. Only samples with an RNA integrity number (RIN) ≥ 8 were used for microarray experiments.

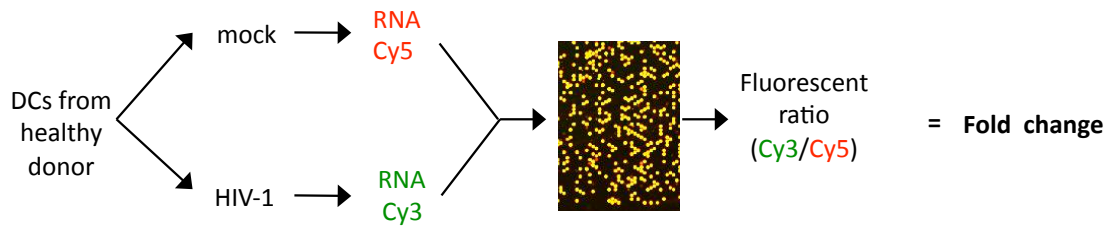


Figure 3.6: Workflow of OGT miRNA microarrays using a dual colour setup. RNA extracted from HIV-1-infected and uninfected DCs is labelled with Cy3 and Cy5 dyes, respectively, and hybridised onto the microarray together. Fold changes are obtained by calculating fluorescent intensity ratios.

were first assessed to ensure low background noise and even distribution of the sample across the array. Data was then normalised and fluorescent ratios were calculated using Genespring GX. This dual colour setup was chosen, as it allows matched sample pair analysis on the same array and minimises experimental variability.

The results of the bioinformatic analysis are shown in Figure 3.7. When comparing the miRNA expression profiles of HIV-1-infected and uninfected DCs, only few and very small changes were observed (Figure 3.7(A)). Using a cut-off of 1.2-fold, six miRNAs were found to be significantly up regulated in HIV-1-infected DCs as compared to uninfected DCs across all three donors ($p < 0.05$) (Figure 3.7(B) and (C)). One of the identified miRNAs, miR-560, is likely to be a fragment of an rRNA transcript, not a miRNA, and was therefore excluded from further analysis [Landgraf *et al.*, 2007]. None of the remaining five miRNAs - miR-663, miR-508-3p, miR-609, miR-650 or miR-150* - had been previously associated with the innate immune response or viral infection. In addition, microarray analysis revealed no changes in the expression of PRR-responsive miRNAs, such as miR-155 or miR-146a, which have been shown to be induced during the antiviral response of DCs (see Section 1.3.4).

Most gene expression data is considered significant above a fold change of 2-fold, and it may therefore be argued that the cut-off chosen in this experiment is rather generous. However, due to the early time point chosen, the inherently low sensitivity of microarrays and, more importantly, the high consistency between the three biological replicates and resulting statistical significance ($p < 0.05$) of the data, I decided to continue with the validation process.

3.3.8 Setup of miRNA-specific quantitative PCR

Microarrays provide a powerful platform for the assessment of global miRNA regulation. However, it is essential to validate array findings by additional methods. For this purpose, a specialised stem-loop qRT-PCR method has been developed which enables the specific

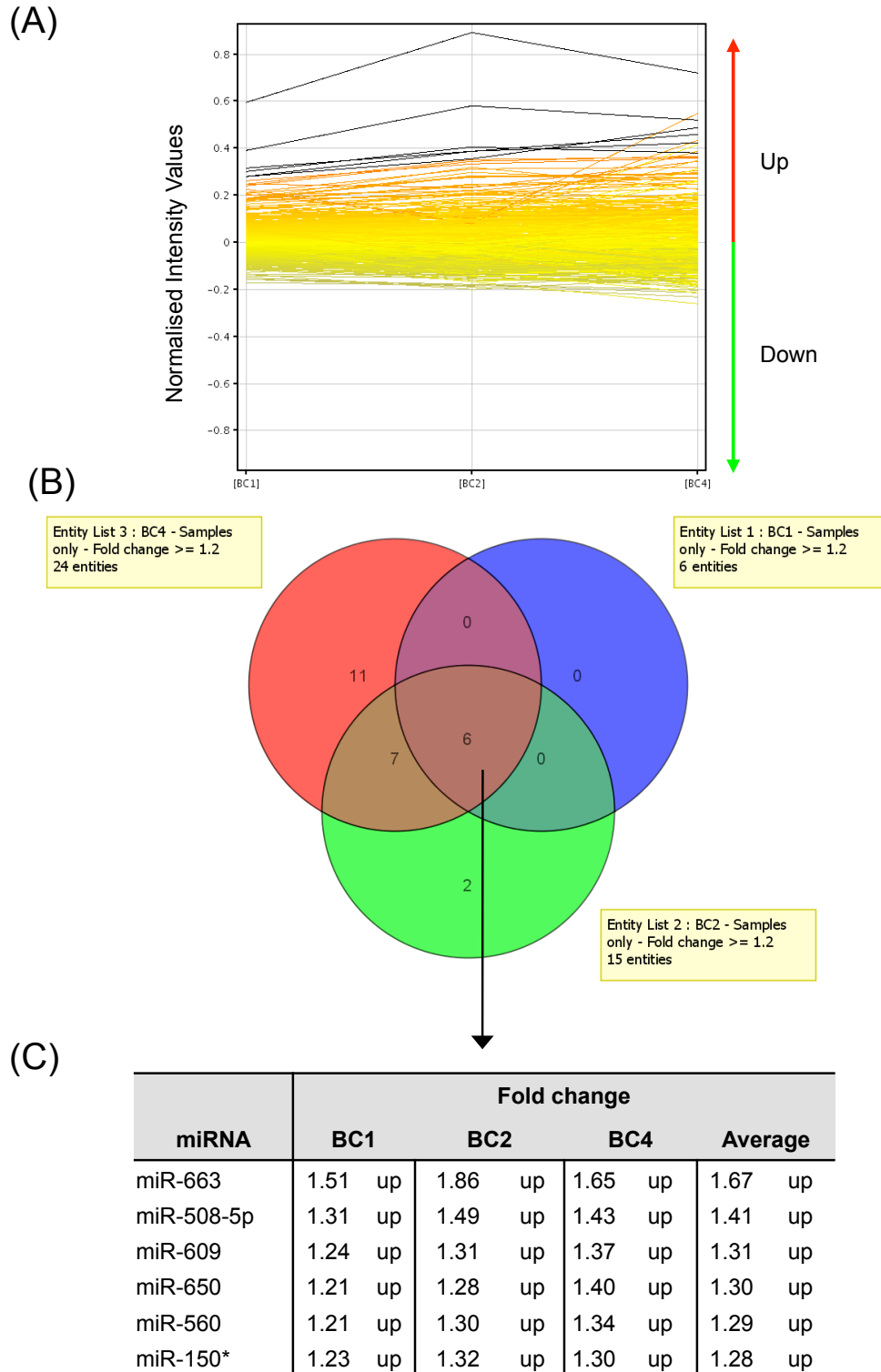


Figure 3.7: Summary of miRNA microarray displaying differentially regulated miRNAs in response to HIV-1 infection in primary DCs. DCs of three different buffy coats (BC1, 2 and 4) were infected with HIV-1 for 2 hrs or left uninfected. RNA was extracted and hybridised onto miRNA microarrays using a dual colour setup. Raw data was normalised and analysed using Genespring GX. **(A)** Profile plot showing normalised intensity ratios of HIV-1-infected vs. uninfected DCs. Highlighted in black are all miRNAs that were differentially expressed in all three donors (fold change ≥ 1.2 and $p < 0.05$). **(B)** Venn diagram showing differentially regulated miRNAs common to all three donors (fold change ≥ 1.2 and $p < 0.05$). **(C)** Table listing the six differentially expressed miRNAs common to all three biological replicates with fold changes.

detection of individual mature miRNA species [Cheng *et al.*, 2009] (Figure 3.8). Briefly, mature miRNAs are reverse transcribed individually using specific stem-loop primers. Each primer is designed to contain a short single-stranded region complementary to the 3' end of the miRNA and a stem-loop region containing a universal primer-binding site. The resulting cDNA is then used as a template for quantification by standard Taqman PCR with a miRNA-specific primer and a second universal primer. For each miRNA, raw C_t values are converted into relative fold changes using the comparative $\Delta\Delta C_t$ method (see Section 2.6.7).

Relative quantification of miRNA expression by stem-loop PCR requires normalisation to an appropriate endogenous control. The use of conventional housekeeping genes, including GAPDH, is not recommended, as they do not share similar properties to miRNAs like stability or size. More importantly, they are not amenable to the miRNA assay design. Hence, a small group of non-coding RNAs, including small nuclear RNAs (snRNA) and small nucleolar RNAs (snoRNA), are commonly used as normalization controls [Kiss, 2002]. As the expression of small non-coding RNAs can vary greatly between different cell types and treat-

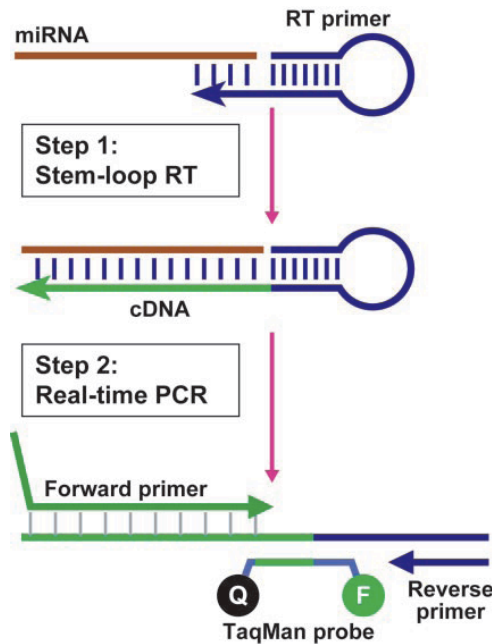


Figure 3.8: Schematic diagram of miRNA-specific stem-loop qRT-PCR. Step 1: Mature miRNAs are reverse transcribed using a miRNA specific stem-loop primer. Step 2: cDNA is quantified using standard Taqman quantitative PCR with a forward primer which is complementary to the mature miRNA sequence, and a reverse primer which binds to the universal stem-loop sequence. Figure taken from Applied Biosystems.

ment conditions, careful selection is crucial. Confidence in the data can also be improved by normalizing against more than one endogenous control. Amongst the different candidates available snoRNAs, RNU44, RNU48 and RNU6B were tested across a range of different uninfected and HIV-1-infected samples at various different time points (Figure 3.9). Out of these, RNU48 has the lowest C_t value and is therefore most abundant in DCs. Furthermore, amplification of RNU48 was most consistent as shown by closely overlapping fluorescent traces and lowest standard deviation for the C_t values across the different conditions tested. Therefore, RNU48 was chosen as normalisation control for all miRNA qRT-PCR expression data presented here.

3.3.9 Validation of microarray data by miRNA-specific quantitative PCR

The validation of array findings was extended to include additional time points, which are thought to represent DCs at three different phases during HIV-1 infection (Figure 3.10(A)):

1. after viral uptake (Day 1).
2. when transmitting HIV-1 particles to CD4⁺ T cells (Day 2).
3. when HIV-1 is actively replicating (Day 6).

As a comparison, expression of the selected miRNA was also measured in DCs stimulated with ssRNA40. Finally, I decided to monitor the expression of the PRR-responsive miRNA miR-155, which was not differentially regulated by HIV-1 infection, but has been shown to be induced in response to a variety of different inflammatory stimuli [Teng & Papavasiliou, 2009].

Briefly, 3×10^6 immature DCs were infected with HIV-1, or left uninfected. Unbound virus was washed off after 2 hrs and DCs were resuspended in 3 ml R10. Cells were harvested after 1, 2 and 6 days post-infection and total RNA was isolated (Figure 3.10(A)). In addition, DCs were stimulated with 10 $\mu\text{g}/\text{ml}$ ssRNA40 for 24 hrs prior to RNA isolation. I sought to measure the expression of miR-663, miR-508-3p, miR-609, miR-650 and miR-150* using the miRNA-specific stem-loop PCR workflow described above. Unfortunately, miR-663 has a GC content $> 90\%$ and primer design was therefore unsuccessful. Surprisingly, no PCR amplification was observed for miR-508-5p or miR-609 suggesting that neither of these miRNAs is expressed in human monocyte-derived DCs.

Figure 3.10 shows the expression profiles of miR-155 and miR-650 in response to HIV-1 infection and TLR8 stimulation. As expected, infection of DCs with HIV-1 had no effect on

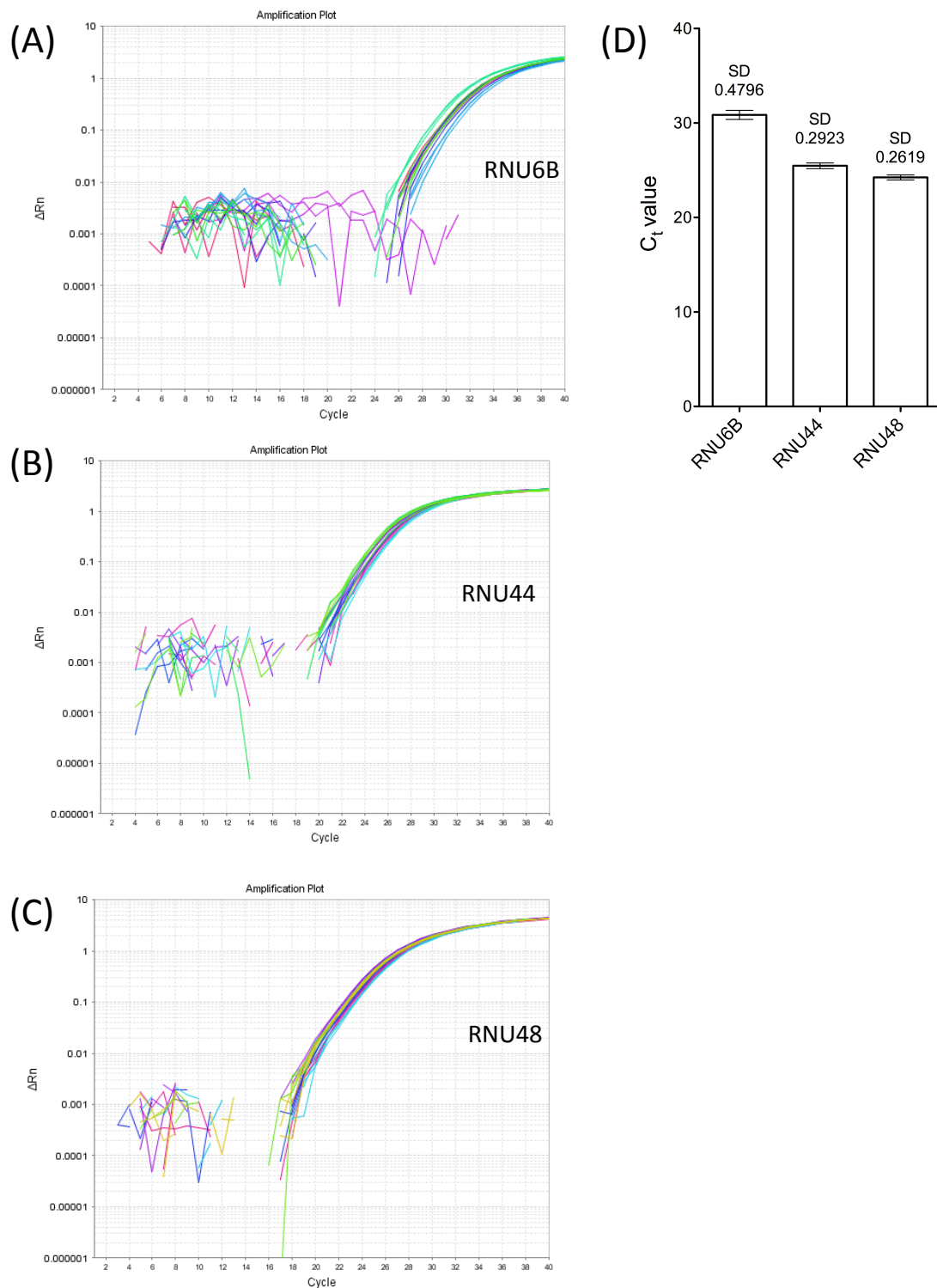


Figure 3.9: Validation of endogenous housekeeping controls for miRNA qRT-PCR. Fluorescent traces showing the expression of (A) RNU6B, (B) RNU44 and (C) RNU48 in DCs infected with HIV-1 for 6 days and in uninfected DCs (6 replicates for each condition). The same amount of input RNA was used for cDNA synthesis (10 ng). (D) Plot showing the C_t values corresponding to the fluorescent traces. Data from 12 replicates shown as mean $\pm$ SD. RNU48 showed lowest C_t value, i.e. highest expression, and lowest SD and was therefore chosen as normalisation control.

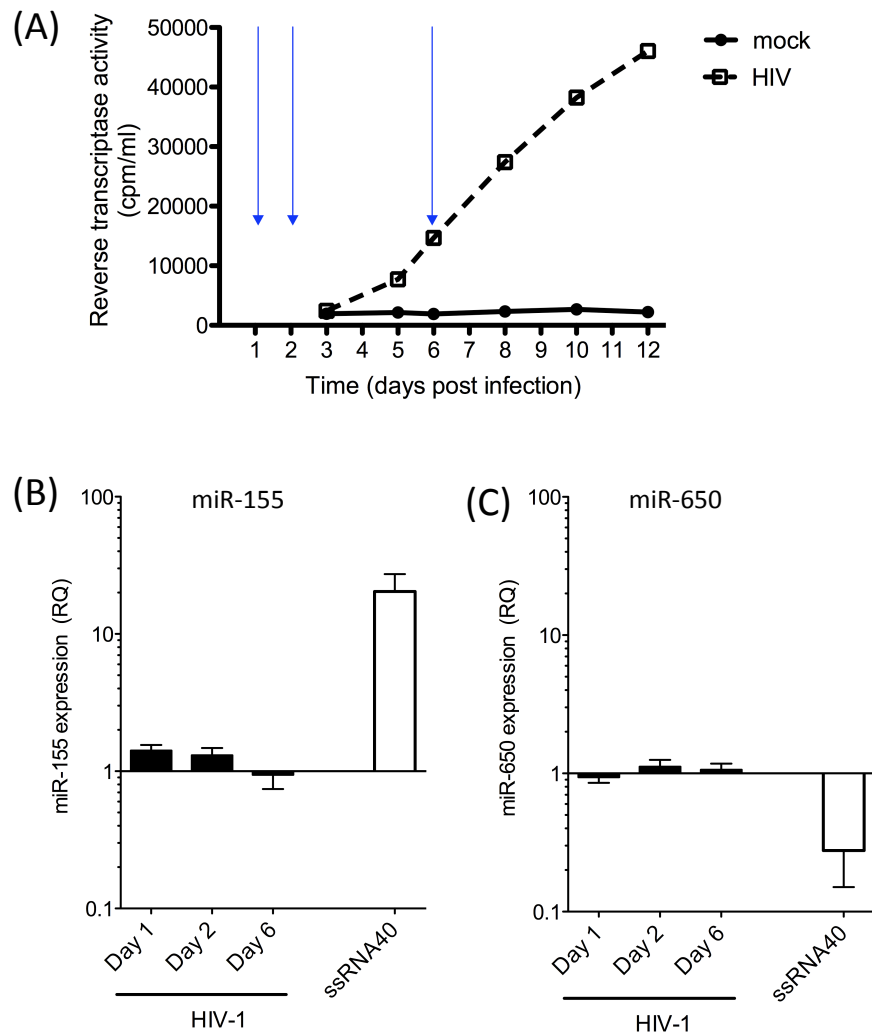


Figure 3.10: Validation of array findings by stem-loop qRT-PCR. (A) Time course of reverse transcriptase activity in culture supernatants of one representative donor indicating time points chosen for validation. Expression of (B) miR-155 and (C) miR-650 on Days 1, 2 and 6 post HIV-1 infection and following stimulation with ssRNA40 (10 μ g/ml) for 24 hrs. Expression is normalised against RNU48 and is shown relative to uninfected control. Data from three donors shown as mean $\pm$ SEM.

the expression of miR-155 (Figure 3.10(B)). In contrast, stimulation of DCs with ssRNA40 resulted in induction of miR-155 expression as has been previously observed for other TLRs (see Section 1.3.5). I was unable to observe any significant changes in expression of miR-650 in response to HIV-1 infection (Figures 3.10(C)). Further investigations revealed an inherent dye bias within the array, i.e. the differences in spot intensity between the two samples were attributed to the different dyes instead of being due to a true difference in miRNA expression. A dye bias can occur across an entire array or, as shown here, only for selective probes, and is normally not removed by array normalization.

It is interesting to note that maturation of DCs with ssRNA40 resulted in decreased expression of miR-650 (Figures 3.10(C)). This is an interesting finding, which provides the basis for the remaining chapters of this thesis.

3.3.10 Additional microarray analysis of HIV-1-infected DCs 24 hrs post-infection

Due to the failure of the microarray, the initial question as to whether HIV-1 infection alters the miRNA expression profile of DCs remains unanswered. I therefore performed additional array analyses. In order to circumvent similar technical problems, a different microarray platform was chosen. The Agilent miRNA microarray includes 866 human and 89 viral miRNA sourced from Sanger miRBase (release 12.0). In addition, a different dual colour setup was chosen which does not allow for dye bias (Figure 3.11); all samples were labelled with the same dye (Cy3) and hybridised onto separate arrays. An internal RNA spike-in (Cy5) was included as a normalization control, which allows for the comparison of samples across different arrays. Finally, preliminary setup experiments showed that miR-155 induction by ssRNA40 peaked around 24 hrs post stimulation (Figure 3.12); as a consequence, microarray analysis was performed at 24 hrs post-infection.

On Day 5, immature DCs were infected with HIV-1 as described above. To confirm that DCs from all four donors were infected, 10^6 DCs were kept in 1 ml R10 and supernatants were tested for reverse transcriptase activity at various time points (Figure 3.13(A)). At 24 hrs RNA was extracted, quality controlled, labelled and hybridised onto Agilent arrays. Raw data were analysed using Genespring GX and the results are displayed in Figure 3.13(B). It is evident that not many differentially regulated miRNAs were detected. Only miR-1305 was both greater than 2-fold regulated and found to be statistically significant ($p < 0.05$). MiR-892b was also more than 2-fold regulated but was not found to be statistically significant ($p > 0.05$) as the regulation of this miRNA varied greatly among donors. Unfortunately,

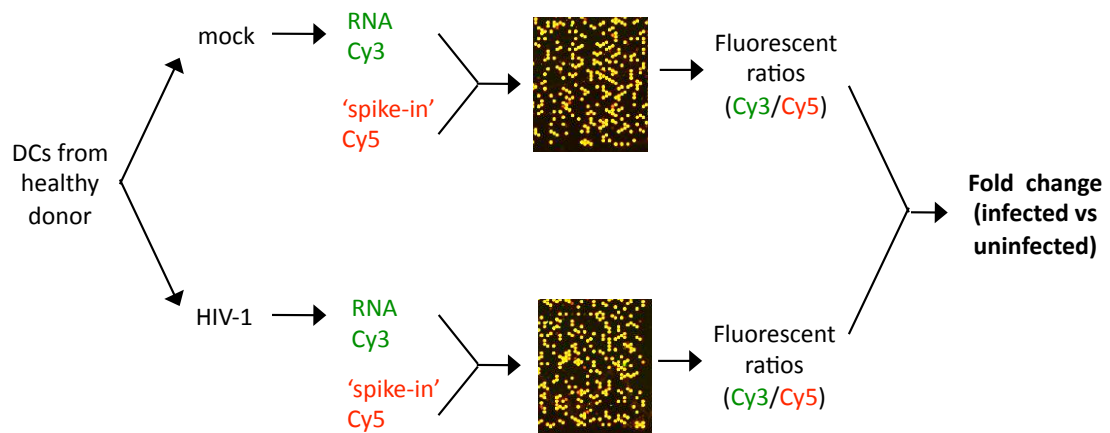


Figure 3.11: Workflow of Agilent miRNA microarray with modified dual colour setup. RNA from HIV-1-infected and uninfected DCs is labelled with the same dye and hybridised onto separate arrays in combination with a 'spike in' RNA control which is used for standardisation.

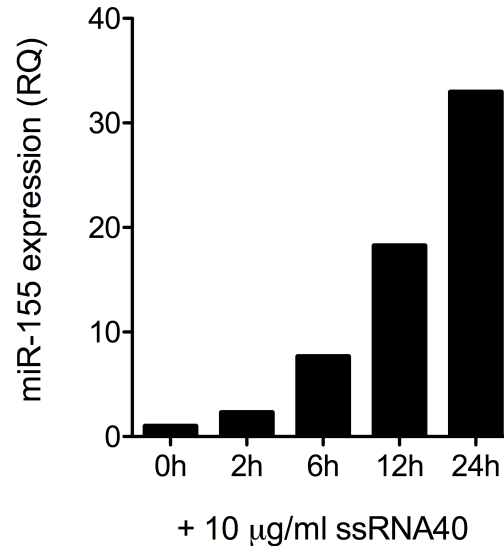


Figure 3.12: qRT-PCR analysis of miR-155 expression in DCs stimulated with ssRNA40. Immature DCs were stimulated with ssRNA40 (10 µl/ml) and cells were harvested at different time points. Total RNA was extracted and miR-155 expression was measured by qRT-PCR. Expression is normalised against RNA48 and is shown relative to an unstimulated control. Results from one representative donor shown.

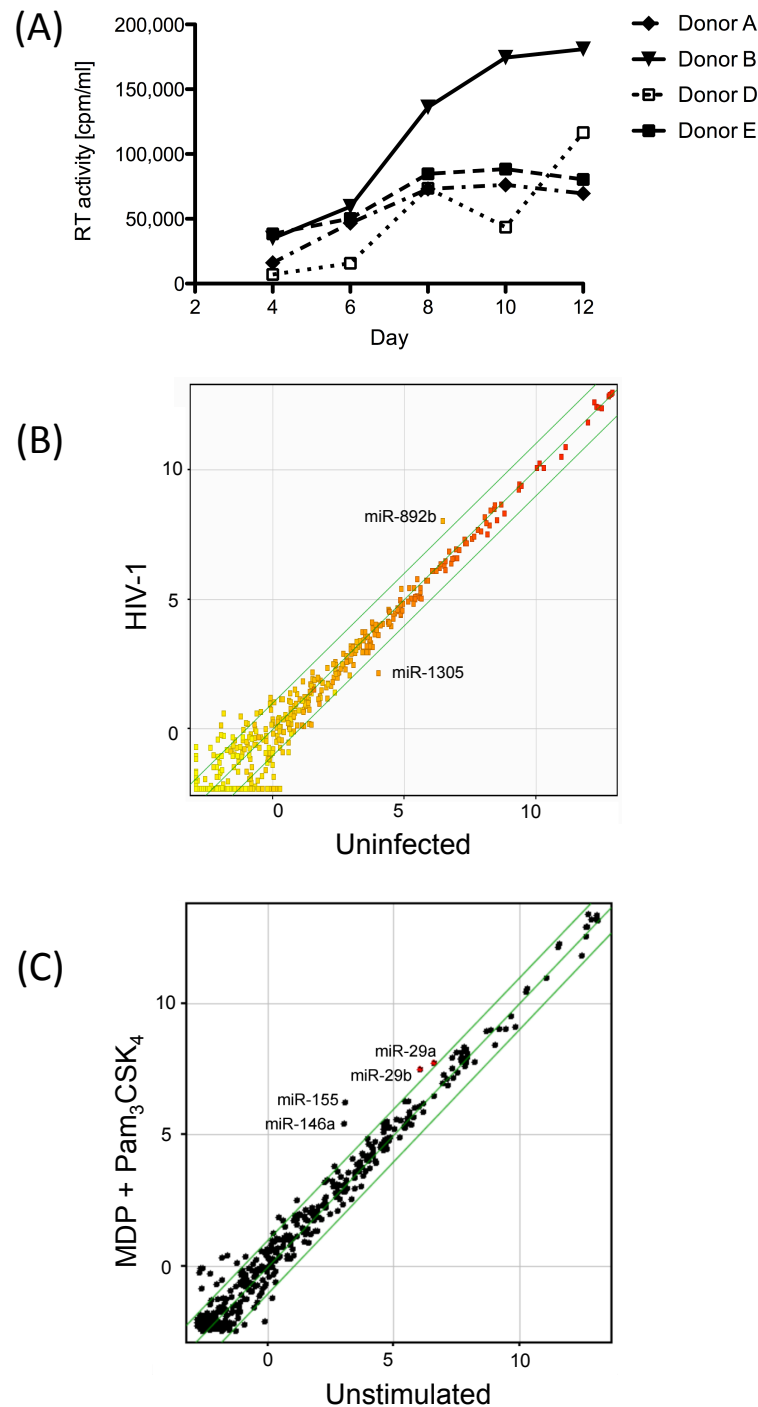


Figure 3.13: Agilent miRNA microarray analysis of DCs infected with HIV-1 for 24 hrs. DCs derived from four different donors were infected with HIV-1 (50 ng p24 gag/ 10^6 cells) for 24 hrs. Total RNA was extracted, labelled and hybridised to Agilent miRNA microarray. **(A)** Infection was confirmed by keeping 10^6 DCs in 1 ml R10 and testing supernatants for reverse transcriptase activity. **(B)** Raw data was analysed using Genespring GX and plot shows more than 2 fold regulation following HIV-1 infection, compared with uninfected DCs. Only miR-1305 was found to be significantly down regulated (fold change ≥ 1.2 and $p < 0.05$). **(C)** Agilent miRNA microarray analysis of DCs stimulated with MDP ($1\mu\text{g/ml}$) and Pam₃CSK₄ ($1\mu\text{g/ml}$) for 24 hrs. Plot shows normalised intensity values and highlights miRNAs more than 2 fold up-regulated by dual stimulation, compared with unstimulated DCs [Dr. Oliver Brain, unpublished data].

validation of miR-1305 by qRT-PCR revealed that the expression of this miRNA is almost undetectable in DCS (C_t value > 38). This made it impossible to follow up this finding.

In a parallel experiment which investigated the changes in miRNA expression in response to dual stimulation with two bacterial PAMPs, MDP and PAM₃CSK₄, differential changes in miRNA expression were observed (Oliver Brain, unpublished data) (Figure 3.13(C)). As this experiment used the same cells and the identical microarray platform, it can be concluded that both were functional.

In summary, despite ensuring that DCS were responsive and infected with HIV-1, and that the array platform was functional, no changes in miRNA expression could be detected. This indicates that HIV-1 infection has no significant effect on the cellular miRNAome of DCS.

3.4 Discussion

This chapter describes the first investigation into the effect of HIV-1 infection on the miRNAome of primary human DCS. In summary, this genome-wide miRNA expression profiling study was unable to reveal any differences between the miRNA profile of HIV-1-infected and uninfected DCS.

It is well-known that HIV-1 hides from innate immune responses by compartmentalisation, and actively subverts innate signalling through DC-SIGN and TLR8 [van der Vlist *et al.*, 2011]. These signalling pathways seem to have a profound impact on the host environment by altering gene expression [Hodges *et al.*, 2007] [Gringhuis *et al.*, 2010]. It is therefore surprising that HIV-1 infection did not seem to have any impact on miRNA expression levels. However, this study was hampered by technical problems as well as by the complexity of the system under investigation. Some of the problems are discussed below.

It is generally well-accepted that exposure of monocyte-derived DCS to HIV-1 *in vitro* fails to induce maturation. However, there is still a lot of remaining controversy surrounding their susceptibility to HIV-1 infection and the subsequent kinetics of replication. The HIV-1 infection time-course presented in this chapter is displayed in (Figure 3.14). It identifies at least four different phases which are characterised by:

1. capture of HIV-1 particles (within 2 hrs).
2. detection of early viral replication in DCS (from ~ 6 hrs).
3. transmission of HIV-1 from infected DCS to CD4⁺ T cells in trans (from $\sim$ Day 2).

4. release of newly synthesised viral particles by HIV-1-infected DCs (from $\sim$ Day 4).

It was therefore very difficult to choose a single time-point for the miRNA profiling study. It may be worth investigating a range of different time-points or adding a few more controls, so ensure that PRR receptors are indeed being triggered by the virus (for example TLR8 as reported in [Gringhuis *et al.*, 2007]). Adding to the overall complexity, it is unclear how much virus has been taken up by the DCs and which fate it encounters, i.e. how much of the captured virus infects the DCs, is degraded, or remains intact and is passed on to CD4⁺ T cells. It is therefore possible that HIV-1 infection of DCs *in vitro* gives rise to a rather heterogeneous mixture of cells.

Overall, I think this data needs to be considered with some caution. It needs to be ensured that the failure to induce differential miRNA regulation represents an active subversion mechanism instead of merely a lack of stimulation as a consequence of low PAMP concentration or compartmentalisation. This could be addressed by using a higher dose of virus or by manipulating its life-cycle in order for it to become more visible to the innate immune system. Harman *et al.*, for example, used a significantly higher dose of HIV-1 to

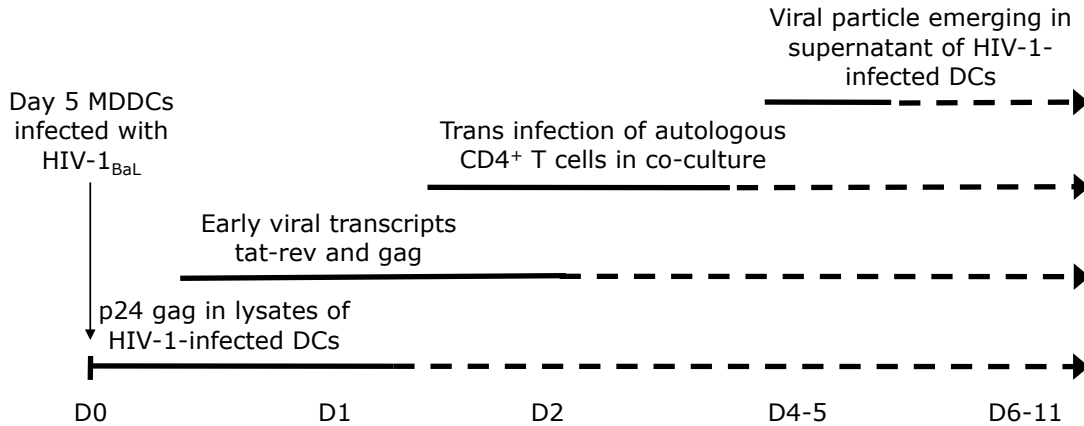


Figure 3.14: Schematic diagram showing the different stages of HIV-1 infection of DCs *in vitro*. Primary monocyte-derived DCs were infected with HIV-1 and uptake was confirmed after 2 hrs. Co-culture experiments demonstrate the internalised virus remains infectious and is transferred to PHA-activated autologous CD4⁺ T cells within the first 2 days of infection. When examining DCs, transcription of early viral gene products (tat-rev) can already be detected within the first 6 hrs after infection, suggesting that DCs are productively infected. However, when monitoring culture supernatants for HIV-1 reverse transcriptase activity, newly synthesised progeny virus can only be detected as early as 4 days after infection.

infect DCs, which resulted in accelerated replication kinetics, with newly synthesised viral particles emerging around 24 hrs post-infection [Harman *et al.*, 2011]. In this case, HIV-1 infection was shown to induce a distinct subset of interferon-stimulated genes, without triggering production of type I or II IFNs [Harman *et al.*, 2011]. This high dose of HIV-1 may not reflect the physiological events, however it increases the proportion of DCs productively infected. Similarly, knockdown of TREX1, which leads to the accumulation of HIV-1 cDNA within the cytoplasm, could be used to induce an HIV-1-mediated innate immune response [Yan *et al.*, 2010]. It would be interesting to explore the miRNA expression profile following forced recognition of the virus.

3.5 Conclusion and future outlook

In summary, the work described in this chapter is supportive of all those studies showing that HIV-1 fails to mature DCs thereby evading the innate immune response. However, due to the lack of knowledge describing the complex interaction between HIV-1 and DCs in this system, and the uncertainty of the events *in vivo*, no convincing conclusions can be drawn from this data.

Nevertheless, it is worth highlighting one interesting finding that has emerged from this study: stimulation of DCs with ssRNA40 reduced the expression of miR-650 (Figure 3.10(C)). This finding is interesting especially considering that, to date, only few PRR-responsive miRNAs have been described that are down regulated on stimulation (see Section 1.3.4). The following chapters focus on this finding in greater detail; Chapter 4 explores the regulation of miR-650 in DCs stimulated with a variety of PAMPs, and Chapters 5 and 6 attempt to identify miR-650 targets and investigate the potential function of this miRNA in the inflammatory response of DCs.

Chapter 4

Regulation of miR-650 by pattern-recognition receptor signalling

4.1 Introduction

As shown in Chapter 3, stimulation of human DCs using the TLR8 ligand ssRNA40 decreases the levels of miR-650. This chapter aims to take this observation further and investigate if miR-650 is indeed a novel PRR-responsive miRNA.

Genomic location and function of miR-650

Human miR-650 was originally identified from human colorectal cells by miRNA serial analysis of gene expression (miRAGE), an approach combining aspects of miRNA cloning and SAGE (Serial Analysis of Gene Expression) [Cummins *et al.*, 2006]. It was mapped to chromosome 22 within the immunoglobulin lambda light chain variable (*IGVL*) genes (miR-BASE). More specifically, multiple copies of miR-650 are found in the human *IGVL* locus, all of which show the same overlap with the leader exon of clan II *IGVL* genes (Figure 4.1) [Das, 2009]. This is rather unusual, as previous genome-wide screens indicate that most of the known mammalian miRNAs are located within intergenic regions or introns of protein-coding or non-coding transcriptional units [Rodriguez *et al.*, 2004]. Phylogenetic analysis provides evidence that miR-650-bearing clan II *IGVL* genes originated in eutherian mammals and were afterwards lost in certain eutherian lineages, including rodents [Das, 2009]. This restricts the expression of miR-650 to primates and humans.

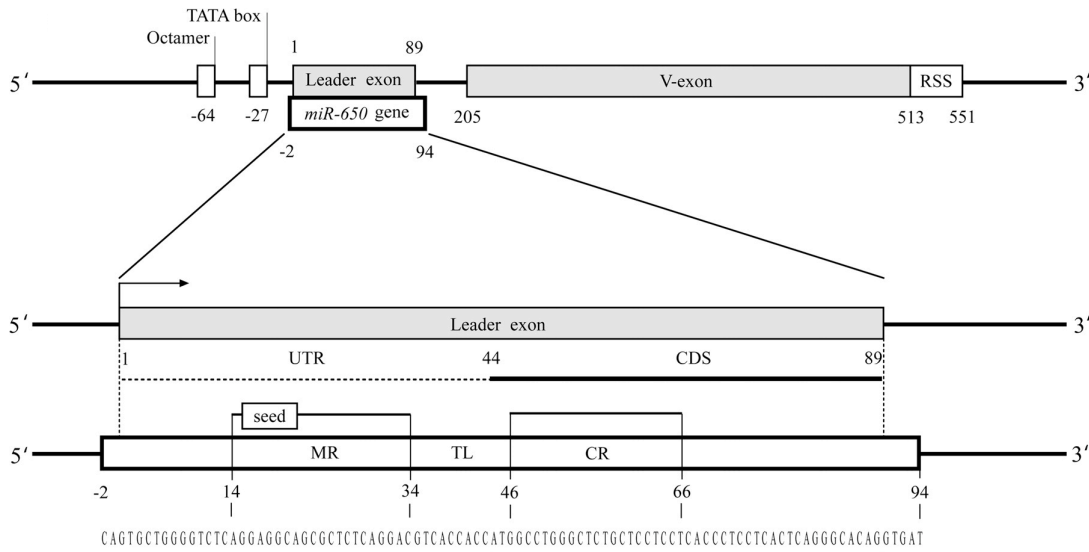


Figure 4.1: Genomic location of the *miR-650* gene. The *miR-650* gene overlaps with the leader exon of the *IGVL* gene. The exact nucleotide sequence of the *miR-650* gene that overlaps with the coding sequence of the leader exon of the *IGVL* gene is underlined. Figure taken from [Das, 2009].

The biological function of miR-650 in humans has as yet to be determined. A recent study which set out to specifically quantifying the expression of miR-650 in 60 paired gastric cancer tissue biopsies indicates that miR-650 is associated with lymphatic and distant metastasis in human gastric cancer [Zhang *et al.*, 2010]. Furthermore, over expression of miR-650 was shown to promote clonogenicity *in vitro* and tumour growth *in vivo*. A separate investigation used a global screening approach to investigate the differences in miRNA expression profile in various clinical melanoma subtypes and found that expression of seven miRNAs, including miR-650, was significantly elevated in acral as compared to non-acral melanoma [Chan *et al.*, 2011]. Challenging its potential role as a cancer promoting 'oncomiR', a different study showed that miR-650 expression is induced by over expression of p16^{INK4a} [Chien *et al.*, 2011], a tumour suppressor that, among others, inhibits cell cycle progression [Serrano *et al.*, 1993] and induces senescence [Hara *et al.*, 1996].

miR-650 - a novel PRR-responsive miRNA?

The finding that miR-650 expression is down regulated upon TLR8 signalling in DCs suggests a novel link between miR-650 and the innate immune response. As reviewed in Section 1.3.4, PRR signalling modulates the expression of various miRNAs. A small group of miRNAs - miR-155, miR-146 and miR-21 - have emerged as the strongest targets of PRR signalling

and are ubiquitous [Quinn & O'Neill, 2011]. Other PRR-responsive miRNAs show a more specific regulatory pattern depending on the stimulus and cell type used [O'Neill *et al.*, 2011].

This chapter aims to expand this preliminary finding by characterising miR-650 expression in DCs matured with a panel of different PRR ligands. In addition, as triggering individual receptors using synthetic ligands may not entirely reflect the complex events that occur during recognition of an intact live pathogen, DCs were exposed to Influenza A virus which contains multiple PAMPs and triggers a range of different PRRs.

Influenza virus infection triggers multiple PRRs

The Influenza virus is a single-stranded RNA virus that infects both upper and lower respiratory tracts of humans. A range of innate immune cells have been shown to play a key role in Influenza A infection [McGill *et al.*, 2009]. These include natural killer cells, alveolar macrophages and alveolar as well as interstitial DCs, which reside in the respiratory tract [Vermaelen & Pauwels, 2005]. DCs are susceptible to Influenza A infection and have been shown to induce potent virus-specific CD8⁺ T cell responses *in vivo* [Bhardwaj *et al.*, 1994] [McGill *et al.*, 2009]. Following recognition of Influenza A virus, DCs initiate a strong pro-inflammatory response which is characterised by secretion of pro-inflammatory cytokines IL-12, TNF- α , IL-6, CXCL10, MIP-1 β and most importantly type I IFNs [Ronni *et al.*, 1995] [Banchereau & Steinman, 1998]. Type I IFNs activate the transcription of a range of different ISGs, such as MxA and IFITM3, which possess antiviral activity critical to the control of Influenza A virus infection [Stetson & Medzhitov, 2006] [Brass *et al.*, 2009].

Pre-activation of innate immune responses have been shown to be protective against lethal challenge with highly virulent Influenza viruses [Wong *et al.*, 2009]. However, a strong innate response, if unregulated, can also exacerbate Influenza-induced disease and immunopathology in some cases [Le Goffic *et al.*, 2006]. Hyperinduction of proinflammatory cytokines and chemokines, including TNF- α , IL-6, IFN- β , CXCL10 and CCL5, can lead to tissue damage and is characteristic for infection with highly virulent Influenza strains like 1918 H1N1 and H5N1 [Chan *et al.*, 2005].

As seen for other viruses, PRR signalling is an important feature of the innate immune response to Influenza infection [Kawai & Akira, 2007] [Wu *et al.*, 2011]. Influenza virus can be sensed by PRRs of all three different groups, TLRs, NLRs and RLHs, at different points throughout its life-cycle. It is therefore likely that different receptors and downstream signalling pathways act together to shape the immune response against the virus.

Innate immune responses against Influenza virus are primarily initiated through recog-

nitiation of viral nucleic acids by endosomal TLR3 and TLR7/8 as well as cytosolic RIG-I. TLR3 recognises dsRNA replication intermediates delivered to the endosome [Alexopoulou *et al.*, 2001]. Recent studies indicate that TLR3 is expressed on pulmonary epithelial cells and can therefore direct the immune response of respiratory epithelial cells [Guillot *et al.*, 2005]. TLR7/8 recognises the viral ssRNA genome segments in late endosomes [Lund *et al.*, 2004b] [Diebold, 2008]. Both TLR3 and TLR7/8 induce a strong proinflammatory response characterised by the secretion of IL-6, IL-12, TNF- α , CXCL10 and type I IFNs.

The role of TLR3 and TLR7/8 in Influenza infection *in vivo* remains controversial. Even though TLR3 deficient mice display reduced inflammatory mediators CCL5, IL-6 and IL-12 as well as lower numbers of CD8⁺ T cells in the lung when compared to WT mice, they show increased survival rates [Le Goffic *et al.*, 2006]. In contrast, intranasal pre-treatment of mice with the TLR3 ligand Poly(I:C) provides a high level of protection against lethal challenge with avian as well as seasonal Influenza strains [Wong *et al.*, 2009]. Similarly, mice deficient in MyD88 were more susceptible to primary Influenza infection but were fully protected against secondary homologous challenge [Seo *et al.*, 2010]. Further work investigating the role of these TLRs in Influenza infection *in vivo* showed that neither TLR3 nor TLR7/8 play an important role in T cell activation and effector function upon infection with Influenza A virus [Heer *et al.*, 2007]. However, TLR7 signalling was critical for regulating anti-Influenza B cell antibody isotype switching.

In addition to TLRs, cytosolic RIG-I (but not Mda-5) detects the viral ssRNA in infected cells, leading to a strong induction of type I IFNs [Kato *et al.*, 2006] [Le Goffic *et al.*, 2007]. It has been shown that RIG-I recognises RNA bearing 5' triphosphates produced during virus replication [Pichlmair *et al.*, 2006]. However, there is still conflicting data about whether, during natural infection *in vivo*, RIG-I associates with the full-length viral genome or short RNA fragment [Rehwinkel *et al.*, 2010] [Baum *et al.*, 2010].

Finally, the Influenza M2 protein, a selective ion channel important for viral pathogenesis, induces a disturbance of cytosolic ionic concentrations which function as DAMPs and trigger the NLRP3 inflammasome [Allen *et al.*, 2009] [Thomas *et al.*, 2009] [Ichinohe *et al.*, 2010]. NLR deficient mice show reduced levels of virus-induced inflammation in the lung and increased mortality [Thomas *et al.*, 2009]. In contrast, a separate study failed to show a link between NLRP3 and levels of survival [Ichinohe *et al.*, 2010]. The secretion of IL-1 β and IL-18 is important for the recruitment of neutrophils and monocytes into the lung to control infection. The importance of these cytokines is highlighted by the increased mortality rate in IL-1 receptor knockout mice [Schmitz *et al.*, 2005]. Similarly, IL-18 deficient mice showed increased mortality and higher viral loads after respiratory challenge with Influenza

A/PR/8/34 virus [Liu *et al.*, 2004].

Evidence for miRNA-mediated host-pathogen interaction in Influenza virus infection

There is limited knowledge about the involvement of cellular miRNAs in the pathogenesis of Influenza virus. Bioinformatic analysis shows that miR-507 and miR-136 have conserved binding sites in Influenza Polymerase B2 (PB2) and Hemagglutinin (HA) genes, respectively [Scaria *et al.*, 2006]. Microarray analysis shows that miR-136 is expressed in the lung, the main site of Influenza virus replication [Lu *et al.*, 2005]. A separate analysis revealed putative binding sites for miR-92a, miR-145 and miR-150 in the HA and PB2 genes of Swine-Origin 2009 A/H1N1 Influenza virus. Similarly, these miRNAs are highly expressed in human lung and trachea tissue. Correlation between the location from which the virus was isolated and sequence conservation implies that host miRNA expression patterns may direct the location specificity [He *et al.*, 2009]. However, so far none of these direct interactions between cellular miRNAs and Influenza virus have been experimentally confirmed. A separate study showed that miR-323, miR-491 and miR-644 inhibit the replication of H1N1 Influenza A virus through binding to the PB1 gene resulting in its degradation [Song *et al.*, 2010]. However, in this case nothing is known about the expression level of these miRNAs in various cell types or their regulation throughout infection. Both studies provide evidence for anti-Influenza miRNAs expressed by the host, however their physiological significance and impact on disease progression remain to be explored.

In order to explore the role of miRNA expression on disease progression of different Influenza strains, Li and colleagues used mice as *in vivo* infection model. Global miRNA profiling of lung tissue revealed that miR-200a and miR-233 were differentially regulated in mice infected with the virulent 1918 H1N1 virus, compared to mice infected by a nonlethal seasonal Influenza virus A/Texas/36/91. A search for miRNA targets in matching gene expression data suggested that this miRNA cluster might contribute to the extreme virulence of the pandemic 1918 Influenza virus [Li *et al.*, 2010b]. However, the exact link between miRNAs and the virulence of different Influenza A virus strains remains to be explored.

4.2 Aims

This Chapter aims to:

- Investigate whether miR-650 expression is indeed regulated by TLR8 signalling triggered by viral nucleic acid.
- Investigate whether regulation of miR-650 expression is ubiquitous or limited to specific PRR signalling pathways and thus to a selective group of pathogens.
- Investigate whether infection of DCs with a live pathogen, in this case Influenza A virus, leads to differential miR-650 expression.

4.3 Results

4.3.1 Investigating the effect of TLR8 signalling on miR-650 expression

In order to investigate whether TLR8 signalling leads to down regulation of miR-650, DCs were stimulated with varying doses of ssRNA40, ssRNA41 or LyoVec (Figure 4.2). As mentioned in the previous chapter, ssRNA40 is a short RNA fragment derived from the HIV-1 LTR, which has been shown to signal through TLR8 [Heil *et al.*, 2004] [Alter *et al.*, 2007]. In contrast, ssRNA41 is derived from ssRNA40 by replacement of all U nucleotides with A and subsequently fails to induce DC maturation; hence, ssRNA41 serves as negative control. An additional control used in this experiment is LyoVec, a cationic lipid that is complexed to both ssRNA40 and ssRNA41. LyoVec protects the ssRNA from degradation and facilitates its cellular uptake.

Expression of miR-650 and miR-155 was measured by qRT-PCR 24 hrs post-stimulation and the results are displayed in Figure 4.3. As mentioned previously, miR-155 expression has been shown to be induced downstream of a variety of PRR signalling pathways [O'Neill *et al.*, 2011]. Therefore, stimulation of DCs with ssRNA40 for 24 hrs leads to induction of miR-155 expression in a dose dependent manner (Figure 4.3(A)). As indicated in Chapter 3, stimulation of DCs with ssRNA40 resulted in the down regulation of miR-650 expression in a dose dependent manner (Figure 4.3(B)). Both the induction of miR-155 and the down regulation of miR-650 observed in these experiments are TLR8-specific, as incubation of DCs with either ssRNA41 or Lyovec for 24 hrs has no effect on the expression of miR-155 or miR-650.

(A) ssRNA40 5'-GCCCCGUCUGUUGUGUGACUC-3'

(B) ssRNA41 5'-GCCCCGACAGAAGAGAGACAC-3'

Figure 4.2: TLR8 ligands. (A) ssRNA40: U-rich ssRNA derived from the HIV-1 LTR, and (B) ssRNA41: derived from ssRNA40 by replacement of all U nucleotides with A.

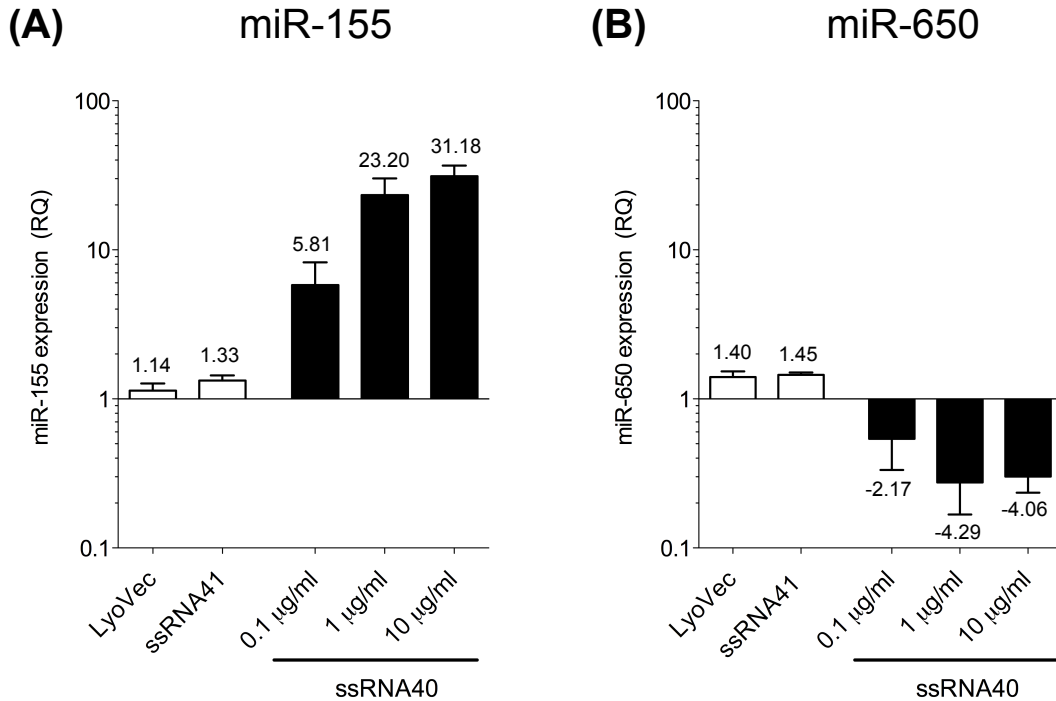


Figure 4.3: Expression of miR-155 and miR-650 in DCs stimulated with TLR8 ligand ssRNA40. DCs were stimulated for 24 hrs with ssRNA40 (0.1 µg/ml, 1 µg/ml, 10 µg/ml), ssRNA41 (10 µg/ml) or LyoVec (10 µg/ml). Expression of (A) miR-155 and (B) miR-650 was measured by qRT-PCR. Expression is normalised against RNU48 and is shown relative to unstimulated DCs. Numbers above bars represent fold changes. Data is from three different donors and is shown as mean ± SEM.

In order to confirm that miR-650 down regulation is indeed dependent on TLR8, DCs were incubated with chloroquine and mianserin hydrochloride to abrogate signalling via endosomal TLRs, including TLR8 [Hart *et al.*, 2005] (Figure 4.4). Chloroquine is thought to be a lysosomotropic agent that prevents endosomal acidification and therefore blocks signalling

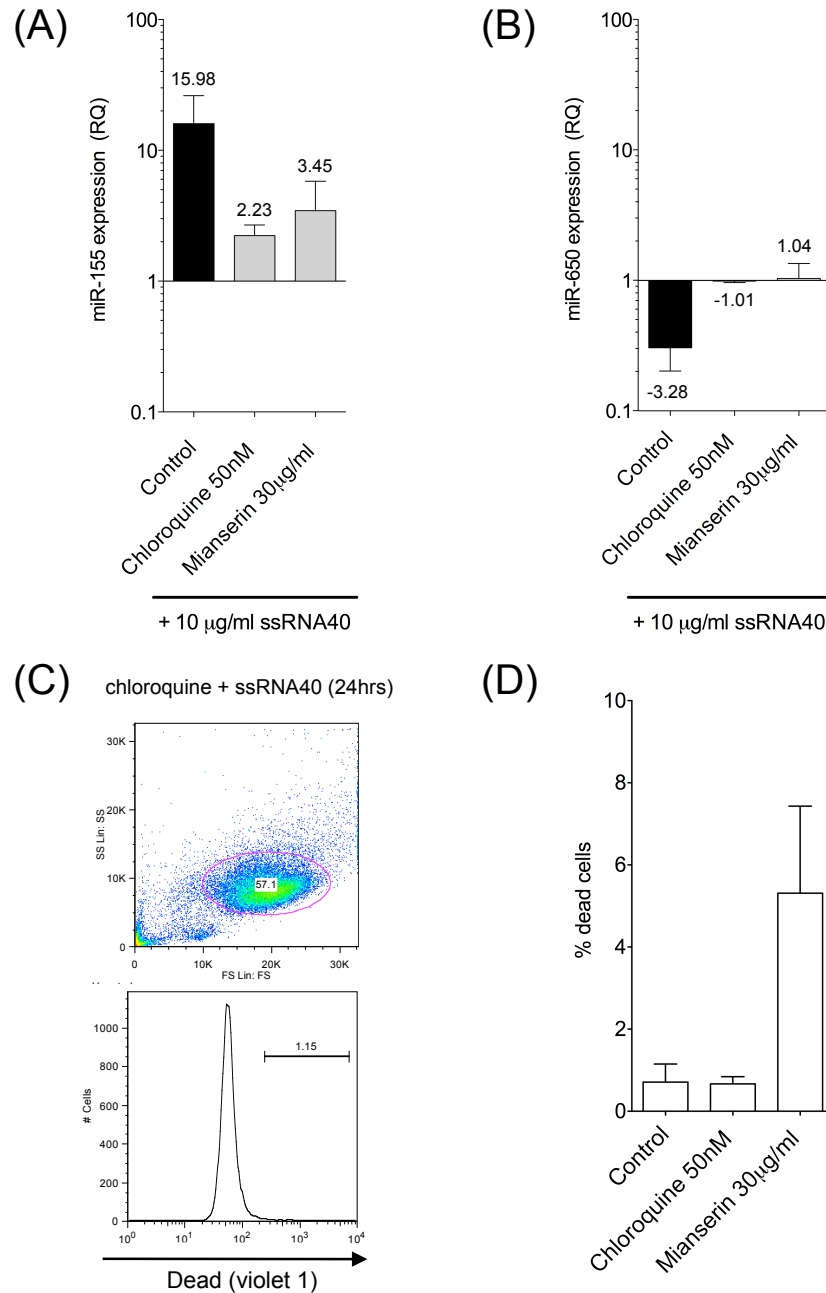


Figure 4.4: Exploring the effect of TLL8 inhibition on downstream miRNA regulation. DCs were pre-incubated with chloroquine (50 nM) or mianserin hydrochloride (30 µg/ml) for 30 min prior to stimulation with ssRNA40 (10 µg/ml). After 24 hrs the expression of **(A)** miR-155 and **(B)** miR-650 was measured by qRT-PCR. Expression is normalised against RNU48 and expressed relative to DCs incubated with LyoVec (10 µg/ml). Numbers above bars represent fold changes. In **(C)** and **(D)** cell death was assessed after 24 hrs by flow cytometry using the LIVE/DEAD Viability Violet kit (Invitrogen). DCs were gated according to size, and dead DCs were identified as violet 1 positive population. While chloroquine treatment did not impact on cell viability, mianserin induced a mild level of cell death. Data is from three different donors and is shown as mean $\pm$ SEM.

of endosomal TLRs [Steinman *et al.*, 1983]. In addition, a recent report suggests that chloroquine inhibits the signalling of nucleic acid through endosomal TLRs by masking the PAMP [Kuznik *et al.*, 2011]. While it inhibits nucleic acid-induced signalling, it augments activation of TLR8 by the small synthetic compound R848. Mianserin exhibits anti-inflammatory activity *in vivo* [Leitch *et al.*, 1995] and abrogates signalling via endosomal TLRs *in vitro* [Sacre *et al.*, 2008]. More specifically, it competes with R848 and therefore blocks signalling at the receptor level; however, its exact mechanism of action remains unknown. Pre-treatment of DCs with 50 μ M chloroquine or 30 μ g/ml mianserin hydrochloride for 30 minutes (according to [Sacre *et al.*, 2008]) prior to the addition of ssRNA40 prevents down regulation of miR-650 expression and inhibits the induction of miR-155 (Figure 4.4(A) and (B)). To confirm that the loss of miRNA regulation is not due to extensive cell death, flow cytometry was used to monitor the proportion of dead cells in each sample. Figure 4.4(C) and (D) show that chloroquine treatment did not result in significant cell death, while treatment with mianserin increased the total proportion of dead cells to approximately 5%. However, this is not enough to account for the above observed effect. Together, these data indicate that signalling via TLR8 increases the levels of miR-155 while simultaneously decreasing expression of miR-650 in monocyte-derived DCs.

4.3.2 Characterization of miR-650 expression in DCs stimulated with a panel of different pathogen-derived PAMPs

As mentioned previously, miR-155 expression is induced in an NF- κ B dependent manner and therefore controlled by a variety of different inflammatory stimuli. In order to investigate whether the decrease in miR-650 expression is limited to sensing viral ssRNA via TLR8 in the late endosome, or whether it is downstream of various PRR signalling pathways, DCs were exposed to a panel of bacterial and viral PRR ligands (for concentrations see Section 2.5.6). After 24 hrs, DC maturation was monitored by flow cytometric measurement of activation markers CD80, CD83 and CD86. In addition, RNA was extracted and miR-650 expression was measured by qRT-PCR. Figure 4.5 shows that miR-650 down regulation is not limited to TLR8 signalling. In fact, miR-650 levels were reduced in DCs stimulated through a variety of different PRRs, irrespective of their cellular localization (cell surface, late endosome or cytoplasm) or ligand source (bacterial or viral).

Due to the different ligand concentrations used, the nature of the derivatives and their stimulatory capacity, a direct quantitative comparison is rather difficult. Stimulation with LPS and ssRNA40 induced the strongest response (Figure 4.5(A)). This was not surprising as

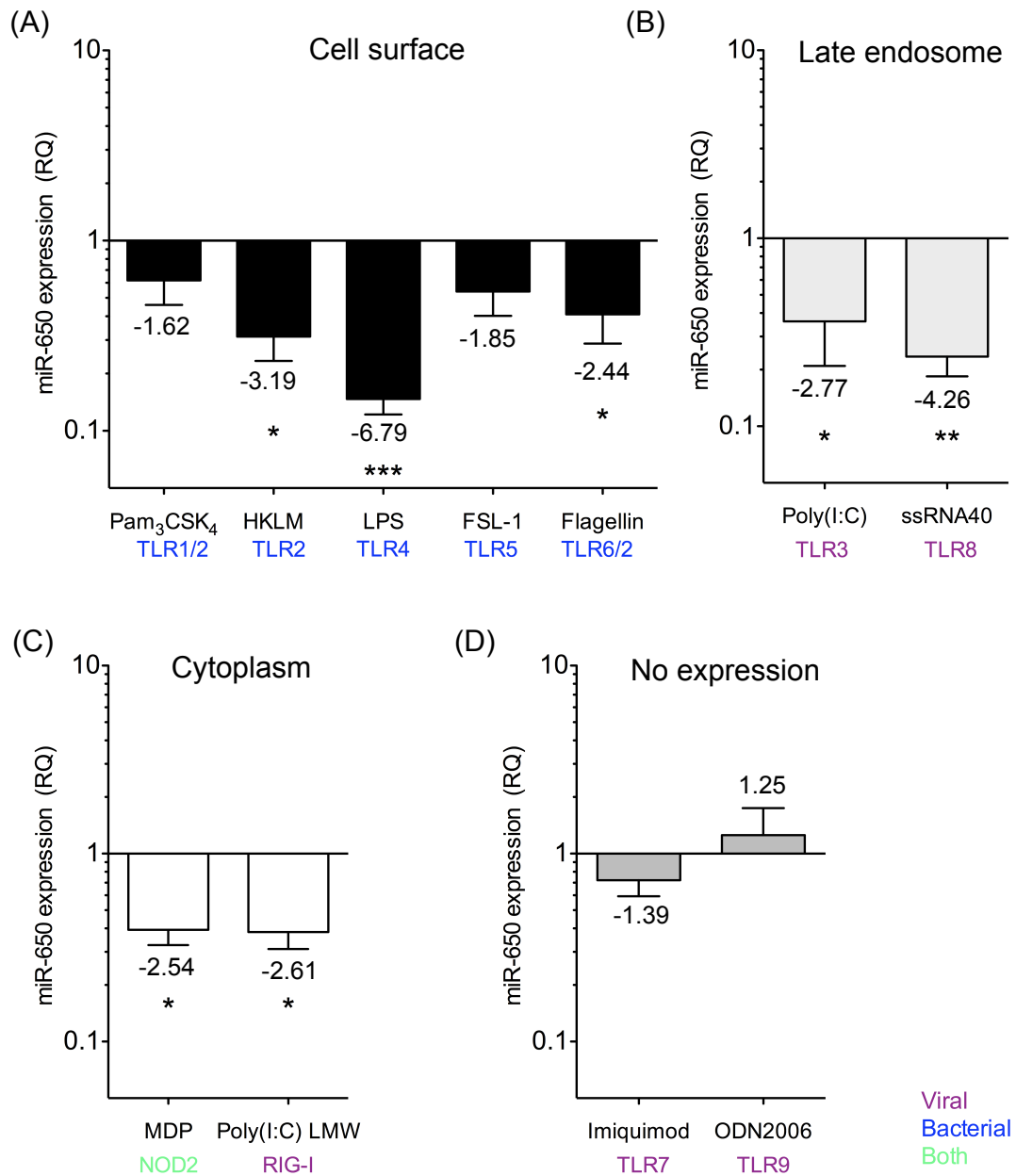


Figure 4.5: Measuring miR-650 expression in DCs stimulated with a panel of different PAMPS. DCs were stimulated with a panel of different PAMPS (for concentrations see Section 2.5.6) and miR-650 expression was measured after 24 hrs by qRT-PCR. Reduced miR-650 expression was observed in response to signalling through a range of PRRs expressed located (A) on the cell surface, (B) in the endosome or (C) in the cytoplasm. (D) Stimulation of DCs with TLR7 and TLR9 ligands had no effect on the level of miR-650 expression. Expression is normalised against RNU48 and is shown relative to unstimulated DCs. Data are from three different donors and are shown as mean $\pm$ SEM. Statistical analysis was performed using a paired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

these two ligands are generally the most potent inducers of DC maturation in this system. In contrast, stimulation with Imiquimod and CpG DNA (ODN2006) failed to regulate miR-650 expression (Figure 4.5(D)). Again, this result was expected as monocyte-derived DCs lack expression of TLR7 and TLR9, and therefore fail to respond to Imiquimod or CpG DNA, respectively [Gorden *et al.*, 2005].

Further analysis was carried out to explore the relationship between miR-650 expression and DC maturation state. In each instance, the relative miR-650 expression on the X-axis is plotted against the expression of DC maturation markers CD80, CD83 or CD86 as measured by flow cytometry (Figure 4.6). The data, shown in Figure 4.6, indicates an inverse correlation between DC maturation and miR-650 expression. This implies that miR-650 is down regulated in a dose dependent manner; the greater the effective concentration of a ligand, the higher the expression of maturation markers, and the lower the expression of miR-650.

These data clearly show that triggering of DC maturation via a range of different PRRs reduces the expression of miR-650, confirming that this effect is not restricted to sensing of viral nucleic acids via TLR8.

4.3.3 Investigation into the molecular mechanism controlling miR-650 expression downstream of PRR signalling

The transcription of miRNA genes is regulated similarly to that of protein-coding genes, i.e. through the action of transcription factors and other regulatory proteins. The expression of most PRR-responsive miRNAs described so far is regulated on a transcriptional level by NF- κ B activity. For example, infection of human epithelial cells with *Cryptosporidium parvum*, a parasite that causes intestinal disease, reduces the levels of primary let-7i and mature let-7 in an NF- κ B dependent manner [Chen *et al.*, 2007]. Similarly, down regulation of miR-125b in response to TLR4 signalling is suggested to be under control of NF- κ B [Tili *et al.*, 2007]; however, this remains controversial [O'Neill *et al.*, 2011]. In light of these findings, I sought to explore whether miR-650 is regulated on a transcriptional level, and whether down regulation is dependent on NF- κ B activation.

Firstly, qRT-PCR was used to assess whether the primary miR-650 transcript, which contains the mature miR-650 sequence, follows a similar expression pattern to its mature form. Unfortunately however, amplification of the primary transcript by qRT-PCR failed. As for many miRNAs, the exact pri-miRNA sequence for miR-650 is undefined. However, the primers used in this experiment are commercially available and were designed to closely flank

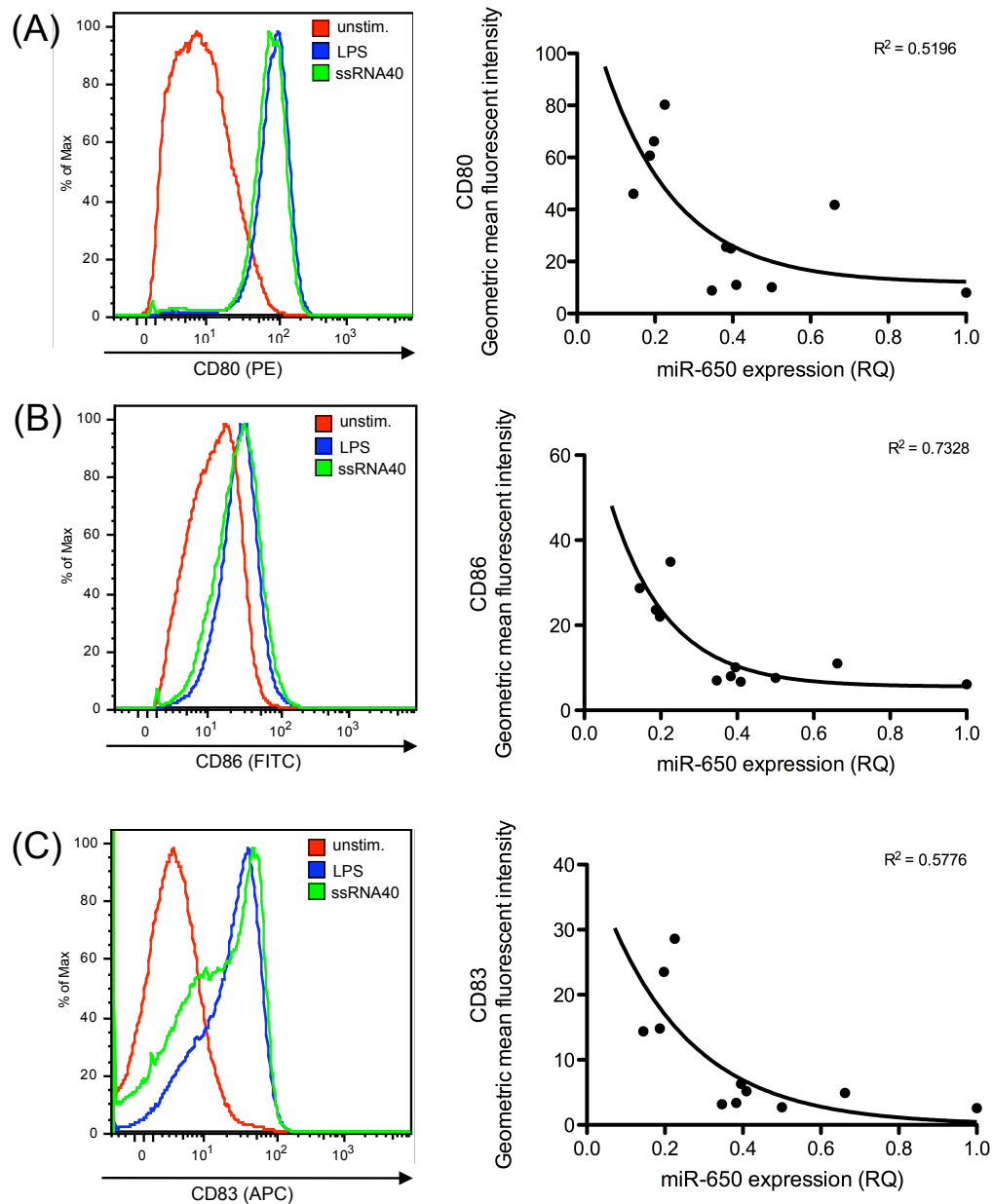


Figure 4.6: Correlation between miR-650 and the maturation state of the DC, as characterised by the expression of maturation markers on the cell surface. DCs were stimulated with a panel of PRR ligands (see Figure 4.5) and cell surface expression of (A) CD80, (B) CD83 and (C) CD86 was measured by flow cytometry. Each panel shows an example FACS plot displaying the expression of the relevant maturation marker on the surface of DCs stimulated with LPS, ssRNA40 or left unstimulated. Finally, the mean fluorescent intensity for each condition (11 in total) is plotted against the relative miR-650 expression. Non-linear regression shows an inverse correlation between the expression of maturation markers on the surface of DCs and the relative level of miR-650 expression. Data is representative of one out of three donors.

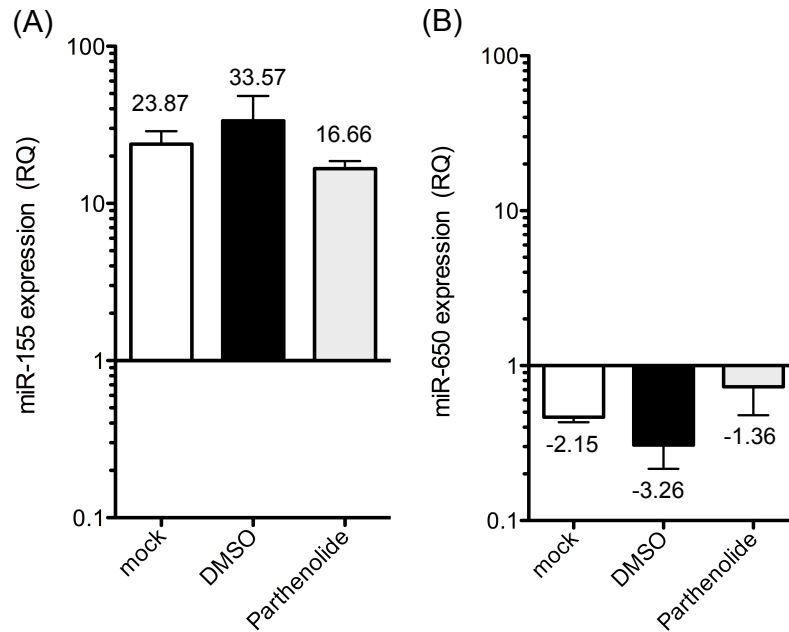


Figure 4.7: Investigating the effect of NF- κ B inhibition on TLR8-induced miRNA regulation. DCs were pre-incubated in Parthenolide (10 μ M in DMSO) or the equivalent amount of DMSO for 1 hr prior to stimulation with ssRNA40 (10 μ g/ml). After 24 hrs the expression of (A) miR-155 and (B) miR-650 was measured by qRT-PCR. Preincubation of DCs in Parthenolide partially inhibits miRNA regulation downstream of TLR8 signalling. Expression is normalised against RNU48 and expressed relative to DCs incubated with LyoVec (10 μ g/ml). Numbers above bars represent fold changes. Data is from three different donors and is shown as mean $\pm$ SEM.

the mature miRNA sequence (Applied Biosystems). Nevertheless, due to the lack of positive controls as well as the lack of publications using these specific primers, it is difficult to assess whether they are actually functional. In addition, rapid processing of the pri-miRNA might make it difficult to detect this transcript.

As a consequence another approach was taken; DCs were incubated with the NF- κ B inhibitor Parthenolide (10 μ M in DMSO) for 1 hr prior to stimulation with ssRNA40 (10 μ g/ml). At 24 hrs post-stimulation, DCs were harvested and miRNA expression was measured using qRT-PCR. The results displayed in Figure 4.7 indicate that treatment with Parthenolide inhibits TLR8-mediated miRNA regulation by approximately 50%, compared to the DMSO control. The remaining regulation may be a result of incomplete NF- κ B inhibition or may indicate an additional regulatory mechanism.

In addition to transcriptional control, it may be possible that PRR signalling activates the turnover of mature miR-650. While much is known about the transcriptional regulation

of miRNA expression, less is known about miRNA decay. Similar to mRNAs, miRNAs are subject to post-transcriptional regulatory mechanisms that affect their stability. In contrast to mRNA and pri-miRNAs, mature miRNAs as well as pre-miRNAs lack the 5' m7GpppN cap structure and the 3' poly(A) tail. These unprotected ends may render them susceptible to cleavage by exoribonucleases. The small RNA degrading nuclease (SDN1) has been implicated in the degradation of mature miRNAs in plants [Ramachandran & Chen, 2008]. Similarly, a study in *C. elegans* showed that the exoribonuclease XRN-2 is involved in the degradation of mature miRNAs [Chatterjee & Grosshans, 2009]. However, its role in human miRNA turnover has not been fully investigated yet. The possible involvement of XRN-2 in PRR-mediated down regulation of miR-650 expression was investigated by transfecting DCs with XRN-2 specific siRNAs 24 hrs prior to stimulation with ssRNA40 (10 μ g/ml). However, as shown in Figure 4.8(A), TLR8-mediated down regulation of miR-650 was still observed despite 75% knockdown of XRN-2 Figure 4.8(B).

These results indicate that miR-650 expression is controlled, at least in part, on a transcriptional level in an NF- κ B dependent manner. Furthermore, levels of mature miR-650 are not susceptible to post-transcriptional regulation by the exoribonuclease XRN-2.

4.3.4 Exploring the effect of Influenza A virus infection on miR-155 and miR-650 expression in DCs

The data shown so far demonstrate that PRR signalling leads to increased levels of miR-155 and decreased expression of miR-650 in monocyte-derived DCs. Moreover, this effect is, at least in part, dependent on the activation of NK- κ B.

In order to verify these findings in the context of an intact pathogen which has the capability of triggering multiple PRRs, an *in vitro* infection model of DCs with Influenza A/Puerto Rico/8/34 (PR8) was chosen. This Influenza strain has been shown to infect DCs and trigger maturation, characterised by the up regulation of MHC, adhesion and co-stimulatory molecules as well as the rapid production of type I IFNs and MxA expression [Cella *et al.*, 1999]. In order to monitor the effect of Influenza A virus infection on miRNA expression, 3×10^6 immature DCs were resuspended in 300 μ l RPMI supplemented with Trypsin and were infected with three different doses (0.04, 4 and 400 HAU) of PR8 virus. Unbound virus was washed off after 2 hrs and DCs were resuspended in complete media at 10^6 cells/ml R10. After 48 hrs the proportion of viral hemagglutinin (HA) expressing DCs were assessed by flow cytometry (Figure 4.9). As shown in Figure 4.9(A), the proportion of HA positive cells was dependent on the viral dose used. After assessing the different levels of

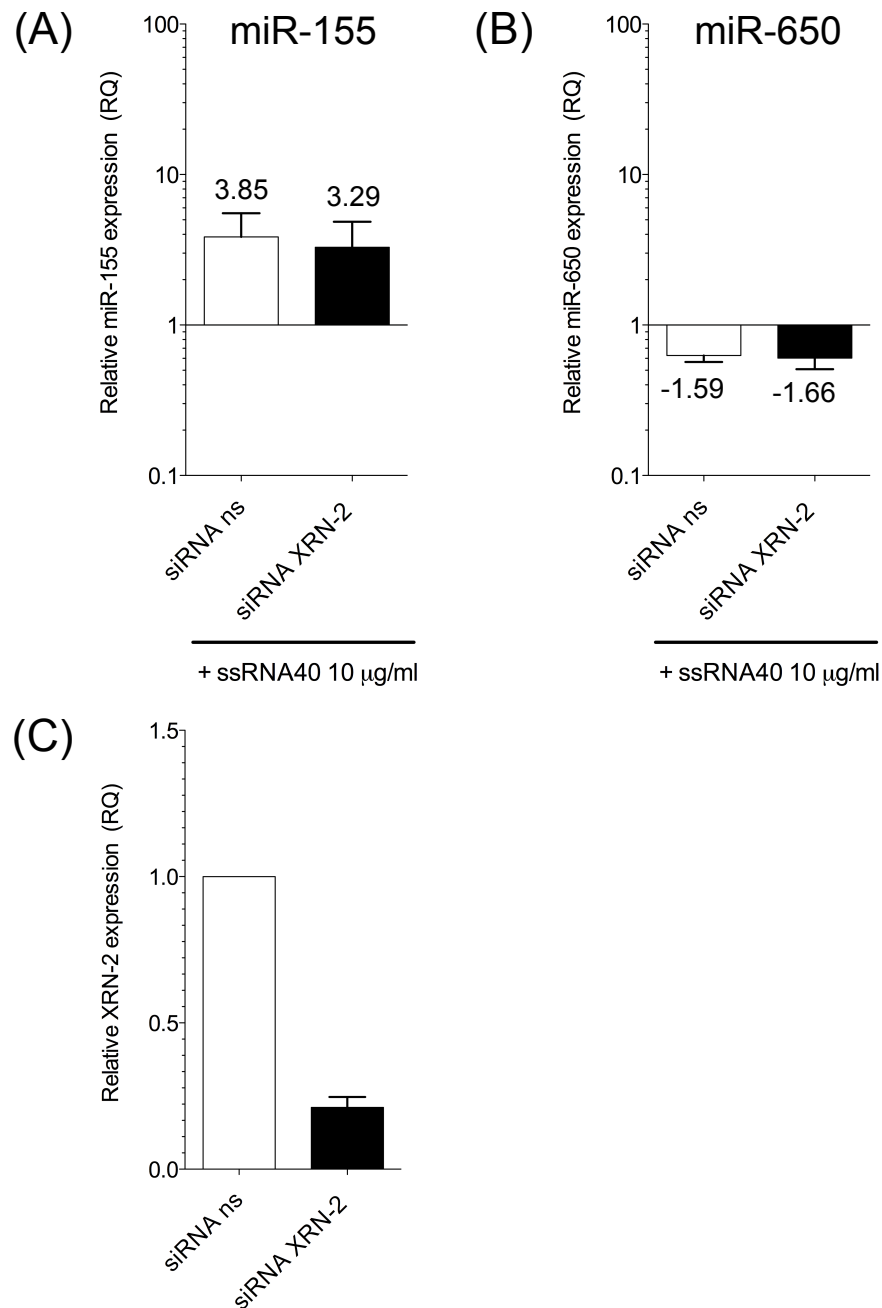


Figure 4.8: Investigating the effect of XRN-2 on TLR8-induced regulation of miR-155 and miR-650. DCs were transfected with XRN-2 siRNA using nucleofection 24 hrs prior to stimulation with ssRNA40 (10 μ g/ml). 24 hrs after stimulation the expression of **(A)** miR-155 and **(B)** miR-650 was assessed by qRT-PCR. Expression is normalised against RNU48 and expressed relative to DCs incubated with LyoVec (10 μ g/ml). Numbers above bars represent fold changes. **(C)** qRT-PCR of XRN-2 mRNA 24 hrs after siRNA transfection. Expression is normalised against GAPDH. Data is from three different donors and is shown as mean $\pm$ SEM.

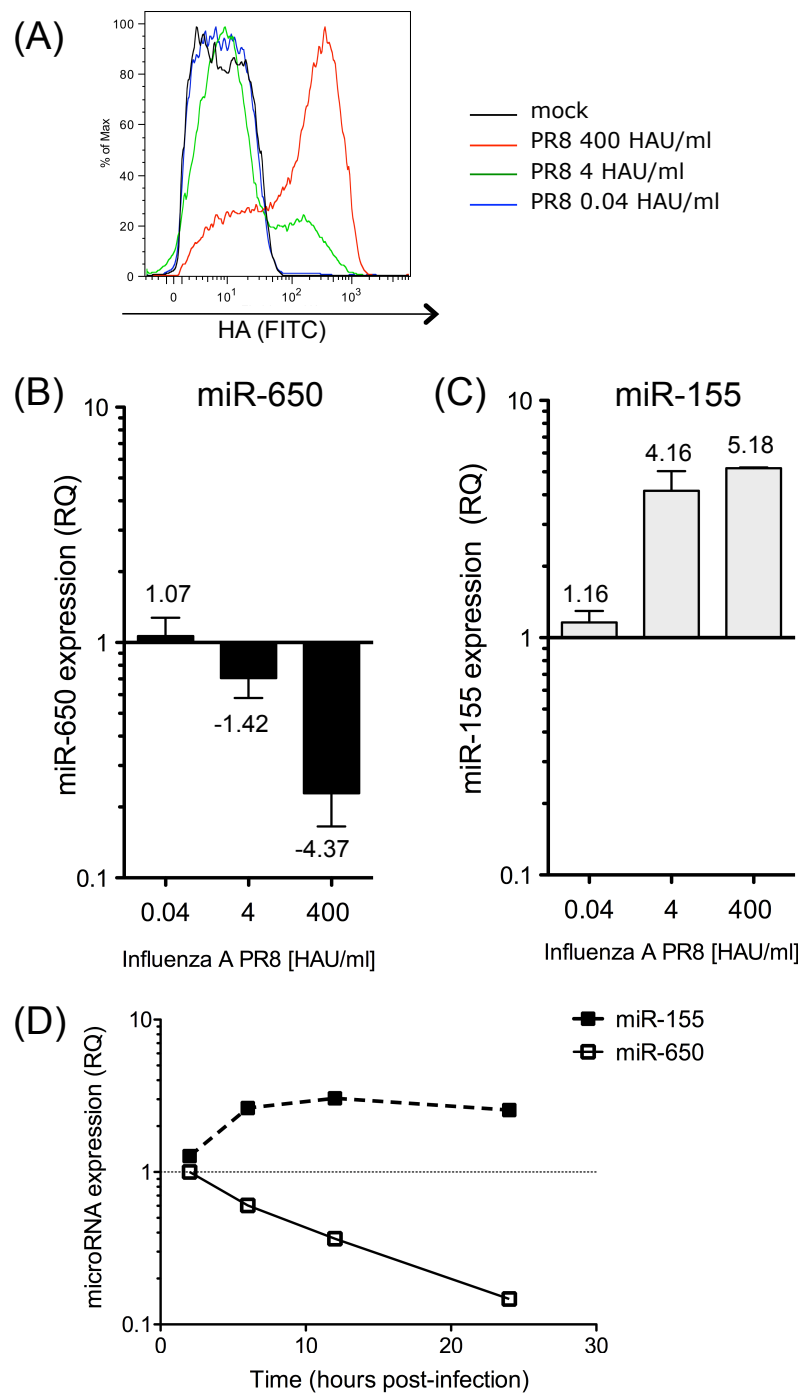


Figure 4.9: Exploring the effect of Influenza A virus infection on the expression of miR-155 and miR-650 in DCs. DCs were infected with three different doses of Influenza A/Puerto Rico/8/34. **(A)** Viral hemagglutinin expression was measured by flow cytometry 48 hrs post-infection. Simultaneously expression of **(B)** miR-650 and **(C)** miR-155 was measured by qRT-PCR. Expression is normalised against RNU48 and expressed relative to uninfected DCs. Numbers above bars represent fold changes. Data is from three different donors and is shown as mean $\pm$ SEM. **(D)** Time course of miRNA regulation in DCs infected with Influenza A virus (40 HAU/ml). Expression is normalised against RNU48 and expressed relative to uninfected DCs. One representative donors is shown.

infection, RNA was extracted for miRNA quantification by qRT-PCR. In agreement with the data shown above, infection with Influenza A virus induced the expression of miR-155 and reduced the levels of miR-650 in a dose dependent manner (Figure 4.9(B) and (C)). Hence a relationship between the fraction of infected cells and the strength of miRNA was observed.

Similar to other PRR responsive genes, miRNAs can be classified into early and late response genes [O'Neill *et al.*, 2011]. To assess the kinetics of miRNA induction, DCs were infected with Influenza A virus at a concentration of 40 HAU/ml and miRNA expression was followed over a 48 hrs time period using qRT-PCR. Figure 4.9(D) displays the relative changes in miR-155 and miR-650 expression over time. Whereas miR-155 expression reaches a plateau after approximately 6 hrs, the expression of miR-650 continues to decline throughout the measured time period.

These experiments confirm that miR-650 is down regulated in response to Influenza A virus infection of DCs *in vitro*. However, the exact PRR repertoire involved in Influenza A virus-induced regulation of miRNAs remains to be defined.

4.3.5 Assessing into the effect of miR-650 on cytokine secretion

After establishing that DCs stimulated with a variety of PAMPs express lower levels of miR-650, I decided to investigate whether miR-650 plays a role in the outcome of DC maturation by exploring the effects of miR-650 on cytokine secretion.

The function of a specific miRNA can be explored by manipulating its expression in the biological system under investigation. For this purpose, specific miRNA mimics and inhibitors have been developed: miRNA mimics represent short dsRNA fragments which are identical to the mature miRNA duplex, whereas inhibitors are short antisense oligonucleotides with perfect complementarity to the mature miRNA target sequence (also referred to as antagomiRs). Binding of an antisense inhibitor to its complementary mature miRNA renders the miRNA unavailable for target binding and targets it for degradation. In order to exclude non-specific off-target effects, oligonucleotides with nonsense sequence, which exhibit no complementarity to any known miRNA, are used as control.

The impact of miR-650 expression on cytokine and chemokines secretion was investigated by transfecting immature DCs with either miR-650 mimic or inhibitor (82.5 nM final concentration) using the Amaxa nucleofection system as described in [Cooney *et al.*, 2010]. Briefly, DCs were harvested at day 5 and 3×10^6 DCs were resuspended in 100 μ l transfection solution. After addition of the miR-650 mimic or inhibitor, DCs were transfected with the nu-

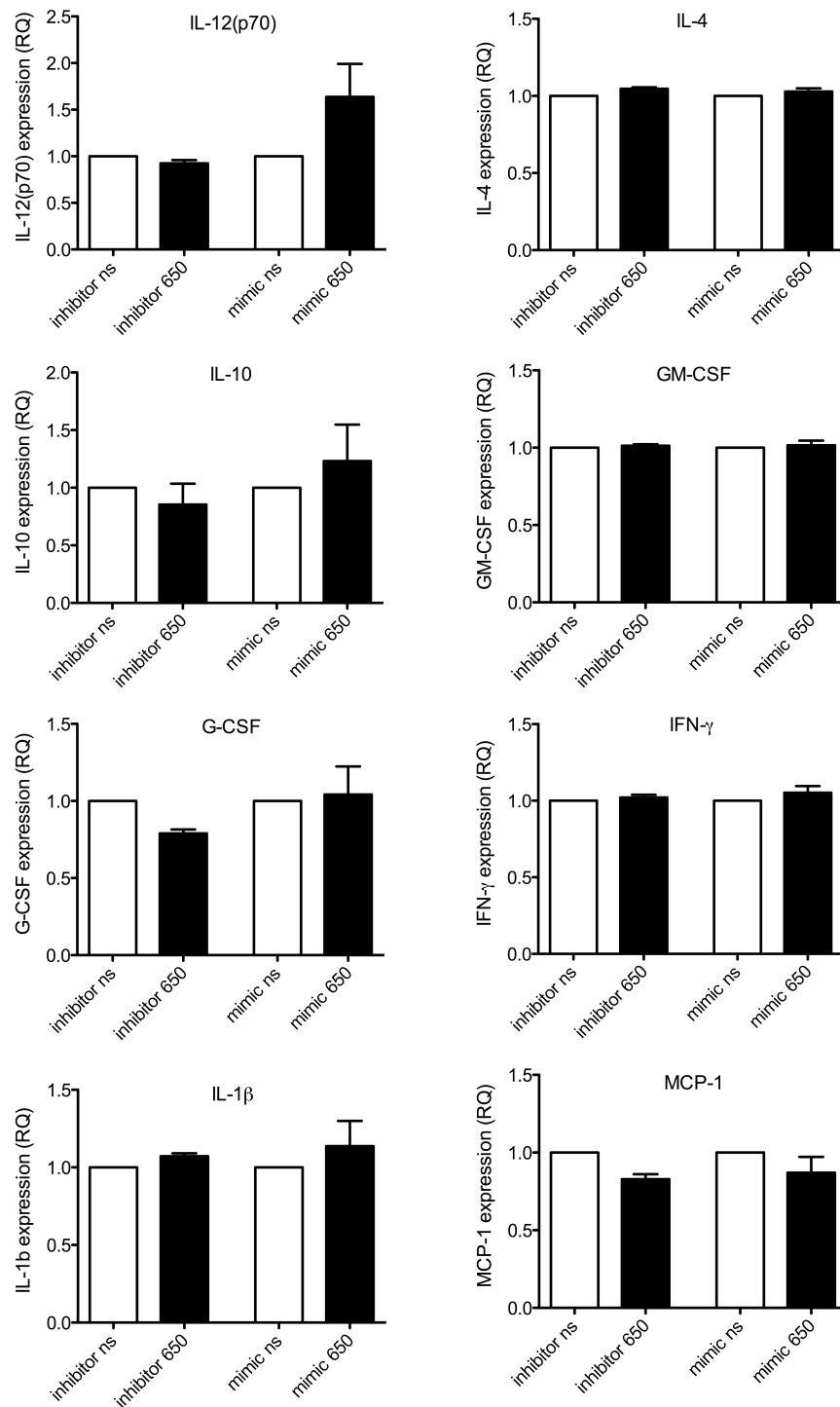


Figure 4.10: Effect of miR-650 expression on the secretion of selected cytokines as measured by Bio-Plex assay. DCs were transfected with miR-650 inhibitor or miR-650 mimic or respective nonsense controls (82.5 nM final concentration) 24 hrs prior to stimulation with ssRNA40 (10 μ g/ml). After 24 hrs supernatants were collected and tested for selective cytokines by Bio-Plex assays. Cytokine levels are shown relative to DCs transfected with control mimic or inhibitor. Data is from two different donors and is shown as mean $\pm$ SEM.

cleofector system using program U-02. Directly after electroporation, DCs were resuspended in 3 ml warm R10 medium. After 24 hrs, transfected DCs were washed and stimulated with 10 $\mu\text{g}/\text{ml}$ ssRNA40. Supernatants were collected at 24 hrs post-stimulation and a variety of cytokines were measured by Luminex assay. Figure 4.10 summarises the results obtained for the inflammatory mediators which were in the detectable concentration range. The secretion of many cytokines, such as IFN- γ , GM-CSF, IL-4 is not affected by modulation of miR-650 expression. Moreover, certain cytokines, such as IFN- γ and GM-CSF, have not shown to be linked to the maturation of monocyte-derived dendritic cells. The expression of MCP-1 was mildly reduced in supernatants of DCs transfected with miRNA mimic as well as miRNA inhibitor. However, a group of cytokines, including IL-10, IL-12(p70) and G-CSF, indicated reciprocal expression in response to miR-650 over expression and knockdown. The expression of these cytokines is slightly elevated in the supernatant of cells transfected with miR-650 mimic, compared to miR-650 inhibitor. Although no statistically significant changes were observed (paired t-test), it is highly speculative to assume that PRR-mediated down regulation of miR-650 could be involved in a negative feedback loop to control the expression of IL-10, IL-12(p70) and G-CSF.

4.4 Discussion

This chapter describes the discovery of miR-650 as a novel PRR-responsive miRNA which is down regulated in monocyte-derived DCs. Expression analysis provides evidence that miR-650 is modulated downstream of a range of different PRR signalling pathways, irrespective of their cellular location or the origin of their ligand.

Due to differences in concentration and stimulatory potential of each ligand, comparisons between the stimulation conditions are not possible. However, when correlating the level of miR-650 to the induction of DC activation markers on the cell surface, an inverse correlation was observed, suggesting a relationship between miR-650 down regulation and the effective dose of the ligand.

Importantly, infection of DCs with an intact pathogen, Influenza A virus, down regulates the levels of miR-650 while inducing the expression of miR-155 and therefore supports the above described finding. Influenza A infection gives rise to a range of different PAMPs throughout its life cycle, and further work is required to define the exact PRR repertoire responsible for modulating miR-650 expression. It is important to note that infection of DCs with Influenza A virus only provides one possible model of viral infection *in vitro*.

Considerably more work will be required to systematically characterise the role of miR-

650 in the innate immune response against invading pathogens. This could potentially include exploring the behaviour of miR-650 expression in different innate immune cells such as macrophages, pDCs or even CD103⁺ mucosal DCs, in response to a variety of inflammatory triggers, including intact pathogens but also other inflammatory mediators such as cytokines and DAMPs.

The exact signalling pathway that regulates miR-650 expression downstream of PRR signalling remains to be defined. Modulation of miR-650 expression occurs downstream of a variety of different signalling pathways thus suggesting a common regulatory mechanism. Preliminary results suggest that miR-650 is, at least partially, regulated on a transcriptional level which involves activity of NF- κ B. The role of NF- κ B in regulating miRNA expression is often investigated by over expressing dominant negative NF- κ B mutants. This approach was unfeasible in primary monocyte-derived DCs, as transfection of plasmid DNA by electroporation resulted in high levels of cell death. As a consequence, additional NF- κ B inhibitors could be used to consolidate this finding. Alternatively, further studies could include a targeted siRNA approach to identify the key players that control miR-650 expression downstream of PRR signalling. As miR-650 overlaps with the leader exon of clan II *IGVL* genes and is thought to use the same promoter [Das, 2009], a promoter analysis may provide further insight into the transcription factors involved in regulating its transcription.

Finally, the main question that arises from these findings is: what function does miR-650 play in the immune response of DCs? A preliminary experiment showed that miR-650 may have a small role in modulating the expression of IL-10, IL-12(p70) and G-CSF. Though effects were very subtle, down regulation of miR-650 resulted in decreased levels of these cytokines potentially providing a negative feedback loop.

4.5 Conclusion

In conclusion, recognition of pathogenic motifs by DCs results in decreased expression of miR-650. This effect is ubiquitous, i.e. not limited to a specific group of pathogens or a particular type of PRR. This observation indicates that miR-650 may play a role in the innate immune response of DCs. However, so far only a single miR-650 target has been validated and the biological function of miR-650 is largely unknown. As a consequence, the next two chapters will focus on identifying targets of miR-650 and cellular pathways regulated by this miRNA.

Chapter 5

Identification and validation of individual miR-650 targets

5.1 Introduction

As explained in Section 1.3.3, miRNAs bind to specific targets sites also referred to as miRNA response elements (MREs), found in mRNAs of specific target genes and regulate their expression on a post-transcriptional level. To understand the biological function of a particular miRNA, it is therefore important to identify its targets. Due to the short length of miRNAs, the limited complementarity with their target sequence and their complex mechanism of action, the development of precise and fast target identification methods has been challenging. As a consequence, prediction and validation of genuine miRNA targets provides the main obstacle for miRNA understanding [Thomas *et al.*, 2010] [Huang *et al.*, 2010].

Principles of miRNA target recognition

MicroRNAs classically bind to complementary sites within the 3'UTR of their target mRNAs. The study of experimentally validated miRNA:mRNA pairs suggests that sequence complementarity is the main determinant governing the interaction between the miRNA and its target mRNA. In plants, miRNAs bind to their target site with perfect sequence complementarity and, as a consequence, targets can be identified with relatively high reliability [Jones-Rhoades & Bartel, 2004]. However, in vertebrates, miRNAs bind to their target sites with partial sequence complementarity. More specifically, miRNA binding is primarily governed by the miRNA seed sequence, which is defined as the 7 consecutive nucleotides starting from either the first or second base at the 5' end of the miRNA, and is required to guide

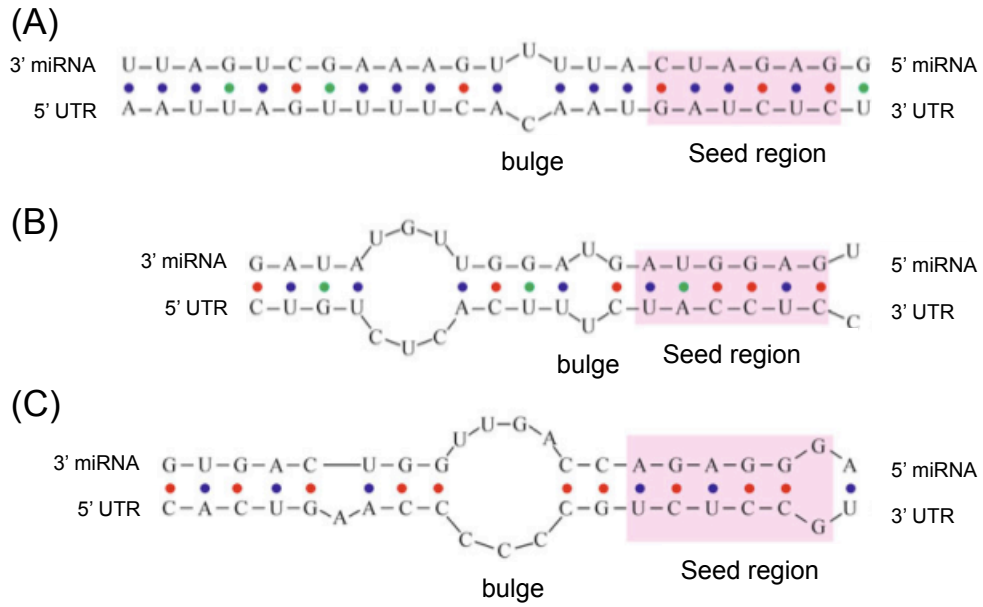


Figure 5.1: Three main types of miRNA:mRNA duplexes. (A) Good binding in the 5' seed region as well as at the 3' end with a characteristic bulge in the middle. (B) Perfect seed complementarity but limited 3' end binding. (C) mismatches in 5' seed which are compensated for by strong binding of the 3' end. Diagram adapted from [Huang *et al.*, 2010].

the RISC complex to its target mRNA [Stark *et al.*, 2003] [Lewis *et al.*, 2003]. In addition, complementarity at the 3' end of the miRNA may also contribute to the binding.

In general, three different types of target sites have been described so far (displayed in Figure 5.1):

- 5' dominant canonical characterised by perfect base pairing at both 5' seed and 3' end of the miRNA with a characteristic bulge in the middle.
- 5' dominant seed only characterised by perfect base pairing at 5' seed but poor 3' end complementarity.
- 3' compensatory which contain a mismatch or wobble in the 5' seed region which is compensated for by extensive complementarity at the 3' end [Brennecke *et al.*, 2005].

Studies in *Drosophila* suggest that the extent of overall complementarity determines the mechanism by which gene expression is silenced [Hutvagner & Zamore, 2002]. If the miRNA binds with perfect or near-perfect complementarity, the target mRNA is cleaved and degraded. On the other hand, partial base-pairing between the miRNA and its target results in inhibition of translation [Bartel, 2004]. However, as will be discussed shortly, recent studies indicate that this mechanism is more complex than initially thought.

***In silico* miRNA target prediction**

Since high-throughput methods for miRNA target identification are currently lacking, computational tools based on sequence complementarity between miRNAs and their respective target sites remain the major source for rapid identification of putative miRNA targets [Bentwich, 2005]. However, due to the short sequence of the miRNA seed, simple seed searches give rise to a large number of putative targets and are prone to high false-positive rates. Furthermore, although seed pairing is the most striking feature, other features have also been shown to affect miRNA targeting. Most bioinformatic tools therefore incorporate additional criteria such as thermodynamic stability of the miRNA:mRNA duplex, evolutionary conservation of the MRE, and mRNA sequence features outside the seed sequence, to maximize specificity and enhance site recognition efficacy [Thomas *et al.*, 2010].

Many web-based miRNA databases have been constructed for computational target prediction. These methods differ in the weight placed on different criteria as well as in the format of data output. Table 5.1 summarizes the main prediction programs currently available; of these, the miRanda algorithm and TargetScan are the most frequently used. These two programs also form the base of the bioinformatic analysis performed in this chapter.

The miRanda algorithm

Target prediction at www.microrna.org is based on the miRanda algorithm which analyses the complementarity between miRNAs and their mRNA target sites using a weighted dynamic programming algorithm [Enright *et al.*, 2003]. It determines the sum of complementary base pairs and mismatches while considering the position within the target site; matches in the seed region (nt 2-8) are weighted higher. The algorithm allows for single G:U wobbles in the seed region and considers additional base pairing at the 3' end of the miRNA [John *et al.*, 2004]. In addition to base pairing, the miRanda algorithm considers the hybridisation energy of the miRNA:mRNA duplex as well as the evolutionary conservation of target sites. As a result of these different features, a down regulation score, termed miRSVR score, is calculated for each miRNA:mRNA interaction [Betel *et al.*, 2010].

TargetScan

The TargetScan prediction program is mainly based on finding complementary seed pairing [Lewis *et al.*, 2003] [Lewis *et al.*, 2005]. It requires perfect Watson-Crick base pairing of the miRNA seed to its targets site and assigns a free energy of binding to each miRNA:mRNA interaction by starting at the complementary seed region and extending in either direction,

Table 5.1: A selection of miRNA target prediction programs currently available.

Method	Type of Method	Reference	Resource
miRanda	Complementarity	[John <i>et al.</i> , 2004]	www.microrna.org
miRanda (miRBase)	Complementarity	[Enright <i>et al.</i> , 2003]	microrna.sanger.ac.uk
TargetScan	Seed complementarity	[Lewis <i>et al.</i> , 2005]	www.targetscan.org
DIANA-microT	Thermodynamics	[Kiriakidou <i>et al.</i> , 2004]	diana.cslab.ece.ntu.gr
PicTar	Thermodynamics	[Krek <i>et al.</i> , 2005]	pictar.mdc-berlin.de
RNAHybrid++	Thermodynamics and statistical model	[Rehmsmeier <i>et al.</i> , 2004]	bibiserv.techfak.uni-bielefeld.de/rmahybrid
MiRTarget2	Support Vector Machine	[Wang & El Naqa, 2008]	mirdb.org
TarBase	Experimentally validated targets	[Sethupathy <i>et al.</i> , 2006]	diana.cslab.ece.ntua.gr/tarbase
miRecords	Metasearch	[Xiao <i>et al.</i> , 2009]	mirecords.biolead.org

stopping at mismatched bases. It distinguishes between three different types of seeds:

- 7mer-1A: exact match to positions 2-7 of the miRNA (the seed) followed by an 'A' (1A anchor)
- 7mer-m8: exact match to positions 2-8 of the miRNA (the seed + position 8)
- 8mer: exact match to positions 2-8 of the miRNA seed (seed + position 8) followed by an A

TargetScan does not consider thermodynamic stability of the miRNA:mRNA duplex but does include target site conservation; however, this is optional. Finally, it assigns a total context score to each miRNA:mRNA interaction [Grimson *et al.*, 2007].

Additional features that influence miRNA target binding

With increasing numbers of experimentally validated miRNA:mRNA pairs, additional contextual determinants are emerging that may impact on miRNA targeting, such as structural accessibility or location of the binding site within the 3'UTR [Grimson *et al.*, 2007]. Site accessibility can be directed by the secondary structure of a given 3'UTR mRNA and has been shown to regulate RISC binding [Zhao *et al.*, 2005]. Moreover, miRNA binding sites are unevenly distributed across 3'UTR sequences and tend to cluster at both ends for long transcripts (> 2000 nucleotides) [Gaidatzis *et al.*, 2007]. For short transcripts, target sites are mostly located near the beginning, starting 15-20 nucleotides downstream of the stop codon [Gaidatzis *et al.*, 2007]. Finally, computational prediction programs focus exclusively on classical action of miRNAs which involves miRNA binding to target sites in the 3'UTR of putative target genes. However, MREs have also been found to exist in coding regions [Forman *et al.*, 2008] and 5' UTRs [Ørom *et al.*, 2008]. Consequently, algorithm design faces the challenge of incorporating these additional features to achieve improved prediction power and accuracy.

Experimental validation of miRNA:mRNA interactions

Even when employing a variety of prediction tools, the discussed limitations mean that it is not possible to judge the accuracy of these forecasts, making experimental target validation essential. To date only few techniques are available to validate direct miRNA:mRNA interactions.

For example, two groups have attempted to isolate miRNAs in combination with their endogenous targets by pulling down RISC complexes and examining eluted mRNAs using

high throughput sequencing [Licatalosi *et al.*, 2008] [Hafner *et al.*, 2010]. This approach has been shown to be very useful for identifying miRNA targets as well as determining the exact miRNA binding site. However, these methods are currently not readily available.

The 3'UTR reporter assay is the most widely adopted method for miRNA target validation, and is capable of mapping the exact MRE [Brennecke *et al.*, 2005] (Figure 5.2). Briefly, the 3'UTR of the putative target gene is inserted downstream of the stop codon of a reporter gene, which in this case is luciferase. This renders reporter gene expression susceptible to over expression of the specific miRNA. Candidate MREs are mapped by testing whether mutations or deletions in the complementary seed region restore reporter gene expression.

The targeted 3'UTR cloning approach is a powerful method for confirming individual miRNA:mRNA interactions. However, it is not amenable for high-throughput screening and requires initial target selection which incorporates a certain degree of subjectivity. Furthermore, validation of individual genes fails to describe the impact of the selected miRNA on global gene expression. This may include direct as well as indirect effects.

Validated targets for miR-650

To date, the biological function of miR-650 is still largely unknown. Bioinformatic analysis identified Inhibitor of Growth 4 (ING4) as a putative miR-650 target [Zhang *et al.*, 2010]. Over expression of miR-650 represses the expression of ING4 on the translational level and promotes tumourigenesis and proliferation of gastric cancer cells. A luciferase reporter assay confirmed a direct interaction between miR-650 and a defined site in the ING4 3'UTR sequence [Zhang *et al.*, 2010]. ING4 is a tumour suppressor protein [Kim *et al.*, 2004] with a PHD-finger motif commonly found in chromatin remodelling proteins. It is involved in p53-dependent signalling and induces G2/M cell cycle arrest [Zhang *et al.*, 2004a]. In summary, increased level of miR-650 in cancer cells represses the expression of the tumour suppressor ING4 and therefore promotes tumourigenesis.

Supporting its function as a tumour promoting 'oncomiR', Feng *et al.* demonstrated that miR-650 is a direct regulator of N-myc downstream-regulated gene 2 (NDRG2), a tumour suppressor gene that is down regulated in colorectal cancer [Feng *et al.*, 2011].

In order to investigate the potential function of miR-650 in the innate immune response, a multi pronged approach was taken which included a bioinformatic search, large scale gene expression profiling and 3'UTR luciferase reporter assays. This chapter focuses on the identification and validation of individual miR-650:target interactions.

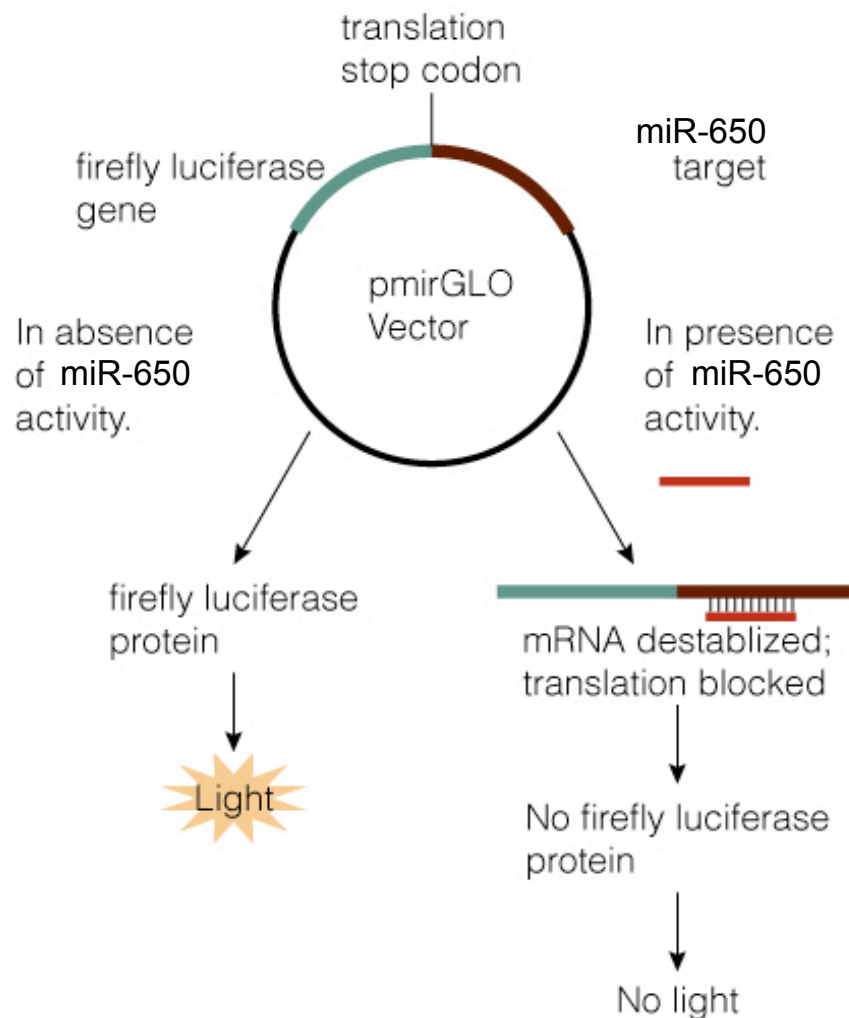


Figure 5.2: Schematic diagram of the 3'UTR luciferase reporter assay. The putative miRNA target sequence is inserted downstream of the stop codon of the reporter gene. Transfection of the reporter vector into cells results in the production of luciferase, which can be quantified by measuring luminescence. If the inserted sequence contains a functional miRNA binding site, luciferase activity is decreased upon over expression of the equivalent miRNA. Figure adapted from pmir-GLO manual (Promega).

5.2 Aims

This Chapter aims to:

- Conduct a comprehensive bioinformatic analysis of putative miR-650 targets.
- Validate a selection of direct targets by 3'UTR luciferase assay.
- Focus on specific targets that may have implications in DC function and the innate immune response.

5.3 Results

In the search for putative miR-650 target sites, a comprehensive bioinformatic analysis was conducted. Figure 5.3(A) shows the sequence for miR-650 and highlights nucleotides 2-8 at the 5' end which form the important seed region.

As already mentioned, there exists a variety of web-based prediction programs which place variable weight on different features of miRNA:mRNA interactions, and vary widely in terms of the output of predicted data. Among these, TargetScan and the miRanda algorithm are widely used. When using online tools, it is important to note that miR-650 belongs to the group of miRNAs which are poorly conserved across different species. Using the miRanda algorithm, a total of 8,766 potential miR-650 targets were retrieved. From the TargetScan database, which uses a more stringent seed search, a total of 5,527 potential miR-650 targets were obtained including 248 conserved and 4,505 poorly conserved sites. The 20 top scoring targets for both programs are shown in Table 5.2.

A rather large proportion of predicted genes (2,527 targets) were shown to be shared between the two programs (Figure 5.3(B)). 75% of all TargetScan predictions overlapped with those made by miRanda and, conversely, 30% of targets obtained by miRanda were also predicted by TargetScan.

5.3.1 Selection of potential miR-650 targets for validation

For experimental validation, it was important to narrow down the large number of potential target genes. Unfortunately, a canonical strategy for filtering out weaker targets, and therefore increasing the confidence level of the selected target pool is currently lacking. However, several studies have tried to assess the performance of different target prediction programs experimentally and these findings were used to generate a customised workflow for initial target selection (shown in Figure 5.4).

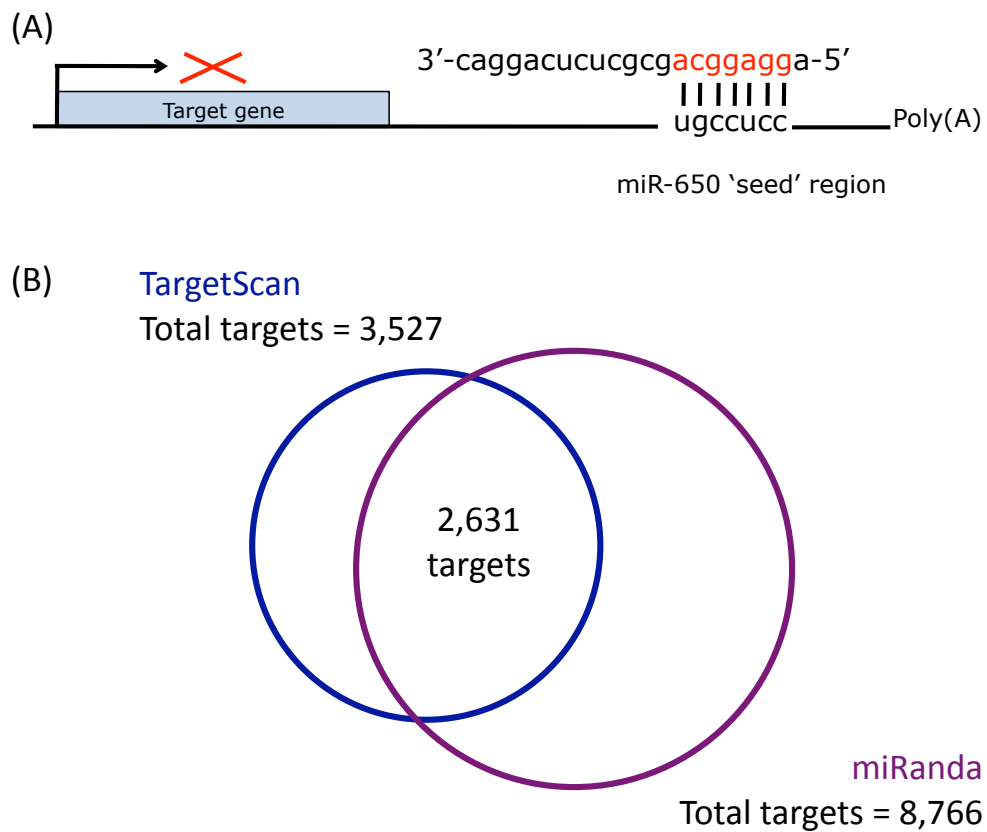


Figure 5.3: *In silico* target predictions for miR-650 according to TargetScan and the miRanda algorithm. (A) The sequence of the miR-650 seed used for computational target site predictions. (B) The total number of predicted miR-650 targets retrieved from TargetScan and miRanda, showing the overlap between the two programs.

Table 5.2: Top 20 predicted miR-650 target according to TargetScan and miRanda.

TargetScan		
GeneID	Name	Total Context score
EBF3	early B-cell factor 3	-0.96
NCOA1	nuclear receptor coactivator 1	-0.92
AGPAT4	1-acylglycerol-3-phosphate O-acyltransferase 4	-0.66
H6PD	hexose-6-phosphate dehydrogenase	-0.64
KRTAP4-12	keratin associated protein 4-12	-0.63
SIRPB1	signal-regulatory protein beta 1	-0.62
SCP2	sterol carrier protein 2	-0.62
F2RL2	coagulation factor II (thrombin) receptor-like 2	-0.61
ZNF488	zinc finger protein 488	-0.59
DLGAP2	discs, large (Drosophila) homolog-associated protein 2	-0.58
IGSF9B	immunoglobulin superfamily, member 9B	-0.58
RNF144B	ring finger 144B	-0.56
MTA2	metastasis associated 1 family, member 2	-0.55
ZNF294	zinc finger protein 294	-0.55
ATP7B	ATPase, Cu++ transporting, beta polypeptide	-0.55
FKBP1A	FK506 binding protein 1A, 12kDa	-0.55
CLLU1	chronic lymphocytic leukemia up-regulated 1	-0.55
SIX5	SIX homeobox 5	-0.55
SAMD12	sterile alpha motif domain containing 12	-0.54
NMT2	N-myristoyltransferase 2	-0.54

miRanda		
Gene ID	Name	mirSVR score
NCOA1	nuclear receptor coactivator 1	-2.59
EBF3	early B-cell factor 3	-2.46
MBD6	methyl-CpG binding domain protein 6	-2.09
KRT6C	keratin 6C	-2.04
PCGF5	polycomb group ring finger 5	-1.97
ASB12	ankyrin repeat and SOCS box containing 12	-1.91
CNTD1	cyclin N-terminal domain containing 1	-1.87
YIPF6	Yip1 domain family, member 6	-1.85
LIMS3	LIM and senescent cell antigen-like domains 3	-1.81
MTA2	metastasis associated 1 family, member 2	-1.78
SIRPB1	signal-regulatory protein beta 1	-1.76
LOC145837	hypothetical LOC145837	-1.38
F2RL2	coagulation factor II (thrombin) receptor-like 2	-1.70
NFKBIL1	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	-1.65
TOP1P1	topoisomerase (DNA) I pseudogene 1	-1.63
RNF160	listerin E3 ubiquitin protein ligase 1	-1.56
WNT1	wingless-type MMTV integration site family, member 1	-1.55
LYPLA2P1	lysophospholipase II pseudogene 1	-1.48
R3HDM1	R3H domain containing 1	-1.45
NHEJ1	nonhomologous end-joining factor 1	-1.44

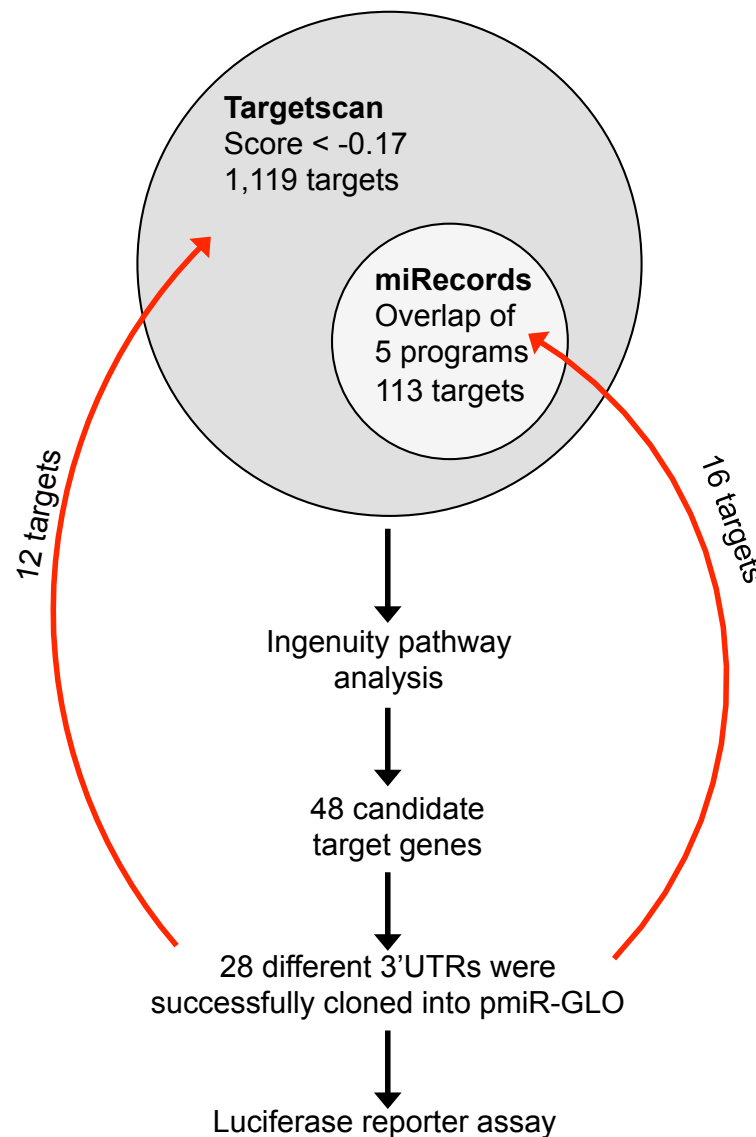


Figure 5.4: Schematic representation of the workflow used to narrow candidate miR-650 targets. Candidate targets genes were narrowed down by two different approaches: 1) by selecting the top third predictions performed by TargetScan (which equates to all targets with a total context score ≤ -0.17) and 2) by using the miRecords metasearch which combines the results from 5 different prediction programs (miRanda, TargetScan, RNAhybrid, PITA and miRTarget2). The latter approach yielded a total of 133 target genes which were all found within the top third TargetScan predictions. All 1,119 putative target genes were analysed by Ingenuity in order to generate a general overview of the function networks they are involved in. This analysis was then used to select 48 target genes for further validation by 3'UTR reporter assay. So far the 3'UTRs of 28 of these putative targets have been successfully cloned into the pmiR-GLO vector and tested for regulation by miR-650.

Studies on miR-181 and miR-233 have tried to evaluate the accuracy of computational predictions by comparing the predictions of various bioinformatic programs with the results obtained from different experimental approaches [Baek *et al.*, 2008] [Selbach *et al.*, 2008]. In summary, it was found that different programs have overall similar target prediction capabilities. However, the top third TargetScan results were shown to correlate significantly better with protein down regulation compared to the bottom third, or indeed predictions from other programs [Baek *et al.*, 2008]. As a consequence, the total predicted target pool for miR-650 was narrowed down to only include the top third predictions made by TargetScan. This corresponded to a total context score of ≤ -0.17 (Figure 5.4(A)). This approach narrowed down the potential target pool from 5,527 to 1,119 predicted targets.

In a separate approach, the miRecords database was used to compile a list of potential targets based on overlapping predictions made by five individual algorithms: miRanda, TargetScan, MirTarget2 [Wang & El Naqa, 2008] [Wang, 2008], PITA [Kertesz *et al.*, 2007] and RNAhybrid [Rehmsmeier *et al.*, 2004]. Combining results from multiple tools is common practice and is often encouraged to increase specificity. However, comparisons are often difficult due to different input parameters as well as different target selection criteria. As a consequence, combinational approaches are accompanied by a loss in sensitivity and therefore often perform worse than a single prediction tool [Alexiou *et al.*, 2009]. Using this combinatorial method, a total of 113 common targets were obtained. Compared to the number of targets predicted by miRanda and TargetScan individually, the number of potential target genes was greatly reduced. Encouragingly, all 133 potential targets were also found among the top third of targets predicted by TargetScan (Figure 5.4(A)). Hence, this combinatorial approach did not identify any additional targets.

In order to interpret the prediction data in the context of biological processes, pathways and networks, all of the top third targets predicted by TargetScan were entered into Ingenuity pathway analysis (<http://www.ingenuity.com/>). The 'Core Analysis' identified a total of 25 different gene networks (Table 5.3). Due to the relative similarity of the number of targets involved in each pathway, it is difficult to predict a dominant cellular function for miR-650. The top scoring functions are post-translational modification, cellular assembly and organisation as well as DNA replication, recombination and repair. The latter group contains the previously validated target ING4 and supports the previous studies which suggest a potential role for miR-650 in cancer. In addition, a number of functions were identified that are related to innate immunity or DC function; these include cellular assembly and organisation, cell morphology, infection mechanism, infectious disease, molecular transport and nervous system development and function. Although these results obtained from pathway

Table 5.3: Ingenuity network analysis of the top third miR-650 targets predicted by TargetScan (top 10 networks shown).

ID	Molecules in Network	Score	Molecules	Top Functions
1	BRPF3, CD59, CDK2AP1, DAG1, DHX15, DOT1L, DPF2, DTX1, DTX3L, DZIP3, GNS, GRB2, HISTONE, ING4, KDM1A, L3MBTL2, LTN1, MBD2, METTL8, MOBK12A, MTA2, MUL1, NAALADL2, OTUD5, PRDM9, RNF4, RREB1, SKAP2, SLC1A2, SMARCD2, SUV39H2, TFCP2L1, TRIM55, UBE2D2, UHRF2	46	34	Post-Translational Modification, Cellular Assembly and Organisation, DNA Replication, Recombination, and Repair
2	ADAM15, ARHGDIA, ARPC5, ASB12, CALML5, CNP, CORO2B, DGKQ, DIAPH2, HCLS1, KIAA1797, KIF1A, LRRC59, LRRN2, LZTR1, NF2, PAPOLG, PCBP4, QKI, RAB11FIP4, RAC1, RACGAP1, SHROOM3, SNX33, SORBS1, TNSI, TRPC3, TRPC5, TXN2, VCL	38	30	Cellular Assembly and Organisation, Cellular Function and Maintenance, Cell Morphology
3	AGPAT4, ANXA6, CDC42SE1, CLDN6, CLDN18, CNTNAP1, CRK, DCP2, DCP1A, DDX6, DLGAP2, DUSP8, ERN1, FGD1, GLTSCR1, GRIN2D, KRT8, KSR2, NECAP1, NES, NFASC, PLEC, REM2, RGS7, SFRP5, SMG1, SP7, VANGL2	38	29	Cell Morphology, Cell-To-Cell Signaling and Interaction, Embryonic Development
4	AKT2, ATG12, CCDC59, CDK18, DBF4B, DDX17, DGCR8, ERBB, EXOSC5, EXOSC6, GPRIN2, HNRNPUL1, IRAK1, KCTD17, KND1, KRTAP4-12, LNX1, MAP3K8, PAK7, PDLIM7, PDS5A, PIM1, RAP1GAP, RAPGEF2, RARRES3, SERBP1, SYNGAP1, TCEB3B, ZNF277, ZNF764	38	29	Cell Morphology, Cellular Assembly and Organisation, Nervous System Development and Function
5	CCDC87, CUL2, ELL, FAF2, FBXW11, FOXF4, GCNLI1, HEXIM1, HOXA11, HOXB3, LCOR, MED20, MED13L, MRPS21, NAB2, NONO, PBX1, PHYHIP, PMP22, POLR2D, POLR2G, RARB, SOCS3, TAF5L, TCF21, TFAP4, UBE3B, VDR, ZFR1	36	29	Embryonic Development, Organ Development, Renal and Urological System Development and Function
6	AHCYL2, ANKRD36B, BCR, CD209, CLEC4M, DAXX, ELMOD3, GUSB, HARS, KDM5B, LTBP2, MACF1, MAP3K14, NCAPH, NEDD9, NUDT10, POLA1, POLE3, POLI, PSD3, PSME3, RAD18, RASSF1, RASSF2, RBM15B, SHOX2, SIRPA, WDR48	33	27	Hepatic System Development and Function, Infection Mechanism, Infectious Disease
7	ATXN1, AXL, BATF2, CBL, CDK5R2, CREB1, CREBZF, CXXC1, DDX28, FSH, GAPVD1, KLHL21, LMNA, MAP1LC3C, MIDN, MITF, MKLN1, PDGF BB, PLEKHA1, PPP1R13B, QRICH1, R3HDM1, S100B, SLC6A6, SQSTM1, TMC6, WNK1, ZNF35, ZNF488	33	27	Cancer, Cardiovascular Disease, Dermatological Diseases and Conditions
8	ANXA2, ATP7A, CCND3, CDH15, CEBPA, CK1, CSNK1G3, DDX21, EPHX1, FGFR2, GCLM, GNPDA1, HES5, HHAT, HLA-DOA, HLA-DRA, LDL, LMAN1, LRP6, MCC, MMRN1, PFAS, TMEM119, VAMP7, WNT1, WNT9B, XBP1	29	25	Nervous System Development and Function, Organ Development, Cellular Development
9	ADAMTSL4, BCKDK, CD274, DLL4, ERBB4, FUBP3, HEY1, KIAA1949, LDLR, LRP, MAML3, NCSTN, NGFR, NOTCH3, NPR3, PCDHGC3, PCNT, PI3K (complex), PI3K p85, PSEN1, PXDN, SALL2, SYNJ2BP, TMED2, TRAF, TRAF1, TRAF4, TYRO3	28	24	Embryonic Development, Organismal Development, Tissue Development
10	CBFA2T2, CSDC2, ELF4, ERK, ETS, GAB2, GTPBP1, HIF3A, ITGA2B, LDL1, LHX3, LHX4, LMO3, MME, MPL, PAX5, PRLR, PURB, RARRES2, RPS6KA5, SERPIND1, SIX5, SPN, SPSB1, SRF, STAT5a/b, TAL1	28	24	Endocrine System Development and Function, Organ Development, Organismal Development

analyses have to be interpreted with caution, they were nevertheless encouraging.

On the basis of this analysis as well as an extensive literature search, 48 candidate targets were taken forward for validation using the 3'UTR luciferase reporter assay system. These genes were randomly distributed within the top third TargetScan predicted targets. While the majority of putative targets were chosen due to prior findings which suggested an involvement in the immune response, others were added speculatively. In the end, the 3'UTR sequences of 28 potential target genes were successfully cloned. While 12 of these genes were among the top third of the predictions made by TargetScan, the other 16 targets were also identified by the combinatorial approach.

5.3.2 Setting up the 3'UTR luciferase reporter assay

The dual luciferase reporter construct pmiR-GLO (Promega) was used for target validation (Figure 2.1). This vector is designed to quantitatively evaluate miRNA activity by measuring expression of the firefly luciferase gene. For this purpose, the 3'UTR sequences of potential target genes were inserted downstream of the firefly luciferase gene; miRNA targeting would therefore result in repression of firefly luciferase activity. The pmiR-GLO has the advantage of a built-in renilla luciferase control which eliminates the transfection of a separate control vector and thus minimizes the variability associated with this assay. Due to the difficulty of transfecting primary DCs, the 3'UTR assay was carried out in HEK293T cells.

The 3'UTR luciferase assay was set up in a 96-well format amenable to high-throughput screening. A setup experiment was required to determine the optimal ratio between pmiR-GLO vector and miR-650 mimic as well as the time point for assessing luciferase activity. For this purpose, HEK293T cells were transfected with different amounts of pmiR-GLO vector (10 μ g, 20 μ g and 50 μ g) and cells were harvested after 24 hrs and 48 hrs. Figure 5.5(A) shows that firefly luciferase expression increased in a dose dependent manner and was significantly higher after 48 hrs compared to 24 hrs. In order to set up the reporter assay, a positive control was required. For this purpose, a short sequence with perfect complementarity to miR-650 was inserted into the firefly luciferase 3'UTR (pmiR-GLO 650) (Figure 5.5(B)). Various amounts of pmiR-GLO 650 vector (10 μ g, 20 μ g and 50 μ g) were co-transfected together with miRNA mimic (25 nM or 50 nM) using Lipofectamine 2000. The different concentrations of miRNA mimic were chosen according to the manufacturer's suggestions for dual transfection and correspond to those used in other studies [Zhang *et al.*, 2010]. Luciferase activity was assessed 24 hrs and 48 hrs post transfection using the Dual Luciferase Reporter Assay System (Promega) which allows sequential measurement of firefly and renilla luciferase activity. As

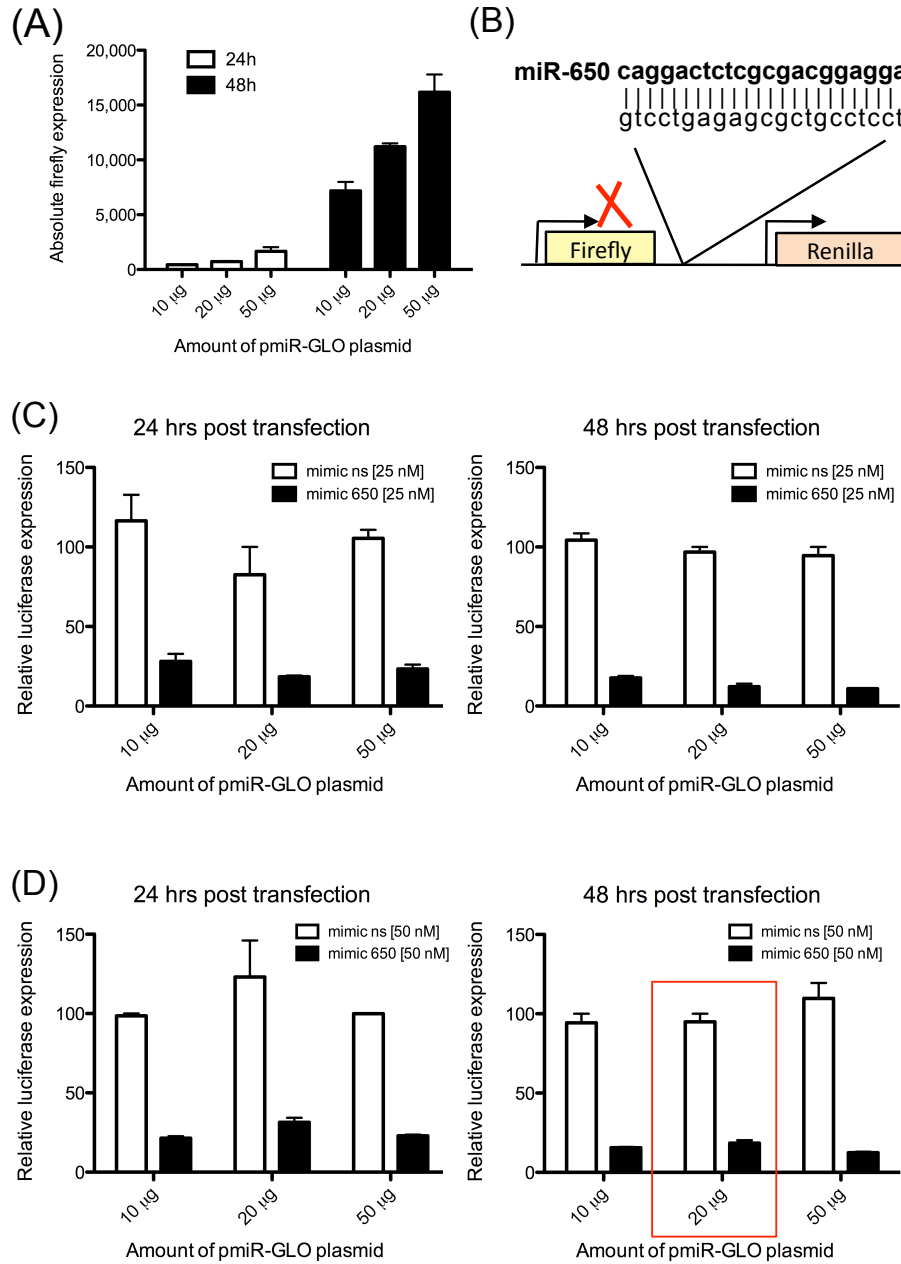


Figure 5.5: Setting up the 3'UTR luciferase reporter assay. (A) 10^5 HEK293T cells were transfected with various amounts of pmiR-GLO vector (10 μ g, 20 μ g and 50 μ g) in a 96-well format using Lipofectamine 2000. Firefly luciferase activity was measured at 24 and 48 hrs post-transfection and absolute values are shown. (B) Construction of a reporter vector (pmiR-GLO 650) which contains the miR-650 complementarity sequence downstream of the luciferase stop codon. In order to set up the relative concentrations of vector and miR-650 mimic, HEK293T cells were co-transfected with p-miR-GLO 650 (10 μ g, 20 μ g and 50 μ g) and nonsense or miR-650 mimic at a final concentration of (C) 25 nM or (D) 50 nM. Firefly luciferase expression was measured 24 hrs and 48 hrs after transfection and normalised against Renilla luciferase activity. Experiments were carried out in triplicates and data is shown as mean $\pm$ SD.

can be seen in Figures 5.5(C) and (D), luciferase activity was significantly reduced in samples transfected with miR-650 mimic compared to its nonsense control.

The reduction in luciferase expression was more pronounced after 48 hrs compared to 24 hrs. Furthermore, repression was independent of the amount of vector as well as of the concentration of miR-650 mimic used. This suggests that the system was saturated. As a result, all subsequent experiments use 20 μg of pmiR-GLO and 25 nM miRNA mimic, and luciferase activity is measured 48 hrs post-transfection (Figure 5.5(E), final condition marked in red).

Figure 5.6 shows the final experimental setup used for the 3'UTR screen. Briefly, HEK293T cells were co-transfected with 20 μg pmiR-GLO construct and either nonsense or miR-650 mimic (25 nM). Luciferase activity was measured 48 hrs post-transfection (Figure 5.6). In order to confirm that the observed changes in luciferase activity are due to over expression of miR-650, an additional control condition was included; HEK293T cells were co-transfected with pmiR-GLO vector, miR-650 mimic as well as a miR-650-specific antisense inhibitor. As mentioned previously, the inhibitor is an antisense oligonucleotide with perfect sequence complementarity to the mature miR-650 strand. Binding of the antisense strand to the mature miRNA is thought to make it unavailable for target binding and targets it for degradation. As shown in Figure 5.6, luciferase expression from pmiR-GLO 650 can be fully rescued upon inhibition of the miR-650 mimic. It is also important to note that co-transfection of the empty pmiR-GLO vector with miR-650 mimic had no effect on luciferase expression. This confirms that the pmiR-GLO vector itself is not targeted by miR-650 and can therefore be used as a negative control in subsequent experiments.

Together, these data suggest that this system is well suited to investigate the interaction between miR-650 and its potential target sequences.

5.3.3 Investigating the interaction of miR-650 and putative target sequences by 3'UTR luciferase reporter assay

In order to investigate whether miR-650 regulates the expression of the selected target genes, their 3'UTR sequences were cloned into the pmiR-GLO vector; the workflow is summarised in Figure 5.7. 3'UTR sequences were retrieved from PubMed. Primers were designed to amplify 3'UTR sequences from genomic DNA and to include a SalI and NheI restriction enzyme site at either end. Following digestion, the 3'UTR sequences were inserted into the multiple cloning site of the pmiR-GLO vector located downstream of the firefly luciferase gene. Since the effect of the exact location of the binding site on miRNA targeting is unclear,

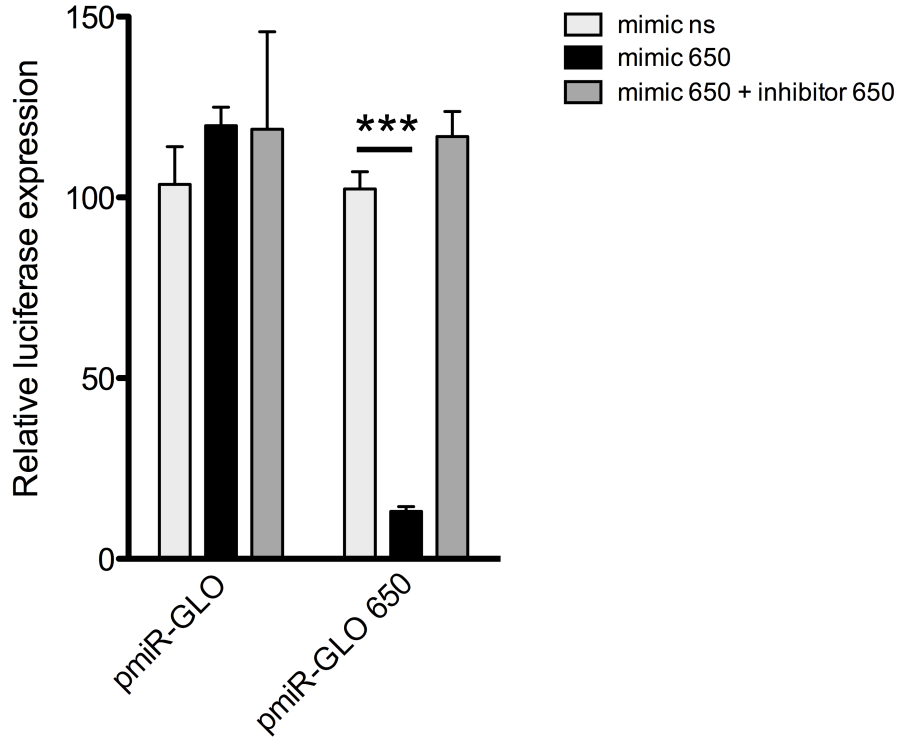


Figure 5.6: Final experimental setup used for all subsequent 3'UTR reporter assays. In a 96-well format, 10^5 HEK293T cells were co-transfected with $20 \mu\text{g}$ pmir-GLO vector and either: 1) nonsense mimic (25 nM), 2) miR-650 mimic (25 nM) or 3) a mixture of miR-650 mimic (25 nM) and an excess of miR-650 antisense oligonucleotide (50 nM). Luciferase activity was measured 48 hrs post-transfection and firefly activity was normalised against renilla expression. Luciferase expression in cells transfected with the empty pmir-GLO vector remains unchanged in response to miR-650 over expression. In contrast, luciferase expression from vector containing the miR-650 complementary site (pmir-GLO 650) is reduced in response to miR-650. Addition of miR-650-specific antisense oligonucleotides effectively rescues luciferase expression. Experiments were carried out in triplicates and data is shown as mean $\pm$ SD. Statistical analysis was performed using an unpaired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

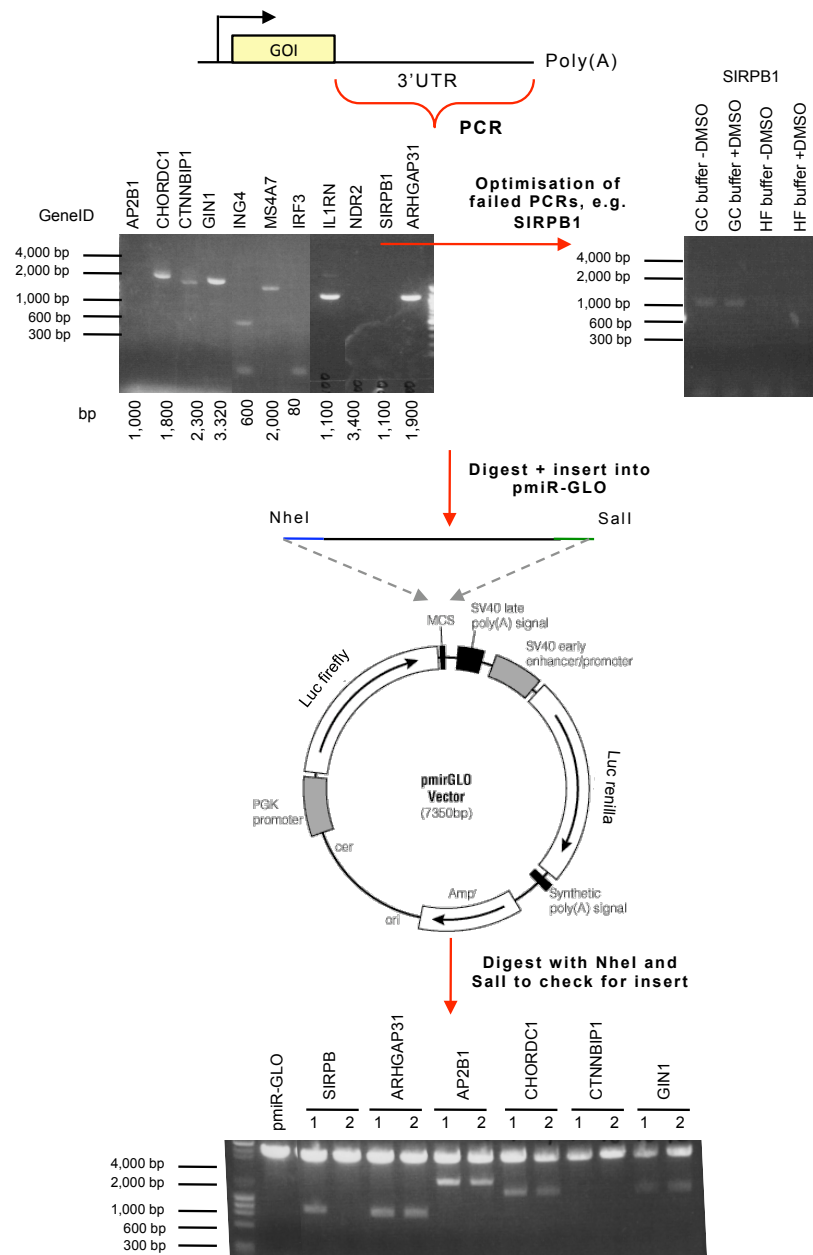


Figure 5.7: Workflow for cloning 3'UTRs sequences of candidate target genes into the pmiR-GLO vector. The 3'UTR sequences from the 48 candidate target genes were amplified from genomic DNA using primers designed according to sequences obtained from PubMed. For sequence which did not amplify in the first round, for example the 3'UTR of SIRPB1, additional rounds of optimisation were performed by varying buffer, DMSO or MgCl₂ concentration. Amplified 3'UTR sequences were digested using NheI and SalI and ligated into the pmiR-GLO vector digested with the same enzymes. Following ligation, plasmids were transformed into competent bacteria. Insertion of the correct 3'UTR sequences was confirmed by restriction enzyme digest and sequencing.

Table 5.4: The 28 putative miR-650 target genes for which 3'UTR reporter assays were performed.

Gene ID	Name	Information	Reference
AP2B1	adaptor-related protein complex 2, beta 1 subunit	Component of the assembly protein complex 2, which serves to link clathrin to receptors in coated vesicles	[Collins <i>et al.</i> , 2002]
ARHGAP31	Rho GTPase activating protein 31	GTPase-activating protein which regulates Cdc42/Rac1 involved in protein trafficking and cell growth	[Tcherkezian <i>et al.</i> , 2006]
ATG12	ATG12 autophagy related 12 homolog (<i>S. cerevisiae</i>)	Ubiquitin-like protein involved in autophagosome formation	[Mizushima <i>et al.</i> , 1998]
CD209	DC-SIGN	Pathogen recognition and cell adhesion receptor; binds to carbohydrate ligands on the surface of microbes and endogenous cells	[Khoo <i>et al.</i> , 2008]
CHORDC1	cysteine and histidine-rich domain (CHORD) containing 1	ADP-dependent HSP90-interacting protein	[Gano & Simon, 2010]
DAGLA	diacylglycerol lipase, alpha	Catalyses the hydrolysis of diacylglycerol (DAG) to 2-arachidonoyl-glycerol (2-AG)	[Bisogno <i>et al.</i> , 2003]
DDX17	DEAD box polypeptide 17	Characterised by the conserved motif Asp-Glu-Ala-Asp (DEAD) is a putative RNA helicases; alternative splicing regulatory factor	[Hönig <i>et al.</i> , 2002]
DHX15	DEAH box polypeptide 15	RNA helicase implicated in pre-mRNA splicing	[Will <i>et al.</i> , 2004]
FKBP1A	FK506 binding protein 1A,	Cis-trans prolyl isomerase involved in immunoregulation and protein folding and trafficking	[Choi <i>et al.</i> , 1996]
GIN1	gypsy retrotransposon integrase 1	Evolved from the integrase domain of an LTR retrotransposon; possibly involved in repressing retrotransposon and retrovirus activity	[Lloréns & Marín, 2001]
ING4	Inhibitor of growth family, member 4	Tumor suppressor protein involved in chromatin remodeling; attenuates NF-kB activity	[Nozell <i>et al.</i> , 2008]
IRF3	interferon regulatory factor 3	A member of the interferon regulatory transcription factor (IRF) family	[Taniguchi <i>et al.</i> , 2001]
MAP1LC3C	microtubule-associated protein 1 light chain 3 gamma	Ortholog of the yeast autophagosome protein Atg8; component of autophagosomal membrane	[He <i>et al.</i> , 2003]
MFN1	mitofusin 1	Mediator of mitochondrial fusion; required for virus-induced redistribution of IPS-1 and IFN production	[Onoguchi <i>et al.</i> , 2010]

Table 5.5: The 28 candidate miR-650 target genes for which the 3'UTR reporter assays were performed (continued).

Gene ID	Name	Information	Reference
MLLT10	ALL1-fused gene from chromosome 10 protein	Transcription factor involved in several chromosomal rearrangements resulting in various leukemias	[Nakamura <i>et al.</i> , 2002]
NOD1	nucleotide-binding oligomerization domain containing 1	Intracellular pattern-recognition receptor that detects bacterial diaminopimelic acid	[Franchi <i>et al.</i> , 2009]
PCSK7	proprotein convertase subtilisin/kexin type 7	Serine protease concentrated in the trans-Golgi network; can rescue unstable MHC class I	[Leonhardt <i>et al.</i> , 2010]
PPP2R1B	protein phosphatase 2, regulatory subunit A, beta	Regulatory subunit of protein phosphatase 2, a Ser/Thr phosphatase implicated in negative control of cell growth and division	[Kamibayashi <i>et al.</i> , 1992]
PTPN7	protein tyrosine phosphatase, non-receptor type 7	MAPK-specific protein tyrosine phosphatase preferentially expressed in a variety of hematopoietic cells	[Eswaran <i>et al.</i> , 2006]
RAB1B	member RAS oncogene family	GTPase that functions in the early secretory pathway; essential for vesicle transport between the ER and Golgi	[Alvarez <i>et al.</i> , 2003]
RARB	retinoic acid receptor, beta	Receptor for retinoic acid, which mediates cellular signalling in embryonic morphogenesis, cell growth and differentiation	[Brand <i>et al.</i> , 1988]
S100B	S100 calcium binding protein B	Engages RAGE (receptor for advanced glycation end products) in a variety of cell types	[Donato <i>et al.</i> , 2009]
SIRPB1	signal-regulatory protein beta 1	DAP12-associated activating receptor expressed in myeloid cells	[Dietrich <i>et al.</i> , 2000]
SOCS3	suppressor of cytokine signalling 3	Cytokine-inducible negative regulators of cytokine signalling	[Yasukawa <i>et al.</i> , 2000]
STX12	syntaxin 12	SNARE involved in neurite outgrowth and endosomal trafficking	[Hirling <i>et al.</i> , 2000]
TGM4	transglutaminase 4	Involved in peptide cross-linking	[Dubbink <i>et al.</i> , 1998]
TMED2	transmembrane emp24 domain trafficking protein 2	Type I transmembrane protein, which is a crucial constituent of the Golgi apparatus; regulator of receptor trafficking	[Luo <i>et al.</i> , 2007]
TRPC5	transient receptor potential cation channel, subfamily C, 5	Membrane protein forming a receptor-activated calcium permeant cation channel; interacts with MxA	[Lussier <i>et al.</i> , 2005]

full-length 3'UTR sequences were inserted in frame with the luciferase ORF. Each construct was digested and sequenced to confirm successful insertion of the correct 3'UTR sequences. Using this method, 28 target genes were successfully cloned; all genes are listed in Tables 5.4 and 5.5.

Each construct was tested for miR-650 binding by luciferase assay using the setup shown in Figure 5.6. The results of the 3'UTR screen are expressed as percentage luciferase expression in response to miR-650 over expression compared to nonsense control. All experiments were done in quadruplicate and the significance of luciferase repression was tested using an unpaired t-test. All results are summarised in Table 5.6.

In order to assess the susceptibility of the target sequence to miR-650, two different criteria were considered: the level of luciferase repression in response to over expression of miR-650 as well as the statistical significance, i.e. the reproducibility of the data (Table 5.6). Figure 5.8 shows the percentage luciferase expression measured in cells transfected with miR-650 mimic, plotted against the p value calculated by unpaired t-test (Figure 5.8). Two cut-offs were set to identify genuine miR-650 targets. Firstly, only results with a p-value below 0.05 were considered statistically significant. Subsequently, a second cut-off, which set the minimum percentage of repression to that measured for the only target validated so far, ING4; this was 90%. From this analysis, targets were divided into three groups: Group 1 (G1) if luciferase expression was repressed below 90% by miR-650 compared to the control and the results were considered significant ($p < 0.05$). Contrary to this, if luciferase activity remained above 90% and the results were not considered statistically significant ($p > 0.05$), targets were defined as non-responders (NR). Finally, there was a group of targets which only fulfilled one of the two criteria showing luciferase activity below 90% but this was considered to be not statistically significant ($p > 0.05$); these targets were defined as Group 2 (G2).

- Group 1 (G1): luciferase expression $< 90\%$ and $p < 0.05$ and (Figure 5.9).
- Group 2 (G2): luciferase expression $< 90\%$ and $p > 0.05$ (Figure 5.10).
- Non-responders (NR): luciferase expression $> 90\%$ and $p > 0.05$ (Figure 5.11).

According to these cut-offs, the 28 targets were divided as follows: 13 in G1, 7 in G2 and finally 8 NRs. The results for the individual genes are shown in Figures 5.9 - 5.11. Importantly, the reduced luciferase activity observed in G1 and G2 in response to miR-650 over expression was successfully rescued upon addition of the miR-650 antisense oligonucleotide, indicating that the repression of luciferase activity was indeed mediated by over expression of miR-650. It is also important to note that I was able to confirm the validated miR-650

Table 5.6: Summary of results obtained from the 3'UTR reporter screen. Each experiment was performed in quadruplicates and statistical analysis was performed using an unpaired t-test.

Gene ID	% luciferase expression compared to ns mimic	<i>p</i> value
AP2B1	82	0.11
ARHGAP31	68	< 0.01
ATG12	75	0.21
CD209	61	0.01
CHORDC1	99	0.93
DAGLA	55	< 0.01
DDX17	82	0.25
DHX15	64	0.01
FKBP1A	74	0.06
GIN1	86	0.04
ING4	90	0.22
IRF3	75	< 0.01
MAP1LC3C	72	0.04
MFN1	117	0.34
MLLT10	124	0.11
NOD1	77	0.08
PCSK7	76	0.05
PPP2R1B	101	0.88
PTPN7	98	0.85
RAB1B	107	0.27
RARB	69	0.06
S100B	124	0.22
SIRPB1	23	< 0.01
SOCS3	100	0.95
STX12	41	< 0.01
TGM4	63	0.02
TMED2	63	< 0.01
TRPC5	51	< 0.01

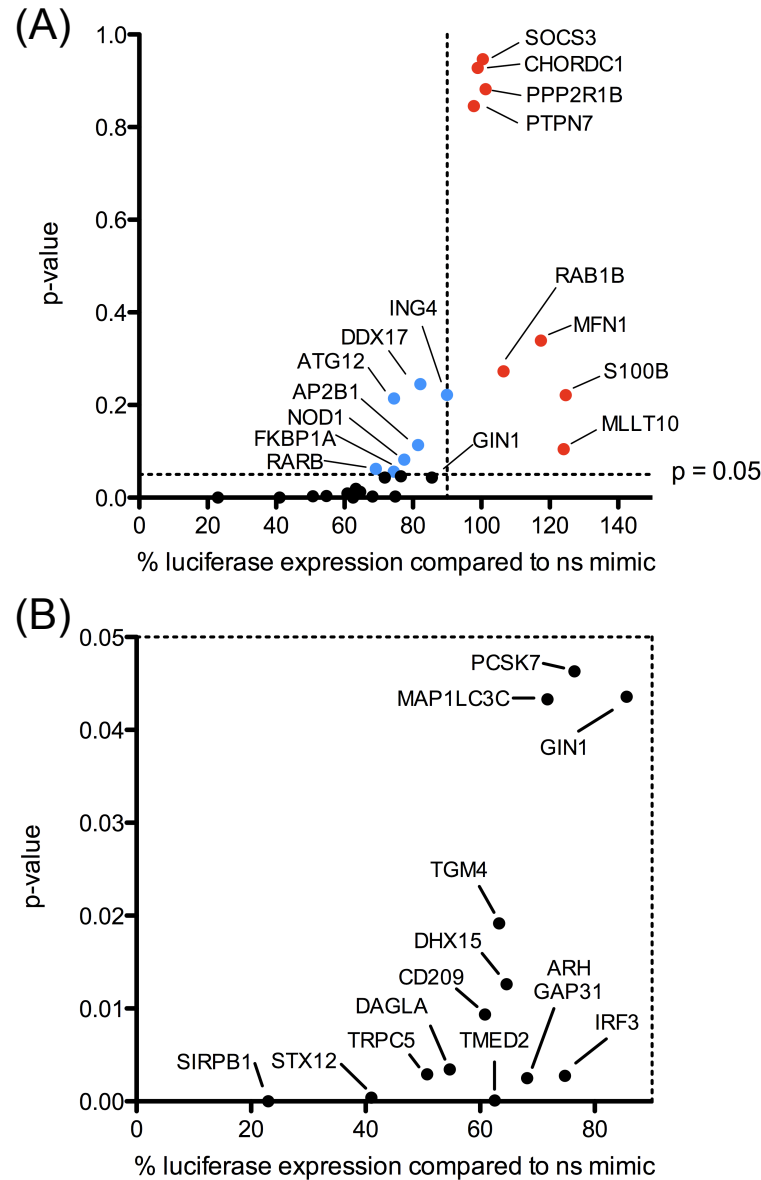


Figure 5.8: Identification of genuine miR-650 targets taking into account the difference in luciferase expression as well as the reproducibility. (A) Plot of the percentage luciferase expression of each target in response to miR-650 mimic compared to nonsense control against the statistical significance of the result (p-value) as determined by unpaired t-test. According to this analysis, tested 3'UTR sequences can be divided into three different groups: Group 1 (black): luciferase expression is repressed below 90% and this is considered to be statistically significant ($p < 0.05$). Group 2 (blue): luciferase expression is repressed below 90% but this is not considered to be statistically significant ($p > 0.05$). The non-responder group (red): luciferase expression is not significantly repressed in the presence of miR-650 ($p > 0.05$). (B) Members of Group 1 displayed in greater resolution.

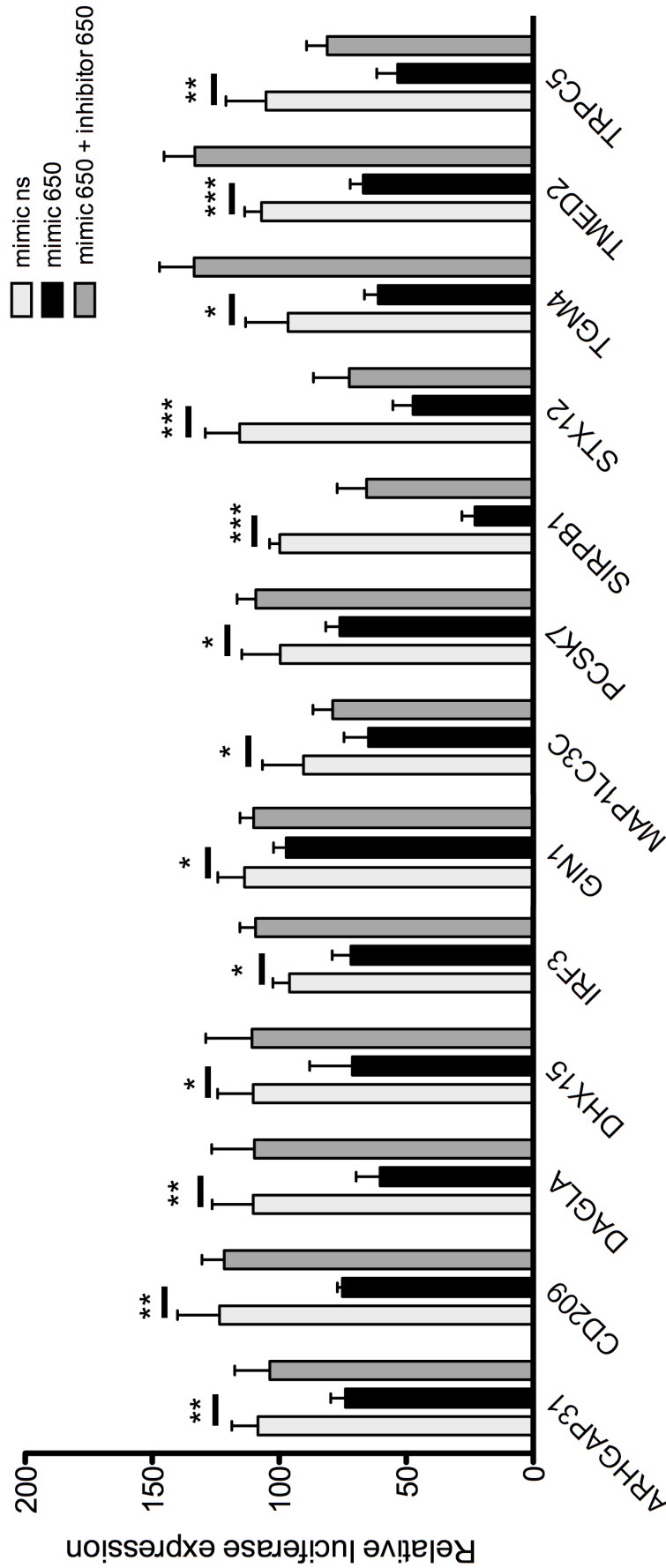


Figure 5.9: Reporter gene expression for candidate targets of Group 1. 3'UTR luciferase reporter assay was performed as explained in Figure 5.6 and luciferase activity was measured after 48 hrs. Candidate genes belonging to Group 1 fulfill two requirements: 1) luciferase expression in the presence of miR-650 mimic was below 90% of the control, and 2) the p -value was below 0.05 as determined by an unpaired t -test. Furthermore, reporter gene expression was recovered by co-transfection of miR-650-specific antisense oligonucleotides. Firefly activity was normalised against Renilla expression. Experiments were performed in quadruplicates and data is shown as mean $\pm$ SD * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

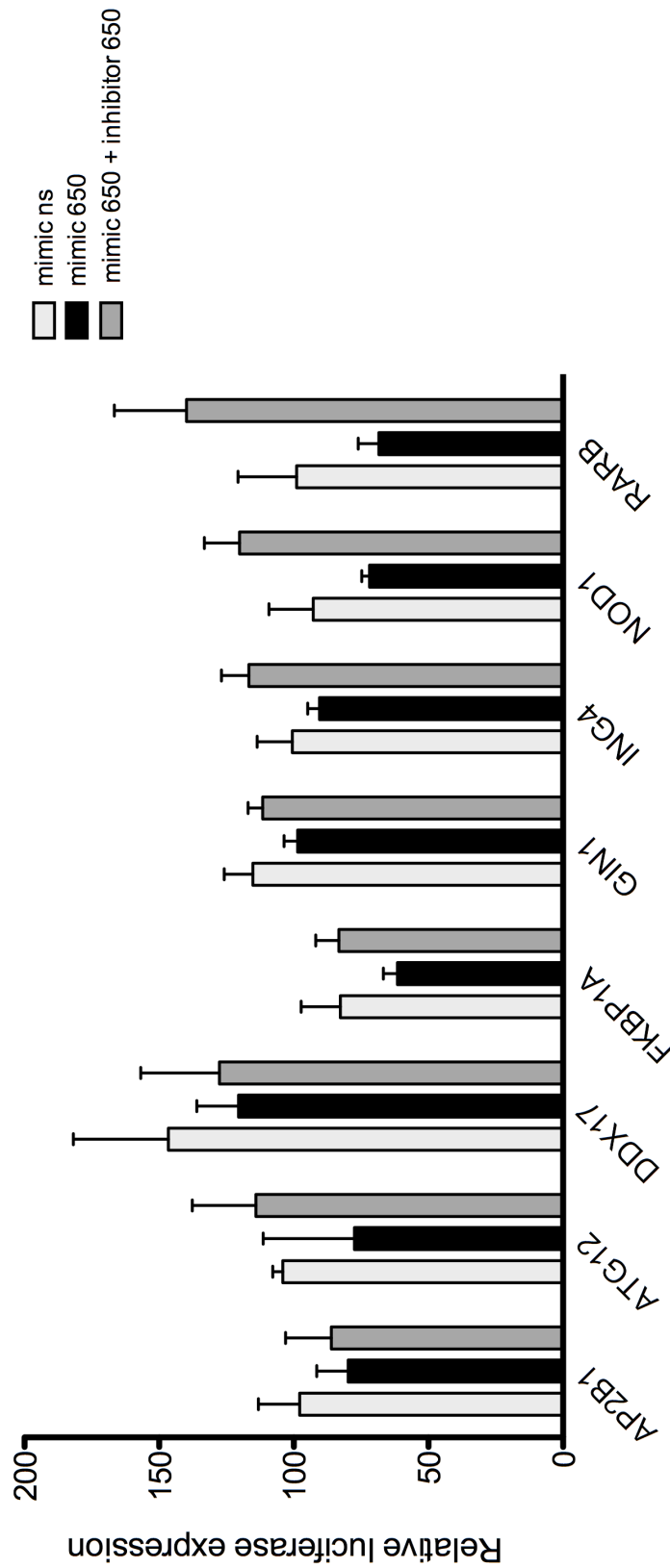


Figure 5.10: Reporter gene expression for candidate targets of Group 2. 3'UTR luciferase reporter assay was performed as explained in Figure 5.6 and luciferase activity was measured after 48 hrs. Candidate genes belonging to Group 2 fulfill only one requirement: luciferase expression in the presence of miR-650 mimic was below 90% of the control. Unfortunately, the reproducibility of the result was low as indicated by a p -value above 0.05. However, reporter gene expression was recovered by co-transfection of miR-650-specific antisense oligonucleotides. Firefly activity was normalised against Renilla expression. Experiments were performed in quadruplicates and data is shown as mean $\pm$ SD * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

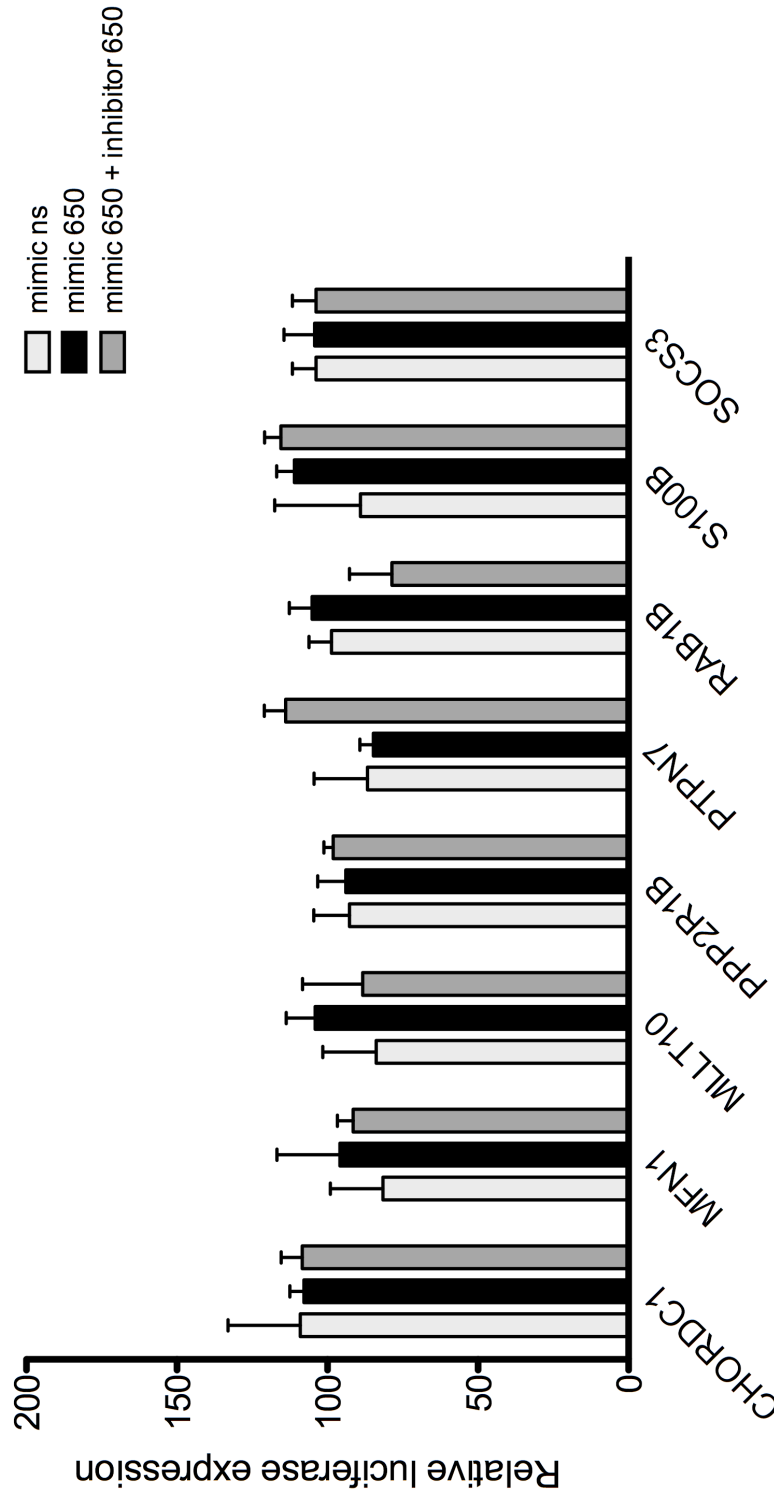


Figure 5.11: Reporter gene expression for candidate targets of the NR group. 3'UTR luciferase reporter assay was performed as explained in Figure 5.6 and luciferase activity was measured after 48 hrs. Candidate genes belonging to the non-responder (NR) group did not fulfill either of the following requirements: luciferase expression in the presence of miR-650 mimic was above 90% of the control and the p -value was above 0.05. Firefly activity was normalised against Renilla expression. Experiments were performed in quadruplicates and data is shown as mean $\pm$ SD * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

target ING4. However, the effect of miR-650 over expression on luciferase expression from the vector carrying the ING4 3'UTR was rather modest and the results were considered not statistically significant due to a high standard deviation. This could, however, be resolved by increasing the number of replicates. In addition to ING4, I was able to identify 19 novel miR-650 targets (G1 and G2 together). In general, the reduction in luciferase expression was not as pronounced as observed for the positive pmiR-GLO 650 control vector (Figure 5.6). This is to be expected as the pmiR-GLO 650 vector contains a sequence with a perfect match for miR-650 and, as mentioned previously, it is thought that perfect complementarity between miRNA:mRNA pairs leads to mRNA cleavage and therefore possibly stronger repression.

5.3.4 Comparison of experimental validation and *in silico* target prediction

As mentioned previously, several studies have tried to evaluate the prediction power of different computational target prediction tools by comparing the results to those obtained from experimental validation [Baek *et al.*, 2008] [Selbach *et al.*, 2008]. Although these studies have provided some important insights, no canonical methods exist to extract genuine targets from *in silico* data sets. Therefore, two different approaches were used to narrow down the pool of predicted targets: firstly, only the top third TargetScan results were chosen (12 targets) and secondly targets were further narrowed down by selecting only the ones predicted by five different programs (16 targets). Figure 5.12 displays the relative luciferase activity of targets derived from the top third predictions made by TargetScan and those predicted by the miRecords metasearch in response to miR-650 over expression. It shows that there is no difference in luciferase expression in response to miR-650 over expression between the members of the two groups. Hence, it can be concluded that there is no added benefit from this combinatorial search.

I already indicated that each prediction program ranks the different targets according to a score representing the different features of the miRNA:mRNA interaction and therefore its quality. It therefore seems logical to be able to use the score to extract genuine targets.

Despite the small size of this data set and the rather subjective criteria used in the initial target selection process, I sought to test this hypothesis by comparing the results obtained from 3'UTR luciferase reporter assay to the information provided by the different target prediction programs. The following analysis will exclusively focus on TargetScan and miRanda, as these were involved in the initial target selection process. Target site information, including the number of binding sites and total binding scores, was retrieved from the relevant

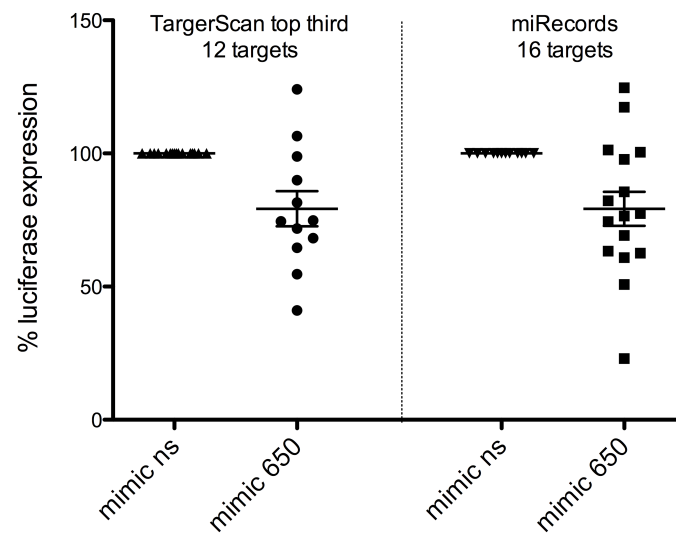


Figure 5.12: Comparing the performance of targets predicted by TargetScan alone and those obtained from the miRecords metasearch. Plot showing the percentage luciferase expression in the presence of miR-650 mimic compared to nonsense control. Putative target genes are divided into two groups: 1) targets which were among the top third TargetScan prediction but not by the combinatorial approach (miRecords), and 2) targets which were among the top third TargetScan prediction and predicted by miRecords. No difference can be observed between the two groups.

Table 5.7: 3'UTR target site information for the 28 candidate miR-650 targets tested by luciferase reporter assay (b.s. = binding site).

Group 1	TargetScan					miRanda	
	8mer	7mer-m8	7mer-1A	Total b.s.	Context score	Total b.s.	mirSVR score
ARHGAP31	1			1	-0.37	2	-0.27
CD209	1	1	1	3	-0.51	6	-0.45
DAGLA		2		2	-0.09	4	-0.46
DHX15		1		1	-0.23	1	-1.32
GIN1	1			1	-0.47	1	-0.79
IRF3	1			1	-0.24	1	-0.28
MAP1LC3C	1			1	-0.24	3	-0.24
PCSK7	2		1	3	-0.53	3	-0.53
SIRPB1	1	2		3	-0.63	4	-1.76
STX12		1		1	0.00	1	-0.25
TGM4	1			1	-0.37	1	-0.97
TMED2	1			1	-0.37	1	-0.81
TRPC5	1			1	-0.44	1	-1.03
Average				1.54	-0.35	2.23	-0.70
Group 2							
	8mer	7mer-m8	7mer-1A	Total b.s.	Context score	Total b.s.	mirSVR score
AP2B1	1			1	-0.27	2	-0.70
ATG12	1			1	-0.34	1	-0.51
DDX17	1	1		2	-0.39	2	-0.47
FKBP1A	2			2	-0.55	3	-0.87
ING4	1			1	-0.35	1	-1.03
NOD1	1	1		2	-0.37	2	-0.24
RARB		1	1	2	-0.40	2	-1.01
Average				1.50	-0.38	1.83	-0.64
NR group							
	8mer	7mer-m8	7mer-1A	Total b.s.	Context score	Total b.s.	mirSVR score
CHORDC1		1		1	-0.22	1	-0.59
MFN1	1			1	-0.45	1	-0.97
MLLT10	1			1	-0.02	1	-0.34
PPP2R1B ¹	1	1		2	-0.49	0	0.00
PTPN7	1	1		2	-0.53	3	-0.20
RAB1B		1		1	-0.05	2	-0.65
S100B	2			2	-0.53	0	0.00
SOCS3	1	2		3	-0.04	3	-0.53
Average				1.57	-0.26	1.57	-0.47

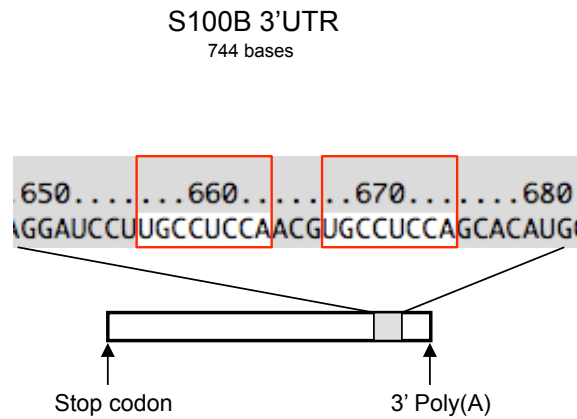


Figure 5.13: miR-650 target sites in the 3'UTR of S100B. The red boxes mark the two miR-650 seed complementary regions in the 3'UTR of S100B. They are located in close proximity to each other, only divided by 3 bp.

websites and is summarised in Table 5.7.

This analysis highlights some common features as well as some important differences between the two prediction programs.

During experimental validation, the Signal-regulatory protein beta-1 (SIRPB1) was shown to be the strongest responding target to miR-650 over expression. Supporting this experimental finding, both TargetScan and miRanda assigns SIRPB1 the lowest, i.e. the best, context score out of all targets analyzed here. Furthermore, miR-650 is the best scoring miRNA among all miRNAs with putative target sites in the 3'UTR of SIRPB1. However, the two prediction programs do not always agree. TargetScan, for example, assigns PPP2R1B a rather good context score, whereas miRanda predicts that this protein does not contain any miR-650 binding site. Further analysis reveals that several different isoforms of this protein exist carrying 3'UTR of varying lengths. Whereas TargetScan uses the transcript from isoform a (NM_02716) which is 3,714 bp in length, miRanda uses the 57 bp short 3'UTR transcript of isoform b (NM_181699). Hence the choice of different input sequences can lead to the observed disagreement between the two prediction programs ². Similarly, TargetScan predicts two miR-650 binding sites in the 3'UTR of S100B whereas miRanda fails to predict any miR-650 binding site. In this case both programs used identical input sequences. However, further analysis reveals that the two 8mer miR-650 binding sites are located in close proximity of each other and are only separated by 3 bp (Figure 5.13). Given that the footprint of miRNA binding is considerably larger than the actual seed sequence, a minimum distance

²The isoform a transcript was cloned in this study but PPP2R1B was not included in further analysis to prevent distortion

of 13 bp is required to prevent steric clashes. In this case, two closely located miRNA binding sites can even cooperate and together enhance target repression [Saetrom *et al.*, 2007]. However, failure of S100B to respond to miR-650 in the 3'UTR luciferase assays indicates that the two miR-650 binding sites may be non-functional.

In order to investigate the correlation between the experimental data and the output parameters generated by TargetScan and miRanda, the total binding scores and the total number of target sites were compared to the repression of luciferase activity; these comparisons are shown in Figure 5.14. In the first row the total binding scores are plotted against the luciferase expression observed in response to miR-650 whereas in the second row scores are grouped according to the three responder types. The last row examines the impact of the number of potential binding sites by grouping them according to the three responder types.

Although the data obtained from TargetScan is fairly widely scattered, a weak correlation can be observed between the total context score as calculated by TargetScan and the percentage luciferase expression. In addition, the NR group shows a lower average total context score compared to G1 and G2. However, no differences in the average number of miR-650 binding sites³ were apparent between the three different groups.

The prediction data calculated by the miRanda algorithm is more uniformly distributed and shows a stronger correlation to the experimental data, both in terms of total mirSVR score as well as total number of miR-650 binding sites. The average mirSVR score of G1 and G2 are lower than that of the NR group. However, none of the differences observed are statistically significant. A similar trend can be observed when comparing the total number of binding sites. Here, the G1 contains the highest average number of potential binding sites while the NR group contains the lowest.

In conclusion, this analysis suggests that there is an overall weak correlation between *in silico* target prediction and experimental validation. Nevertheless, the data output from the miRanda algorithm shows a better correlation with the experimentally observed data compared to TargetScan and may therefore be more powerful in predicting genuine targets.

5.3.5 Further validation of selected target genes

As mentioned above, insertion of the SIRPB1 3'UTR sequence into the pmir-GLO vector resulted in the strongest reduction of luciferase activity in response to over expression of miR-650. This provides evidence for the binding of miR-650 to this target sequence. These

³The total number of binding sites for TargetScan is defined as the sum of 8mer, 7mer-m8 and 7mer-1A sites

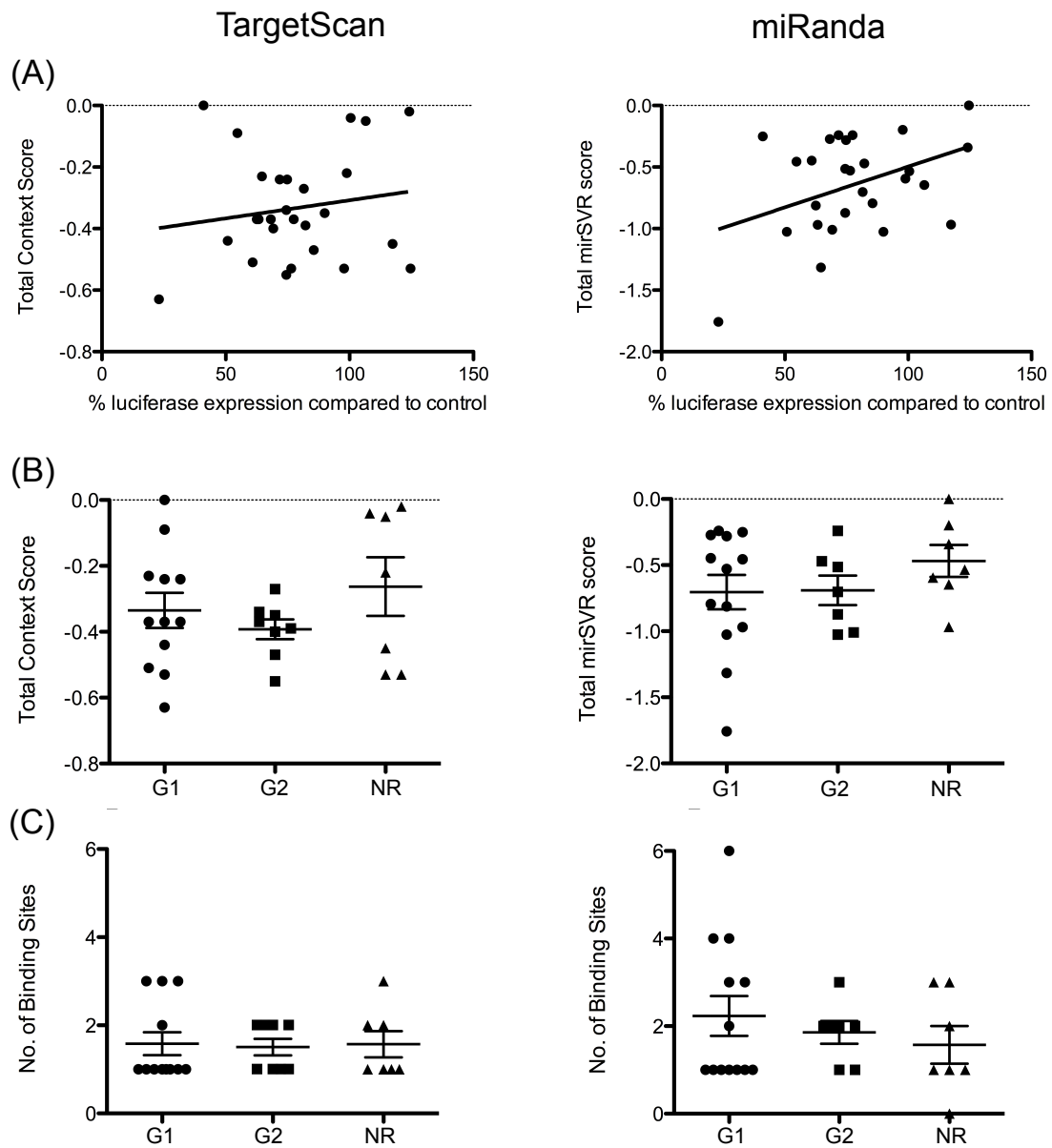


Figure 5.14: Correlations between experimentally observed data and *in silico* target predictions (TargetScan and miRanda). Panel (A) shows the correlation between target scores calculated by TargetScan and miRanda and the percentage luciferase expression measured in the presence of the miR-650 mimic relative to the nonsense control. In panel (B) scores are plotted according to Group 1 (G1), Group 2 (G2) and the non-responder group (NR). In panel (C) the total number of binding sites predicted by TargetScan and miRanda is plotted according to the three different groups. Data in (B) and (C) is shown as mean $\pm$ SEM.

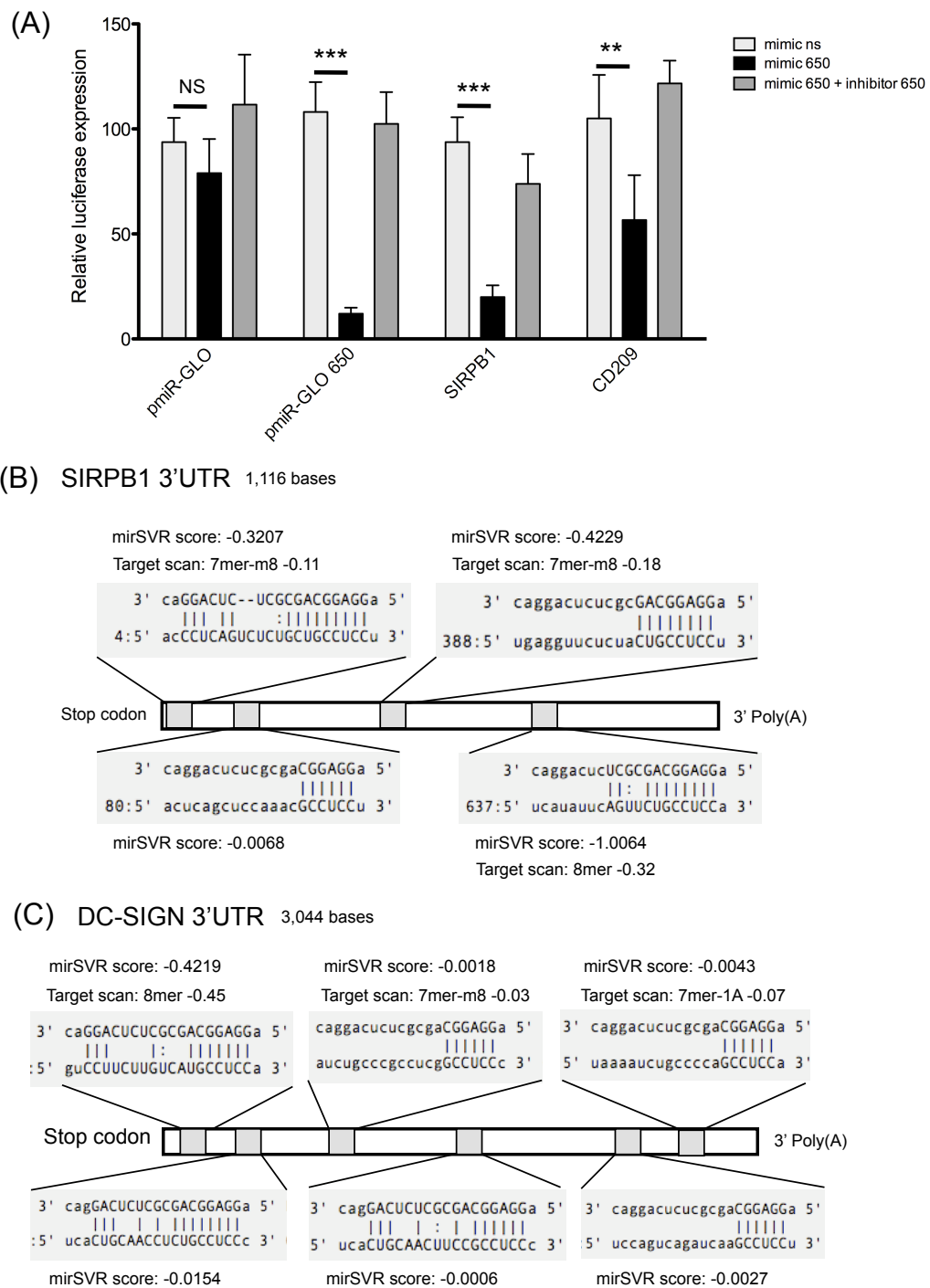


Figure 5.15: Confirming SIRPB1 and CD209 (DC-SIGN) as targets for miR-650 by 3'UTR reporter assay. (A) Additional luciferase reporter assays were performed to confirm SIRPB1 and CD209 as targets for miR-650. Firefly activity is normalised against renilla expression. Data is from 6 experiments and shown as mean $\pm$ SD. Statistical analysis was performed using an unpaired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS = not significant. This figure also shows the predicted miR-650 binding sites in the 3'UTR of (B) SIRPB1 and (C) DC-SIGN with site information derived from TargetScan and miRanda.

results were confirmed by repeating the reporter assay; results are shown in Figure 5.15(A). Overall, a 79% reduction in luciferase activity was measured for cells co-transfected with pmiR-GLO containing the 3'UTR of SIRPB1 and miR-650 mimic. This effect was efficiently rescued by the addition of the miR-650 inhibitor. As shown in Figure 5.15(B), the SIRPB1 3'UTR is 1,116 base pairs in length and contains four putative miRNA binding sites at various locations along the target sequence. While the binding site at position 637 contains a matching 8mer sequence and is assigned the lowest scores, the site at position 80 is only predicted by miRanda and not by TargetScan and is predicted to be the weakest target site.

Another high scoring target is the C-type binding lectin receptor DC-SIGN. Additional experiments confirmed that insertion of the DC-SIGN 3'UTR into the pmiR-GLO vector reduced luciferase expression by 54% (Figure 5.15(A)). The cloned 3'UTR sequence is 3,044 bp in length and is predicted to contain up to six miR-650 binding sites, three with good scores predicted by both TargetScan and miRanda, and three weaker sites predicted by miRanda only (Figure 5.15(C)).

The identification of DC-SIGN as a target for miR-650 was very interesting, especially considering the role of DC-SIGN in infection of various pathogens and its cross-talk with other PRRs [Khoo *et al.*, 2008]. However, at the time the experiments were performed, a study was published demonstrating that up regulation of miR-155 in monocyte-derived DCs decreases the level of DC-SIGN [Martinez-Nunez *et al.*, 2009]. In this case, miR-155 does not bind the 3'UTR of DC-SIGN directly. Instead, miR-155 targets the transcription factor PU.1 which, amongst other effects, leads to reduced expression of DC-SIGN mRNA and protein [Martinez-Nunez *et al.*, 2009]. As miR-155 would antagonise the potential effect of miR-650 on DC-SIGN expression, this target was not followed up any further.

5.3.6 Investigating the potential role for miR-650 in autophagy

Interestingly, two of the targets, Atg12 and MAP1LC3C (also referred to as LC3C), are essential components of the autophagy pathway (Figure 5.16). Autophagy⁴ is an ancient cellular process in which the cell engulfs and digests components of its own cytoplasm. It is characterised by the formation of an isolation membrane within the cytoplasm that engulfs designated cargo into a double-membrane vesicle referred to as an autophagosome. This is followed by the fusion of the autophagosome with the lysosome, resulting in the degradation of the sequestered cargo.

Autophagy occurs in all cells at a low basal level and performs homeostatic functions,

⁴More specifically referred to as macroautophagy

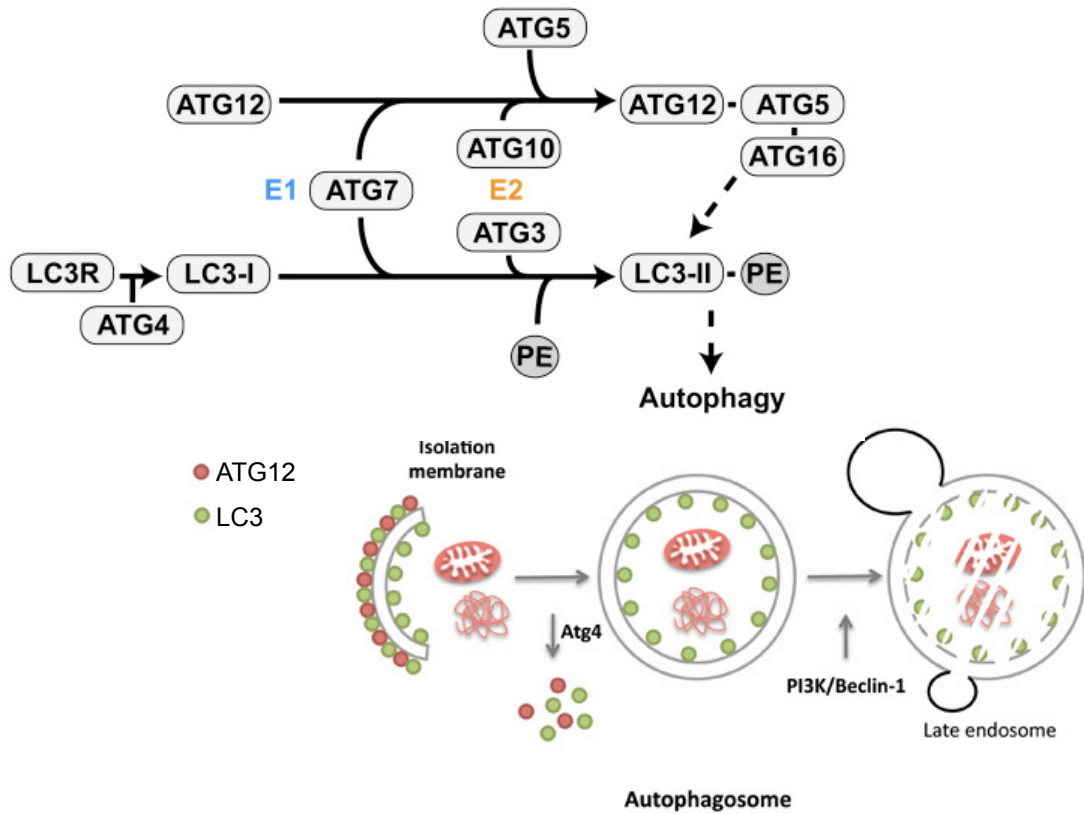


Figure 5.16: Schematic diagram showing the different steps involved in autophagosome formation. Both ATG12 and LC3 are ubiquitin-like proteins that are activated by the E1-like enzyme ATG7. ATG12 and LC3 are then transferred to the E2-like enzymes ATG10 and ATG3 and are conjugated to ATG5 and phosphatidylethanolamine (PE), respectively. LC3-PE, also known as LC3-II is incorporated into the membrane of the forming and completed autophagosome and is subsequently degraded in the lysosome. The ATG12-ATG5 conjugate associates with ATG16 to form an E3-like ligase which is involved in anchoring LC3-PE to the autophagosomal membrane. Figure adapted from [Jaeger & Wyss-Coray, 2009].

such as protein and organelle turnover. In addition, autophagy is a protective mechanism that is rapidly induced under cellular stress to recycle essential nutrients and therefore maintain vital cellular functions. Important for this study is the increasing evidence suggesting that innate immune signalling triggered by PAMP recognition induces the autophagosome formation [Delgado *et al.*, 2008] [Shi & Kehrl, 2008]. The process of autophagosome formation is orchestrated by more than 30 essential autophagy related gene (Atg) proteins, which form two ubiquitin-like conjugation systems. The process of autophagosome formation is described in greater detail in Figure 5.16.

Both ATG12 and LC3C contain a single 8mer miR-650 binding site in their 3'UTR (Figure 5.17). However, while LC3C belongs to G1, Atg12 was grouped into G2 due to the high

standard deviation. Repeat experiments confirmed that the 3'UTR of both LC3C and Atg12 responds to miR-650 in reporter assays with 56% and 60% repression, respectively (Figure 5.17(A)).

Given the potential role of miR-650 in regulating the expression of Atg12 and LC3C, both involved in orchestrating the formation of autophagosomes, it seemed reasonable to hypothesize that miR-650 may have an effect on autophagosome formation. Autophagosome formation can be monitored by immunoblotting or confocal microscopy. Both techniques detect the conversion of cytosolic LC3-I to its lipidated form LC3-II, as it is recruited to the autophagosomal membrane [Tanida *et al.*, 2004]. Detection of LC3 is rather challenging and many studies therefore use a construct expressing an LC3-GFP fusion protein which provides a convenient readout for autophagy induction. However, this has repeatedly been criticised, as over expressed LC3-GFP can spontaneously form aggregates and localise to inclusion bodies in an autophagy-independent manner [Kuma *et al.*, 2007]. Therefore, autophagy induction was measured by staining for endogenous levels of LC3B following the protocol established in [Cooney *et al.*, 2010].

I sought to investigate the role of miR-650 in autophagy by transiently transfecting immature DCs with miR-650 mimic or inhibitor for 24 hrs. DCs were then allowed to adhere to culture slides before they were washed, fixed and stained for endogenous LC3B. As visualised by confocal microscopy, LC3B staining is located in the cytoplasm (Figure 5.18(A)). In the steady state, DCs display a basal level of autophagy characterised by a uniform distribution of LC3B throughout the cytoplasm. However, following knockdown of miR-650 by transfection of antisense oligonucleotides LC3B staining becomes punctate, indicative of the formation of autophagosomes (Figure 5.18).

These results suggest that PRR-mediated down regulation of miR-650 may be involved in autophagy induction in response to invading pathogens.

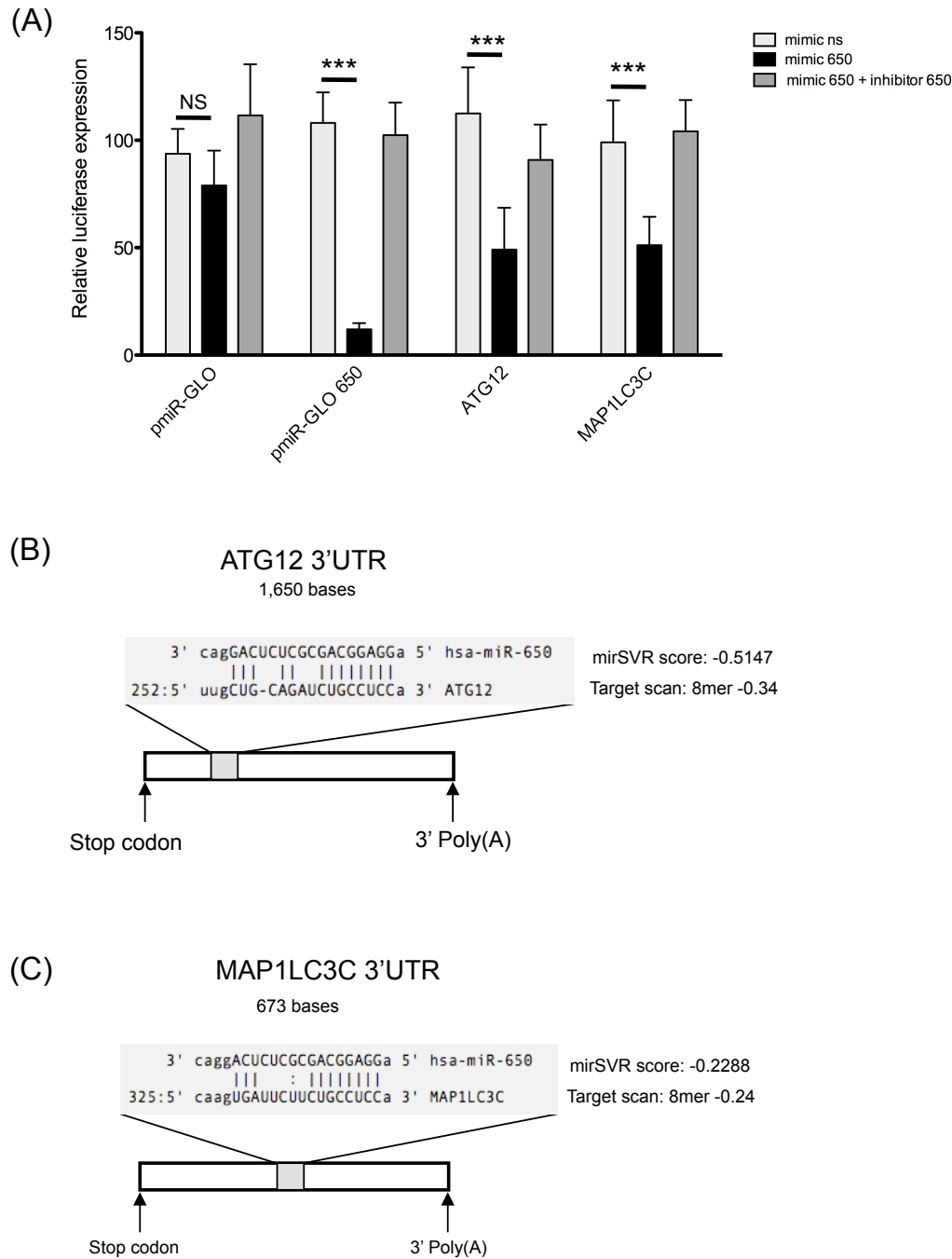


Figure 5.17: Confirming ATG12 and MAP1LC3C as targets for miR-650 by 3'UTR reporter assay. (A) Additional luciferase reporter assays were performed to confirm ATG12 and MAP1LC3C as targets for miR-650. Firefly activity is normalised against renilla expression. Data is from 6 experiments and shown as mean $\pm$ SD. Statistical analysis was performed using an unpaired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS = not significant. This figure also shows the predicted miR-650 binding sites in the 3'UTR of (B) ATG12 and (C) MAP1LC3C with site information derived from TargetScan and miRanda.

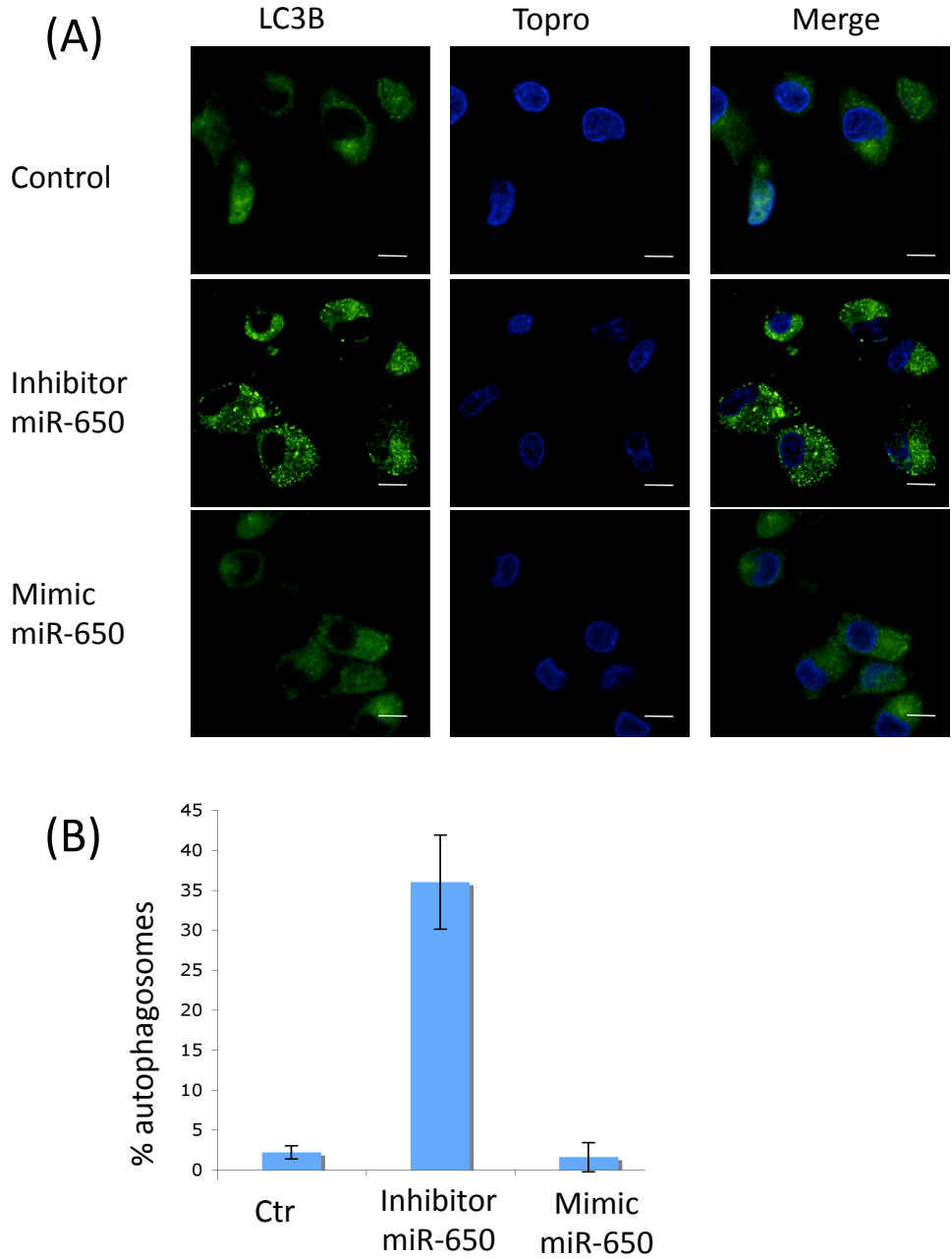


Figure 5.18: Effect of miR-650 expression on autophagosome formation in human DCs. (A) Visualisation of autophagosomes in human DC by staining for LC3B (ee Cooney et al. for experimental setup [Cooney *et al.*, 2010]). DCs were transfected with miR-650 inhibitor of miR-650 mimic and incubated for 24 hrs before staining for LC3B. Nuclei were visualised using TO-PRO stain (Invitrogen). One representative donor out of three is shown. (B) Quantification of the percentage of cells showing autophagosome formation characterised by punctate LC3B staining. Data shown as mean $\pm$ SD.

5.4 Discussion

Using a combination of computational methods and 3'UTR reporter assay, the work in this chapter validates the known miR-650 target ING4 and identifies a number of novel targets. The data demonstrate that miR-650 directly targets a variety of genes involved in immune functions, such as cell-to-cell signalling and autophagy. This is, to my knowledge, the first report linking miR-650 to the immune response.

The divide between *in silico* target predictions and experimental validation of miR-650 targets

As already mentioned, there is currently no canonical way for identifying novel miRNA targets. Computational tools are currently the best starting point for exploring potential miRNA:target interactions. However, due to the lack of knowledge concerning the nature of the interaction, computational predictions yield a significant number of false positive as well as false negative results. Nevertheless, these tools are invaluable for narrowing down potential targets prior to performing 3'UTR assays which currently provide the only method for identifying a direct interaction between the miRNA and its target sequence.

This selection process not only aims at decreasing the number of potential targets but should preferably also lead to enrichment in genuine targets. As previously mentioned, several groups have attempted to evaluate different prediction programs experimentally using various genome-wide screening methods [Baek *et al.*, 2008] [Selbach *et al.*, 2008]. Selbach *et al.* found that the top third predictions obtained by TargetScan correlate significantly better with the experimentally observed data [Selbach *et al.*, 2008]. These criteria were used in this study in order to narrow down the potential miR-650 target sites for validation. However, as the work in this chapter shows, narrowing down candidate target genes to only include the top third of all TargetScan predictions still yields a large number of potential target genes. Furthermore, out of the 28 target genes tested, only 20 could be experimentally validated, thus showing a success rate of approximately 70%. In addition, the 3'UTR of the validated target genes vary greatly in their susceptibility to miR-650 regulation as shown by varying levels of luciferase repression.

Comparison of these experimental data with *in silico* predictions revealed that the information provided by the miRanda algorithm, i.e. both context score and number of binding sites, correlated better with the experimentally observed data than TargetScan. This suggests that the mirSVR score and number of binding sites calculated by the miRanda algorithm may be used as indicators for prediction quality. However, due to the spread in the data,

it is not possible to define a clear cut-off which allows the selection of the most significant targets. Furthermore, it is difficult to draw general conclusions from these data, not only due to the small sample size and prior target selection, but also because it is unclear whether these principles are limited to miR-650 or can also be applied to other miRNAs. Therefore, significantly more work is required to expand this study to include a wider range of candidate target genes and other miRNAs as well as additional target prediction programs.

Candidate miR-650 targets identified by 3'UTR reporter assay

The confirmation of ING4 as a miR-650 target, combined with the finding that the top ranking function of predicted miR-650 targets includes cell morphology and DNA replication, recombination and repair, supports the study by Zhang *et al.* which suggests that miR-650 is associated with cancer [Zhang *et al.*, 2010]. Recent reports highlight a potential role for ING4 in the immune system as it interacts with NF- κ B and suppresses its transcriptional activity [Nozell *et al.*, 2008]. Knockdown of ING4 expression results in enhanced NF- κ B-regulated gene expression leading to increased IL-6 expression [Li & Li, 2010]. So far, the role of ING4 in the innate immune response is not fully defined. On the basis of this study it could, however, be hypothesised that PRR-mediated down regulation of miR-650 might lead to increased ING4 expression, which in turn acts as a negative feedback loop turning off NF- κ B activation. This is in agreement with the data in Chapter 4 showing that down regulation of miR-650 reduces the secretion of selected cytokines and, taken together, these findings suggest an involvement of miR-650 in a negative feedback loop controlling innate immune responses.

The remaining, newly identified miR-650 targets include a number of proteins such as IRF3, NOD1, SIRP β and DC-SIGN, which are known to be expressed in monocyte-derived DCs and have well-known functions in the innate immune response. In contrast, other targets identified in the course of this study have a more speculative function in the innate immune response.

SIRP β is a transmembrane glycoprotein that belongs to the family of signal regulatory proteins (SIRPs) and was the strongest responder to miR-650 in the 3'UTR luciferase assay [Kharitonov *et al.*, 1997]. Human SIRP β has been shown to be expressed in myeloid cells including DCs, monocytes, macrophages, but not T lymphocytes [Seiffert *et al.*, 2001]. It contains three Ig-like domains in its extracellular domain and a short cytoplasmic tail [Tomasello *et al.*, 2000]. SIRP β is an activating receptor that associates with DAP12, an ITAM bearing polypeptide. Ligation of SIRP β results in tyrosine phosphorylation of DAP12

and subsequent recruitment of the SH2-containing protein kinase Syk [Tomasello *et al.*, 2000] [Dietrich *et al.*, 2000]. Engagement of SIRP β by specific monoclonal antibodies promotes phagocytosis and induces the formation of filopodia and lamellipodia in mouse peritoneal macrophages [Hayashi *et al.*, 2004]. In addition, SIRP β has been shown to positively regulate the transepithelial migration of neutrophils [Liu *et al.*, 2005]. Interestingly, a study in mouse microglial cells demonstrates that SIRP β expression is induced by interferons and suppresses LPS-mediated TNF α secretion [Gaikwad *et al.*, 2009]. This again indicates an involvement of miR-650 in a negative feedback loop. However, the ligand for SIRP β is currently unknown and its role in the immune response of DCs has not yet been fully elucidated.

Taken together, it could be hypothesised that PRR signalling down regulates miR-650, which in turn up regulates inhibitory proteins ING4 and SIRP β to prevent over activation and a cytokine storm.

However, as mentioned previously, 3'UTR assays were carried out in HEK293T cells and further work is necessary to confirm regulation of target gene expression by miR-650 in monocyte-derived DCs. Furthermore, these findings need to be put into the context of a complex cellular network. It is therefore necessary to monitor whether the expression of these potential miR-650 targets is indeed regulated during the course of immune activation.

As an example, the 3'UTR screen identifies IRF3 as a target for miR-650. However, IRF3 is not transcriptionally regulated in response to PRR signalling. Its activation is triggered through phosphorylation which promotes dimerisation and nuclear translocation [Hiscott *et al.*, 1999]. Phosphorylated IRF3 is then rapidly degraded via a proteosomal dependent pathway. In light of these findings, miR-650 could merely play a stabilising role, maintaining IRF3 expression during infection.

The targeting of DC-SIGN by miR-650 highlights an even more complex scenario. As already mentioned, DC-SIGN expression is also controlled by miR-155 in an indirect manner [Martinez-Nunez *et al.*, 2009]. Hence, PRR-mediated up regulation of miR-155 might antagonise the action of miR-650. This highlights the potentially complex miRNA regulatory networks used to fine-tune gene expression. Future work on a system level is required to explore the effect of miR-650 on the expression of selected target genes in DCs during the course of an immune response.

The regulation of autophagosome formation by miR-650

The work in this chapter identifies a novel link between miR-650 and the autophagic pathway. Increasing evidence suggests that innate immune signalling triggered by PAMP recognition

induces the autophagic pathway. Stimulation through a number of TLRs, including TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7 and TLR9, results in the induction of autophagy in human macrophages [Delgado *et al.*, 2008] [Shi & Kehrl, 2008]. Among these, TLR7 is the most potent trigger of autophagy, although the reason for this is still unclear. The activation of autophagy by TLRs requires signalling via the adaptor molecules MyD88 and TRIF which interact with Beclin-1, a key regulator of the autophagic pathway. This leads to the dissociation of Beclin-1 from its pro-apoptotic interactors Bcl-2 and Bcl-XL, thus leading to the induction of autophagy [Shi & Kehrl, 2008]. It is important to note that many of these studies were performed in the human Raw264.7 macrophage cell line and await confirmation in primary cells. Other PRRs are also capable of regulating autophagy. Stimulation of human monocyte-derived DCs with the NOD2 ligand MDP, for example, induces autophagy, resulting in bacterial restriction and increased MHC class II antigen presentation [Cooney *et al.*, 2010].

To date only two miRNAs have been implicated in the regulation of autophagy. Zhu *et al.* demonstrated that miR-30a targets Beclin-1, a key autophagy-promoting gene [Zhu *et al.*, 2009]. Over expression of miR-30a reduces expression of Beclin-1 and blunts the activation of autophagy. In a more recent study Frankel *et al.* identified miR-101 as a potential negative regulator of rapamycin-induced autophagy which functions by targeting STMN1, RAB5A and ATG4D [Frankel *et al.*, 2011]. Overexpression of STMN1 could partially rescue cells from miR-101-mediated inhibition of autophagy.

The data presented in this chapter show that miR-650 targets Atg12 and LC3C, and furthermore, that down regulation of miR-650 induces autophagy in DCs. However, it remains unclear whether the expression of ATG12 and LC3C is induced upon PRR signalling and, more importantly, whether increased expression of either of these two proteins is sufficient to trigger the induction of autophagy.

As described previously, both Atg12 and LC3 are essential components of the autophagic machinery that localise to the autophagosomal membrane. LC3 is a structural component of the autophagosome and supports membrane hemifusion during elongation. It also forms an anchor for cargo recruitment [Nakatogawa *et al.*, 2007]. Three isoforms of human LC3 exist, all of which locate to autophagosomal membranes but show distinct expression patterns in different human tissues and also differ in their post-translational modifications [He *et al.*, 2003]. It is at present unclear whether there is redundancy or whether these three isoforms have distinct roles. While this report shows that the expression of LC3C is controlled by miR-650, *in silico* target prediction suggests that the other two isoforms LC3A and LC3B lack miR-650 binding sites. Therefore, further work is required to determine whether manipulation of

LC3C alone is sufficient to manipulate autophagy in dendritic cells, and whether the other LC3 isoforms are possibly regulated by miR-650 in either a direct or indirect manner.

The other autophagy gene identified, Atg12, conjugates to Atg5 and, in a complex with Atg16L, regulates the elongation process of the isolation membrane [Mizushima *et al.*, 1998] and is important for the recruitment of LC3 to the autophagic membrane. A recent study by Jounai *et al.* showed that over expression of the Atg12-Atg5 complex in mouse embryonic fibroblasts (MEFs) suppresses the production of type I IFNs in response to dsRNA [Jounai *et al.*, 2007]. According to the authors, Atg12-Atg5 interferes with dsRNA-mediated signalling by interacting directly with the CARD domains of both IPS-1 and RIG-I. This observation was supported by a more recent study demonstrating that Atg5^{-/-} MEFs show enhanced RLR signalling, produce higher levels of type I IFN and have increased resistance to VSV infection [Tal *et al.*, 2009]. The link between RLR signalling and the absence of autophagy was shown to be due to accumulation of dysfunctional mitochondria and the accompanying build-up of reactive oxygen species. However, the role of Atg12-ATG5 conjugates in the inhibition of type I IFN production is somewhat counterintuitive, as autophagy is commonly thought of as an antiviral mechanism.

In addition to targeting Atg12 and LC3C, miR-650 could potentially also regulate the expression of other proteins involved in autophagosome formation. One candidate identified in this study is the protein FKBP1A, a cis-trans isomerase that belongs to the family of immunophilins. It plays an important role in basic cellular processes involving protein folding and trafficking and binds to the immunosuppressant FK506 [Choi *et al.*, 1996]. Importantly, it has also been shown to interact with rapamycin and the mammalian target of rapamycin (mTOR) [Standaert *et al.*, 1990], one of the key regulators of autophagy. mTOR inhibits autophagy under steady-state conditions and is inactivated in response to cellular stress such as starvation, leading to the induction of autophagy. It is, however, unknown if over expression of FKBP1A affects the activity of mTOR.

Taken together, these data offer multiple potential mechanisms by which miR-650 may be able to induce autophagy in DCs. Further work will be required to see which of these, or potentially other as yet unconfirmed targets of miR-650, are the prime drivers of this biological activity; or whether, as seems more likely given the complex nature of the miRNA network highlighted above, it is the cumulative effect of a number of diverse miR-650 targets.

Finally, considerably more work will be required to investigate the importance of miR-650-induced autophagy in the context of bacterial or viral infection. In recent years, autophagy has been increasingly recognised to play an important role in controlling infections by delivering cytosolic PAMPs to endosomal TLRs and directing intracellular or indigested

pathogens to the lysosomes promoting their destruction [Sanjuan *et al.*, 2009]. The killing of engulfed pathogens is thought to occur via a process referred to as LC3 assisted phagocytosis (LAP) during which LC3 is recruited to the surface of the ingested phagosome and, through an unknown mechanism, promotes maturation of the cargo containing phagosome to lysosomes [Sanjuan *et al.*, 2007][Andrade *et al.*, 2006]. Furthermore, autophagy is involved in feeding antigen to MHC class I [Dengjel *et al.*, 2005] [English *et al.*, 2009] and class II [Nimmerjahn *et al.*, 2003] [Schmid *et al.*, 2007] and therefore promotes the activation of an adaptive immune response. Finally, aberrant regulation of autophagy contributes to human inflammatory disorders such as Crohn's disease.

As Influenza A has been shown to down regulate miR-650 (Chapter 4), this could provide a good starting point for future investigations. Several studies have shown that infection of MDCK cells and the human lung epithelial cell line A549 with different Influenza A strains strongly induces the formation of autophagosomes within infected cells [Zhou *et al.*, 2009] [Gannagé *et al.*, 2009]. However, the role of autophagy in Influenza A virus infection remains controversial. Zhou and colleagues demonstrate that inhibition of autophagosome formation, by depleting LC3 or Beclin-1, impairs the accumulation of viral proteins (M1 and M2) and therefore reduces viral titres. This suggests that the Influenza A virus hijacks the autophagic machinery to increase its replicative capacity [Zhou *et al.*, 2009]. In addition, Gannage *et al.* provided evidence that the M2 protein of Influenza A efficiently blocks the fusion of autophagosomes with lysosomes. Using Atg5^{-/-} MEFs the authors show that loss of autophagy reduces the survival of Influenza A virus-infected cells. This suggests that autophagy may also serve as a cell survival pathway in influenza-infected cells. However, even though the authors reported an accumulation of viral proteins and RNA, they did not detect higher viral titres in the supernatants of autophagy-competent cells.

5.5 Future work and conclusion

In summary, work in this chapter confirms one known and identifies 19 novel miR-650 targets by 3'UTR reporter assay. Although the effect of miR-650 on reporter gene expression was blocked by a specific miR-650 antisense inhibitor, ongoing work is being carried out to identify the exact MRE using site-directed mutagenesis. For 3'UTRs with more than one binding site, this could be used to assess the contribution of individual sites to miR-650 mediated target regulation. The dissection of individual binding sites may be helpful for further unravelling the characteristics of miRNA targeting and therefore serve to improve current prediction algorithms. In addition, further work is needed to confirm the effect of miR-650 on these

targets in monocyte-derived DCs and to elucidate their role in the innate immune response.

As the 3'UTR reporter assay system is not amenable for high-throughput screening, prior selection of target genes was required. Therefore, the targets identified in this work merely provide a small snapshot of the function of miR-650. In order to assess the global effect of miR-650, including direct and indirect effects as well as non-classical miRNA action, large-scale genome-wide approaches need to be considered. This is the focus of Chapter 6.

Chapter 6

Exploring the function of miR-650 using global gene expression profiling

6.1 Introduction

MicroRNAs are complex regulators of gene expression that can, by direct or indirect mechanisms, fine-tune protein synthesis of thousands of genes. Following identification of individual miR-650:target pairs in Chapter 5, this chapter focuses on elucidating both direct and indirect effects, in an attempt to obtain a broad overview of cellular networks regulated by miR-650.

As previously mentioned, endogenous miRNA levels can be manipulated using specific mimics to over express, or antisense oligonucleotides to knockdown the miRNA of interest. Changes in gene expression are then assessed on a global level by high-throughput profiling methods. However, the choice of method is complicated by the complex action of mammalian miRNAs.

The mechanism of miRNA-mediated gene silencing

As described in Section 1.3.3, imperfect sequence complementarity outside the seed sequence is a key characteristic of the interaction between mammalian miRNAs and their targets. This not only complicates computational target prediction, but also dictates the mechanism by which miRNAs execute target silencing. Initial studies in *Drosophila* indicated that the degree of overall complementarity directs the mechanism by which miRNAs mediate silencing

[Förstemann *et al.*, 2007]. It is commonly thought that, if a miRNA binds to its target with perfect complementarity, the miRNA:mRNA duplex is cleaved between nt 9 and 11 of the miRNA by the RISC complex. This inhibits transcription and results in decreased target mRNA levels [Haley & Zamore, 2004] [Martinez & Tuschl, 2004]. Alternatively, if binding occurs with imperfect complementarity, the mRNA is not cleaved. Instead, miRNA binding interferes with either the initiation or elongation step of protein translation [Pillai *et al.*, 2005] [Olsen & Ambros, 1999].

However, it is now generally well-accepted that this simple distinction does not necessarily apply for mammalian miRNAs which, despite binding to their target with imperfect complementarity, have the ability to modulate target expression on an mRNA level [Bagga *et al.*, 2005] [Farh *et al.*, 2005] [Giraldez *et al.*, 2006]. Consequently, several studies have attempted to assess the relative contribution of mRNA degradation and translational repression in mammalian miRNA-mediated gene silencing on a genome-wide level [Baek *et al.*, 2008] [Selbach *et al.*, 2008] [Guo *et al.*, 2010] [Yang *et al.*, 2010b].

Mammalian miRNAs affect target gene expression on an mRNA and translational level

Two independent studies investigated the global effects of miRNA manipulation using Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) combined with state of the art mass spectrometry to measure the overall impact of a particular miRNA on protein production and compared these results to simultaneously performed gene expression microarrays [Baek *et al.*, 2008] [Selbach *et al.*, 2008] [Mourelatos, 2008]. Together, these studies demonstrate that over expression of a single miRNA affects the protein level of several hundred genes. In agreement with the general view that miRNAs fine-tune gene expression, effects were relatively mild as only few proteins decreased more than 2-fold. However, it is important to note that a more dramatic net effect may be observed under physiological conditions as a results of cooperative action of different miRNAs. Furthermore, most targets were repressed at both the mRNA and translational level, with the contribution size of each process to down regulation being dependent on the individual miRNA:mRNA pair. Consistent with the above-mentioned principles, miRNA:mRNA pairs with mismatches between nt 9-11 of the miRNA were mainly repressed at the protein level with little effect on mRNA level. This is in agreement with the idea that these miRNA:mRNA pairs cannot be cleaved by the RISC complex, thus favouring translational inhibition over mRNA degradation. In most cases, the observed changes in mRNA expression did not fully account for the repression observed

at protein level. Furthermore, measurement of mRNA and protein expression over time revealed that mRNA levels are initially stable and match protein levels only at later time points [Selbach *et al.*, 2008]. This supports the view that mammalian miRNAs primarily promote translational repression which, in turn, results in mRNA destabilisation.

These results were later confirmed by studies which failed to show a strong correlation between mRNA and protein expression when comparing the effects of miR-21 and miR-143 over expression on an mRNA as well as on a protein level using gene expression microarrays and proteomic-based approaches, respectively [Yang *et al.*, 2009] [Yang *et al.*, 2010b]. However, a recent study by Guo *et al.* suggests that, similar to the mechanism described in plants, mammalian miRNAs predominantly exert their effects through decreasing the level of mRNA [Guo *et al.*, 2010]. This study used large-scale sequence analysis of both total mRNA and ribosome bound mRNA transcripts to monitor changes induced by miRNA over expression and estimates that approximately 84% of miRNA-mediated target repression is due to decreased mRNA levels. The differences observed between the studies described here may be a consequence of different miRNAs, cell lines or methodologies used. When considering these contradictory findings, the exact mechanism by which mammalian miRNAs mediate gene silencing remains controversial.

In addition to sequence complementarity, these and other studies revealed several other features which may direct the mechanism of mammalian miRNA silencing. Firstly, few selected targets have been shown to be regulated by miRNAs in a cell type specific manner [Inomata *et al.*, 2009] indicating that the exact mechanism of action may be specific for the miRNA and cell type used. Secondly, the level of target gene expression may also play a vital role in determining the mechanism of miRNA action. While Guo *et al.* suggest that mRNA repression is independent of the target expression level [Guo *et al.*, 2010], Baek *et al.* show that targets with lowest mRNA levels are predominantly regulated on a translational level as their mRNA level remains unresponsive to miRNA manipulation [Baek *et al.*, 2008]. Lastly, specific groups of targets may show preference for either transcriptional or translational repression. Lastly, there may be a range of other factors influencing the choice of miRNA silencing mechanisms. In the study by Selbach *et al.*, for example, proteins that are synthesised by ER-associated ribosomes are primarily regulated on a translational level, possibly because they cannot localise to P-bodies unlike transcripts associated with free ribosomes [Selbach *et al.*, 2008].

Genome-wide approaches used to explore miRNA function on a global level

Though it would be highly desirable to address miRNA-mediated target regulation on a protein level, high-throughput quantitative proteomic screens pose several challenges. High proteome coverage and sensitivity are essential but, even with state of the art mass spectrometry, quantification is usually only possible for the most abundant proteins [Baek *et al.*, 2008] [Selbach *et al.*, 2008]. In addition, SILAC requires rapid cell division and is therefore not appropriate when using non-dividing cells such as monocyte-derived DCs.

As mentioned earlier, the most promising approaches combine RISC pull-down and high throughput sequencing (called HITS CLIP and PAR CLIP) comparing cells which express and are depleted of the miRNA of interest [Licatalosi *et al.*, 2008] [Hafner *et al.*, 2010]. In addition to identifying novel miRNA targets on a genome-wide level these approaches can be used to map the exact MRE. However, these methods are not readily accessible and require extensive setup and optimisation.

As a consequence, the effect of miRNAs on translation is often estimated by monitoring changes on the mRNA level using microarray profiling [Lim *et al.*, 2005] [Ceppi *et al.*, 2009]. As mentioned previously, gene expression profiling using microarray technology provides a rapid and powerful tool to analyse thousands of transcripts simultaneously. Even though these studies have been performed successfully in some cases, they carry the risk of a decreased discovery rate as they rely on the assumption that miRNA binding reduces target mRNA stability, and thus neglect the potential of miRNAs to regulate expression exclusively on a protein level by inhibiting translation.

Finally, although global expression profiling is useful for identifying biological networks regulated by an individual miRNA therefore providing a general overview of its biological function, it fails to differentiate between direct and indirect effects. As a consequence, computational tools as well as 3'UTR assays are required to define in which cases direct miRNA:mRNA interactions are involved.

Global effects of miR-155 revealed by expression profiling studies

Due to the key role of miR-155 in a number of different cellular functions, a variety of global screening studies have focussed on this particular miRNA. Both Guo *et al.* and Selbach *et al.* investigated the effect of miR-155 over expression in HeLa cells, which express low levels of endogenous miR-155, by using SILAC and ribosome profiling, respectively. In addition, a recent study by Lössner *et al.* used SILAC to assess the effect of miR-155 in HEK293T cells [Lössner *et al.*, 2011].

Unfortunately, the contradictory nature of miRNA research in general is also true of these studies as, whilst Guo *et al.* found that regulation occurs predominantly on an mRNA level, the other studies suggest that miR-155 repression is mainly mediated by translational repression. In an attempt to elucidate the role of miR-155 in primary innate immune cells, Ceppi *et al.* used microarray technology to measure changes induced by miR-155 knockdown in monocyte-derived dendritic cells [Ceppi *et al.*, 2009]. Down regulation of miR-155 in immature DCs resulted in 177 differentially regulated probe sets (employing a cut-off of 1-5 fold) with 131 up and 46 down regulated genes. Even though gene expression analysis revealed the known miR-155 targets BACH1, CUTL1, and CEBPB [O’Connell *et al.*, 2008], it only led to the validation of one novel target, namely TAB2.

A comparison shows that, despite the fact that each study led to the identification of novel miR-155 targets, the overlap was limited; only approximately one third of the proteins down regulated in HeLa were also quantified in HEK293T cells and none of these proteins have been identified as direct miR-155 targets [Lössner *et al.*, 2011]. Observed differences may stem from different cell types and profiling methods. What is clear, however, is that the methodology used is crucial and that all data obtained will have to be interpreted in the context of the techniques and cell lines used.

Experimental setup

As mentioned previously, endogenous miRNA levels can be selectively manipulated using miRNA mimics or antisense inhibitors. Both miRNA over expression and knockdown have been successfully used for large-scale screening studies [Selbach *et al.*, 2008] [Ceppi *et al.*, 2009] and the choice depends on the individual circumstances. It is worth noting that, as in the case of siRNAs, over expression of miRNAs can lead to non-physiological off-target effects which may be misleading [van Dongen *et al.*, 2008]. Nevertheless, a comparison between the effects triggered by knockdown or over expression of the miRNA let-7b showed that target expression is inversely modulated, suggesting that off-target effects do not necessarily dominate [Selbach *et al.*, 2008]. Furthermore, up regulation of a specific miRNA can saturate the miRNA processing machinery and therefore displace other endogenous miRNAs [Khan *et al.*, 2009].

In this study, I sought to investigate the effects of miR-650 on the global gene expression profile of monocyte-derived DCs. For this purpose, miR-650 was over expressed and mRNA levels were monitored by microarray technology. Since many genome-wide studies have been performed in an attempt to explore the function of miR-155, and many targets have already

been validated for this particular miRNA, over expression of miR-155 served as a positive control. In addition, given the limited overlap between previous profiling studies, this may reveal additional interesting findings concerning the action of miR-155 in primary DCs.

6.2 Aims

This chapter aims to:

- Explore the global effect of miR-650 over expression in DCs on a transcriptional level using microarray technology.
- Discriminate between direct and indirect targets using computational prediction tools and 3'UTR reporter assays.
- Focus on specific targets that may have an effect on the function of DCs and the innate immune response.

6.3 Results

6.3.1 Collecting samples for microarray study

In brief, immature monocyte-derived DCs from three buffy coats were harvested and transfected with oligonucleotides mimicking miR-650, miR-155 and a nonsense control. Transfections were performed by electroporation using the Amaxa nucleofector as previously described in [Cooney *et al.*, 2010]. After 24 hrs, total RNA was extracted, quantified using the Nanodrop UV spectrophotometer and checked on the Agilent 3100 Bioanalyser platform to verify RNA integrity. In light of the results of the 3'UTR reporter assay setup experiment performed in Chapter 5, which demonstrated that miRNA-mediated target repression is more pronounced after 48 hrs, as well as a study by Wang *et al.* showing increased discovery rates at later time points [Wang & Wang, 2006], it would have been preferable to perform the profiling study at a later time point. However, experience in our group has shown that nucleofection of monocyte-derived DCs induces a significant amount of cell death accompanied by a decline in RNA quality. As can be seen from the Bioanalyser traces displayed in Figure 6.1(A), the RNA isolated 24 hrs post nucleofection was of lower quality compared to that obtained from untreated cells (see Figure 6.1). As a result of overall lower RNA integrity, the cut-off was lowered to allow samples with a RIN value above 7.

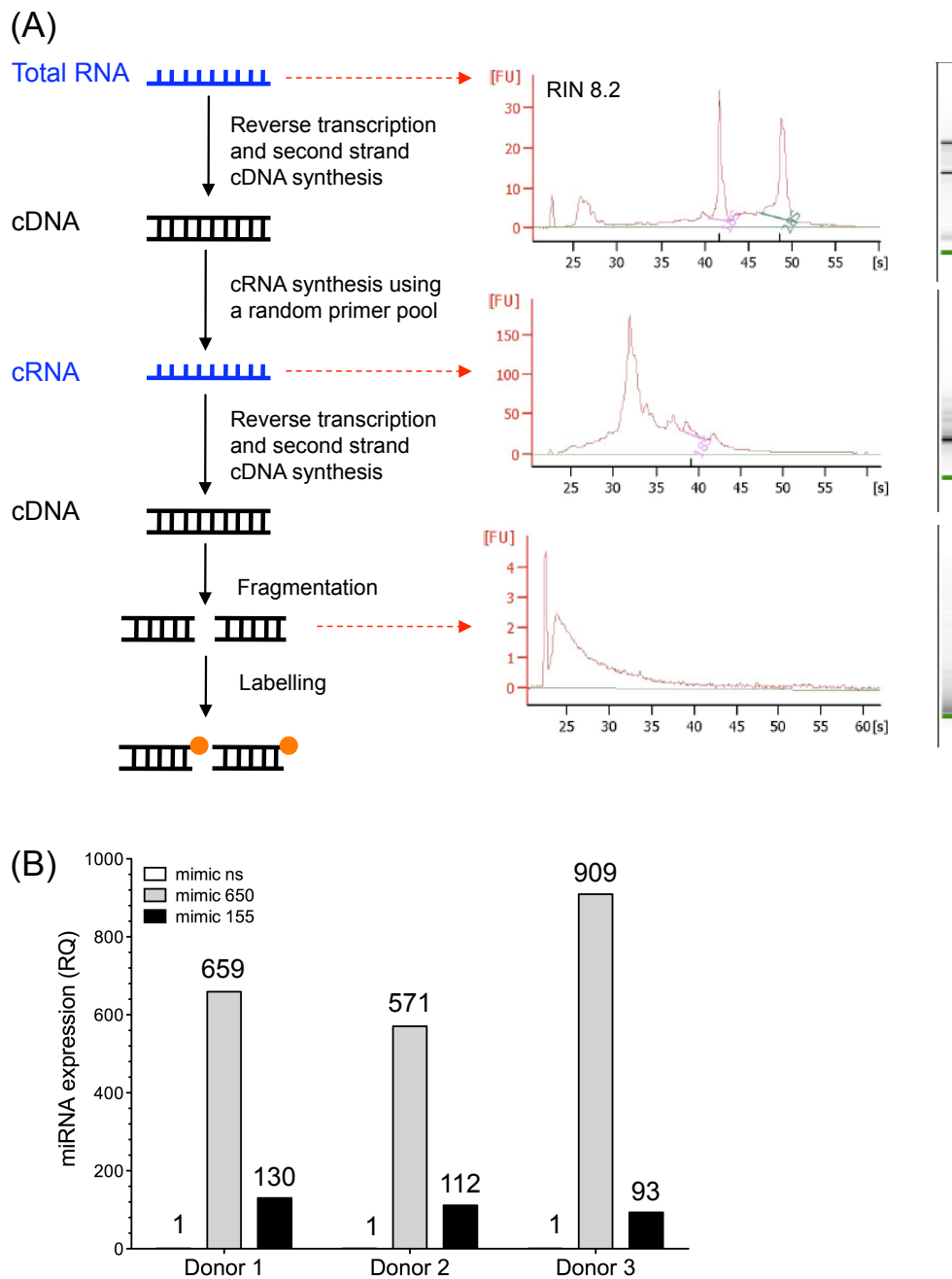


Figure 6.1: Samples collected for Affymetrix microarray analysis. DCs were transfected with either miR-650, miR-155 or nonsense mimic. **(A)** RNA was extracted 24 hrs post-transfection and the quality was assessed using the Bioanalyser 2100. RNA was reverse transcribed into double-stranded cDNA which served as the template for amplification by *in vitro* transcription. The resulting cRNA is reverse transcribed back into cDNA which was finally fragmented, labelled and hybridised onto the Affymetrix microarray. **(B)** Measuring miR-650 and miR-155 expression in cells transfected with the corresponding miRNA mimics. The plot shows the samples from three different donors which were used for array analysis. Expression is normalised against RNU48 and is shown relative to the nonsense control.

Transfection of miR-650 and miR-155 mimics was confirmed by qRT-PCR, shown in Figure 6.1(B). However, despite using the same final concentration of miRNA mimic, over expression of miR-650 was more efficient across all three donors than that of miR-155. In general, up regulation varied from approximately 100-1,000-fold which is significantly higher than what is observed under physiological conditions. However, consideration must be given to the fact that this assay does not provide any information about the cellular location of the mimic. As DCs are highly phagocytic, it is possible that some of the miRNA mimic exists in endosomal compartments and is therefore unable to interact with its targets. The fold changes obtained may therefore not reflect the true level of biologically active miRNA within the cell.

The Affymetrix GeneChip Human Gene 1.0 ST Array, which includes 28,869 gene probes, was used to measure relative mRNA expression levels. I prepared all RNA samples for hybridisation using the GeneChip WT Terminal Labeling Kit (Affymetrix) (workflow is summarised in Figure 6.1(A)). Briefly, RNA was reverse transcribed into cDNA and amplified by *in vitro* transcription. In all cases, cRNA synthesis resulted in the generation of different size RNA fragments mainly between 200 to 2,000 nt in length; this was to be expected (Figure 6.1(A)). In the next step, the cRNA was reverse transcribed back into cDNA and fragmented into shorter cDNA oligonucleotides. Efficient fragmentation of cDNA should generate a single sharp peak when analysed by Bioanalyser. The slightly wider peak observed for the samples processed for this study is indicative of incomplete digestion. However, this is not thought to have an effect on hybridisation. Samples were hybridised onto Affymetrix arrays under guidance of Dr. Dilair Baban (Wellcome Trust Centre of Human Genetics, Oxford) and according to manufacturer's instructions. An initial on chip normalisation was performed to provide raw signal intensities and global scaling was used to compare genes from chip to chip. All data quality controls passed the initial quality guideline set by Affymetrix. Data was analysed using Genespring GX software and differentially expressed genes were considered significant if they passed a 2-way ANOVA test at $p < 0.01$ with a change of greater than 1.5-fold.

6.3.2 Genome-wide profiling of gene expression changes induced by over expression of miR-650 and miR-155

Analysis showed that a total of 289 transcripts were differentially regulated in samples transfected with miR-155 mimic; out of which 164 were up and 125 were down regulated (fold change ≥ 1.5 , $p < 0.01$). A list with the top 20 regulated genes is displayed in Table 6.1.

Table 6.1: Top 20 differentially regulated genes in response to miR-155 over expression.

Down regulated genes		
Fold change	Gene ID	Description
6.77	CCL8	chemokine (C-C motif) ligand 8
6.60	IFITM1	interferon induced transmembrane protein 1
6.41	IFI27	interferon, alpha-inducible protein 27
6.15	APOBEC3A	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A
5.38	CXCL10	chemokine (C-X-C motif) ligand 10
4.03	CXCL11	chemokine (C-X-C motif) ligand 11
3.59	IFIT2	interferon-induced protein with tetratricopeptide repeats 2
3.25	INDO	indoleamine-pyrrole 2,3 dioxygenase
3.14	LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein
3.05	GBP5	guanylate binding protein 5
3.03	GBP4	guanylate binding protein 4
2.89	CCNA1	cyclin A1
2.83	OASL	2'-5'-oligoadenylate synthetase-like
2.69	CCL7	chemokine (C-C motif) ligand 7
2.69	ISG15	Interferon-induced 15 kDa protein
2.68	ADAM19	ADAM metallopeptidase domain 19 (meltrin beta)
2.66	CD38	CD38 molecule
2.65	IFITM2	interferon induced transmembrane protein 2 (1-8D)
2.57	SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1
2.49	ITGB7	integrin, beta 7

Up regulated genes		
Fold change	Gene ID	Description
-2.97	CD14	CD14 molecule
-2.75	GATM	glycine amidinotransferase (L-arginine:glycine amidinotransferase)
-2.74	AMICA1	adhesion molecule, interacts with CXADR antigen 1
-2.49	FCGR1A	CD64 molecule
-2.46	CD302	CD302molecule
-2.18	LTA4H	leukotriene A4 hydrolase
-2.16	SLC46A3	solute carrier family 46, member 3
-2.04	CLEC10A	C-type lectin domain family 10, member A
-2.04	GPSN2	glycoprotein, synaptic 2
-2.04	LPL	lipoprotein lipase
-2.03	FRMD4B	FERM domain containing 4B
-2.01	LDHB	lactate dehydrogenase B
-1.99	PRKACB	protein kinase, cAMP-dependent, catalytic, beta
-1.95	MPEG1	macrophage expressed gene 1
-1.94	GGTA1	glycoprotein, alpha-galactosyltransferase 1
-1.92	EMR4	egf-like module containing, mucin-like, hormone receptor-like 4
-1.91	FBP1	fructose-1,6-bisphosphatase 1
-1.90	PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)
-1.88	OAT	ornithine aminotransferase (gyrate atrophy)
-1.88	AIF1	allograft inflammatory factor 1

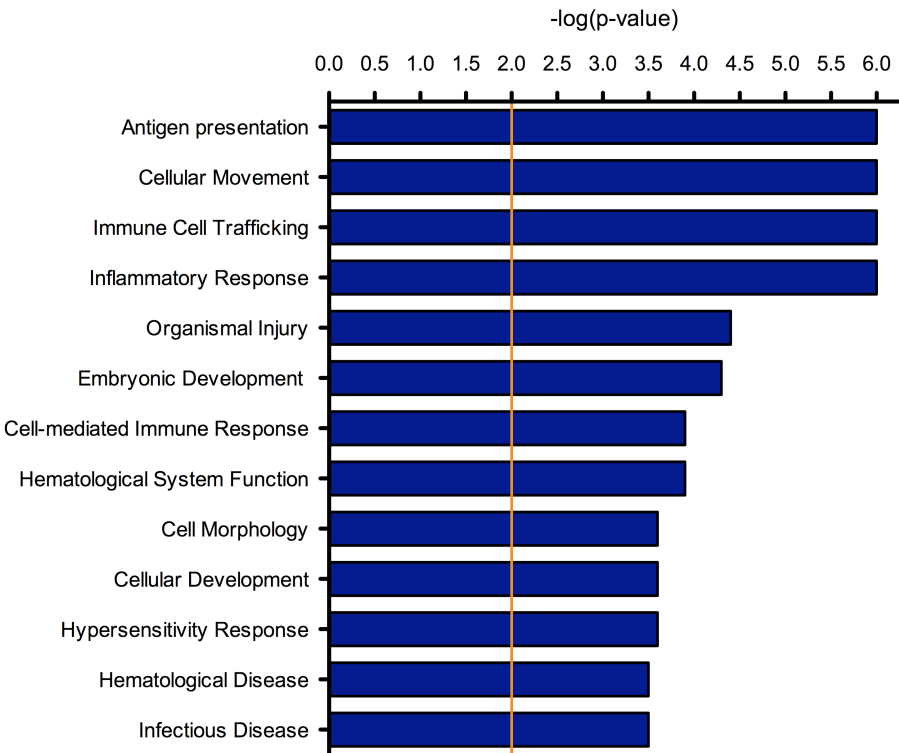


Figure 6.2: Pathway analysis of differentially expressed genes following over expression of miR-155. Differentially regulated genes following over expression of miR-155 detected by microarray were analysed by Ingenuity Core Analysis and the top 13 significant functions are shown ($p < 0.05$).

Given that miRNAs are classically considered to be negative regulators of gene expression, the observation that more than 50% of all transcripts are up regulated was rather surprising. However, this resembles the array analysis performed by Selbach *et al.*, which showed that approximately 66% of all regulated genes were up regulated 32 hrs after miR-155 over expression [Selbach *et al.*, 2008]. The large proportion of up regulated genes is probably indicative of strong secondary effects induced by miR-155. Indeed, miR-155 has been shown to target important negative regulators of gene expression such as SOCS1 [Androulidaki *et al.*, 2009]. It is important to note that miRNAs have also been shown to directly enhance target gene expression through binding to specific sites in the 5'UTR. However, further investigations using a comprehensive search for potential binding sites within full-length mRNA transcripts are required to investigate this possibility.

Pathway analysis of differentially regulated genes shows that miR-155 may be involved in a number of different pathways including antigen presentation, immune cell trafficking and the inflammatory response. This highlights the important role of miR-155 in the innate immune response (Figure 6.2). Furthermore, this analysis identified several genes with known linkage to miR-155, for example, PDCD4. PDCD4 is among the down regulated genes and has been previously shown to be induced in response to miR-155 knockdown [Yamanaka *et al.*, 2009]. These findings confirm that the experimental setup and the microarray functioned. Unfortunately, comparison of these results with the study performed by Ceppi *et al.* was not possible as their full list of differentially regulated genes was not included in their publication.

In contrast to these results, over expression of miR-650 induced more modest changes in gene expression. This was rather surprising, especially given that miR-650 is transfected to a greater efficiency. This may suggest that, compared to miR-155 which has been shown to target key regulators of gene expression thereby inducing strong secondary effects, miR-650 might only modulate gene expression via indirect effects. Alternatively, weak responses of miR-650 on mRNA expression may indicate that this miRNA primarily functions to repress target expression on a translational level.

The choice of a stringent statistical cut-off ($p < 0.01$) allowed the fold change to be lowered to include all transcripts regulated more than 1.2-fold. This analysis identified 80 regulated genes in response to miR-650 up regulation out of which 12 were up and 68 were down regulated (fold change > 1.2 , $p < 0.01$). A list with the top 20 regulated genes is displayed in Table 6.2.

Interestingly, none of the direct targets identified in Chapter 5 were among the differentially regulated genes. Nevertheless, network analysis of all differentially regulated genes identified similar biological function networks as described in Section 5.3.1. This shows a

Table 6.2: Top 20 differentially regulated genes in response to miR-650 over expression.

Up regulated genes		
Fold change	Gene ID	Description
-1.84	CXCL10	chemokine (C-X-C motif) ligand 10
-1.50	IFIT2	interferon-induced protein with tetratricopeptide repeats 2
-1.46	GCH1	GTP cyclohydrolase 1 (dopa-responsive dystonia)
-1.41	CCL8	chemokine (C-C motif) ligand 8
-1.39	FCGR1A	CD64 molecule
-1.38	BIRC3	baculoviral IAP repeat-containing 3
-1.35	PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)
-1.35	INDO	indoleamine-pyrrole 2,3 dioxygenase
-1.33	LTA4H	leukotriene A4 hydrolase
-1.32	PLXNC1	plexin C1
-1.32	GBP5	guanylate binding protein 5
-1.32	CCL5	chemokine (C-C motif) ligand 5
-1.31	COP1	caspase-1 dominant-negative inhibitor pseudo-ICE
-1.31	RNF170	ring finger protein 170
-1.30	GBP3	guanylate binding protein 3
-1.30	GBP4	guanylate binding protein 4
-1.29	OMA1	OMA1 homolog, zinc metallopeptidase (<i>S. cerevisiae</i>)
-1.29	CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
-1.29	SLC39A8	solute carrier family 39 (zinc transporter), member 8
-1.29	OAT	ornithine aminotransferase (gyrate atrophy)

Down regulated genes		
Fold change	Gene ID	Description
1.33	OR52K3P	olfactory receptor, family 52, subfamily K, member 3 pseudogene
1.26	STAC	SH3 and cysteine rich domain
1.22	MOBKL2B	MOB1, Mps One Binder kinase activator-like 2B (yeast)
1.22	RUNX1	runt-related transcription factor 1
1.21	C17orf68	chromosome 17 open reading frame 68
1.20	FAM83G	family with sequence similarity 83, member G

functional overlap between this data and the putative targets predicted by computational methods. The top four pathways identified by this analysis include cell-to cell signalling and interaction, haematological system development and disease, cellular movement and cell morphology as well as a number of pathways associated with cancer. These networks are displayed in Figures 6.3 - 6.6 with differentially regulated genes highlighted in grey.

In order to investigate which of these targets are regulated by direct interaction with miR-650 and which result from secondary effects, miRanda and TargetScan were used to search for potential miR-650 binding sites in the 3'UTRs of these genes. The results together with the fold changes obtained by array analysis are summarised in Figure 6.7. In agreement with the *in silico* target prediction analysis performed in Section 5.3, the overlap between miRanda and TargetScan was generally good, although TargetScan predicted less target sites indicating that it is a more stringent tool. The analysis shows that only a small fraction of regulated genes contain putative miR-650 binding sites in their 3'UTR. More specifically, putative binding sites were only found in the 3'UTRs of IFIT2, FCGR1A, GBP4, IFITM and NUB1. Unexpectedly, RUNX1, which is up regulated in response to miR-650 over expression, contains a potential binding site. This shows that miR-650 binding sites are not exclusively found in down regulated targets. This analysis indicates that the majority of differentially regulated genes are either likely to be the result of secondary effects or just not predicted to contain miR-650 target sites by these two particular computational tools.

6.3.3 Validation of array data by quantitative PCR

Gene expression microarrays are a powerful platform for the assessment of global changes in gene expression, however it is important to validate findings using additional methods. I therefore sought to confirm the array findings for the top three most regulated transcripts in response to miR-650 over expression, CXCL10, GCH1 and IFIT2, using Taqman qRT-PCR (Figure 6.8). I further sought to validate three additional genes with known association to the immune response (Figure 6.9): IFITM1, OASL and IRAK3. While IFITM1 and OASL were regulated reciprocally, the expression of IRAK3 was regulated cooperatively by miR-650 and miR-155. The housekeeping gene GAPDH was used as an endogenous normalisation control and, in each case, fold changes are expressed relative to nonsense mimic control. As shown in Figures 6.8 and 6.9, a good level of correlation is seen between the fold changes measured by qRT-PCR and those obtained by microarray analysis. However, for up-regulated genes, analysis by qRT-PCR showed greater fold changes as compared to the array data. This serves to show that the arrays were functional and strengthens the conclusion that miR-650

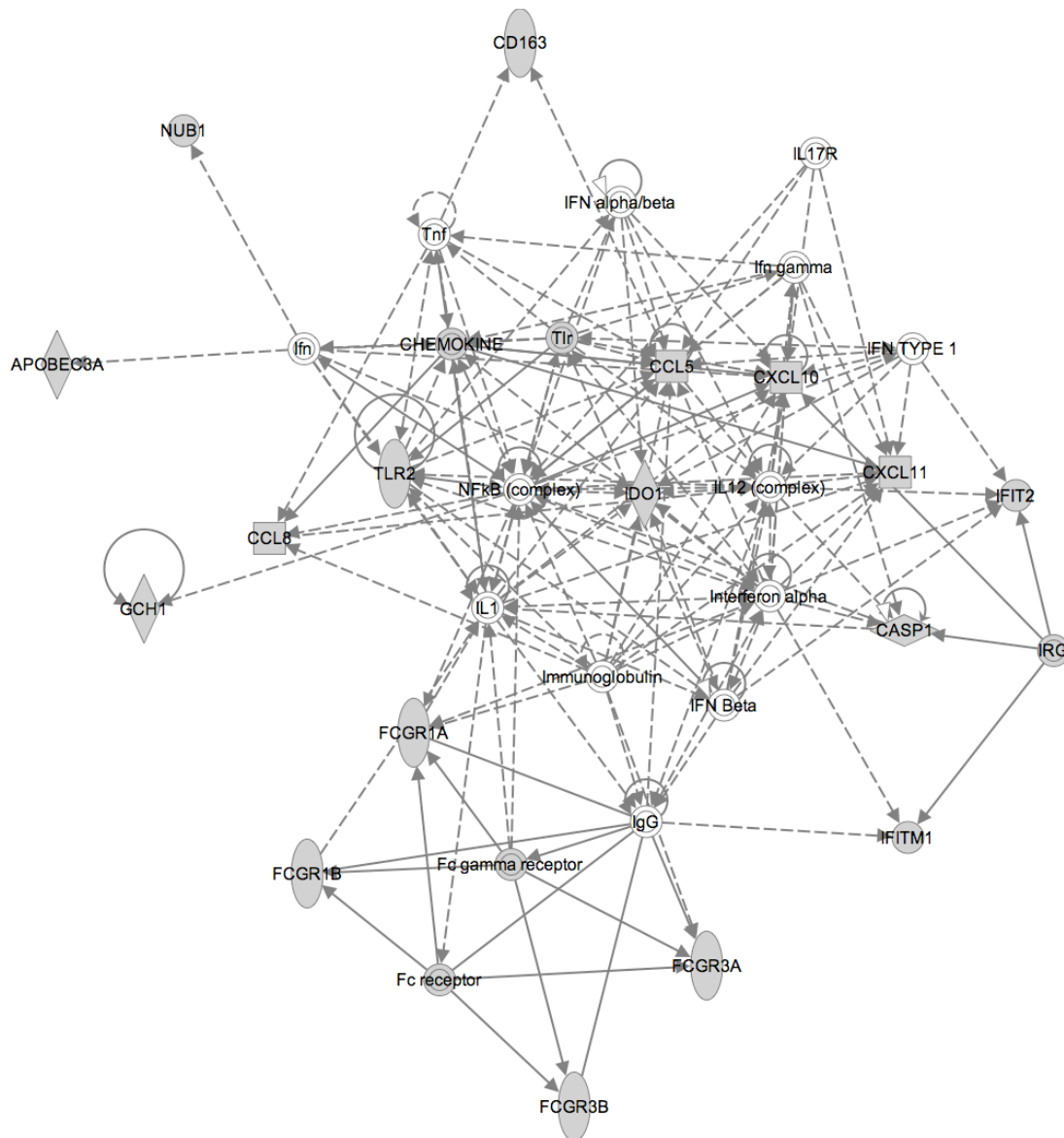


Figure 6.3: Ingenuity network analysis of differentially regulated genes following miR-650 over expression: Network 1. Network 1 includes genes which are involved in cell-to-cell signalling and interactions as well as haematological system development and function. Differentially regulated genes are highlighted in grey.

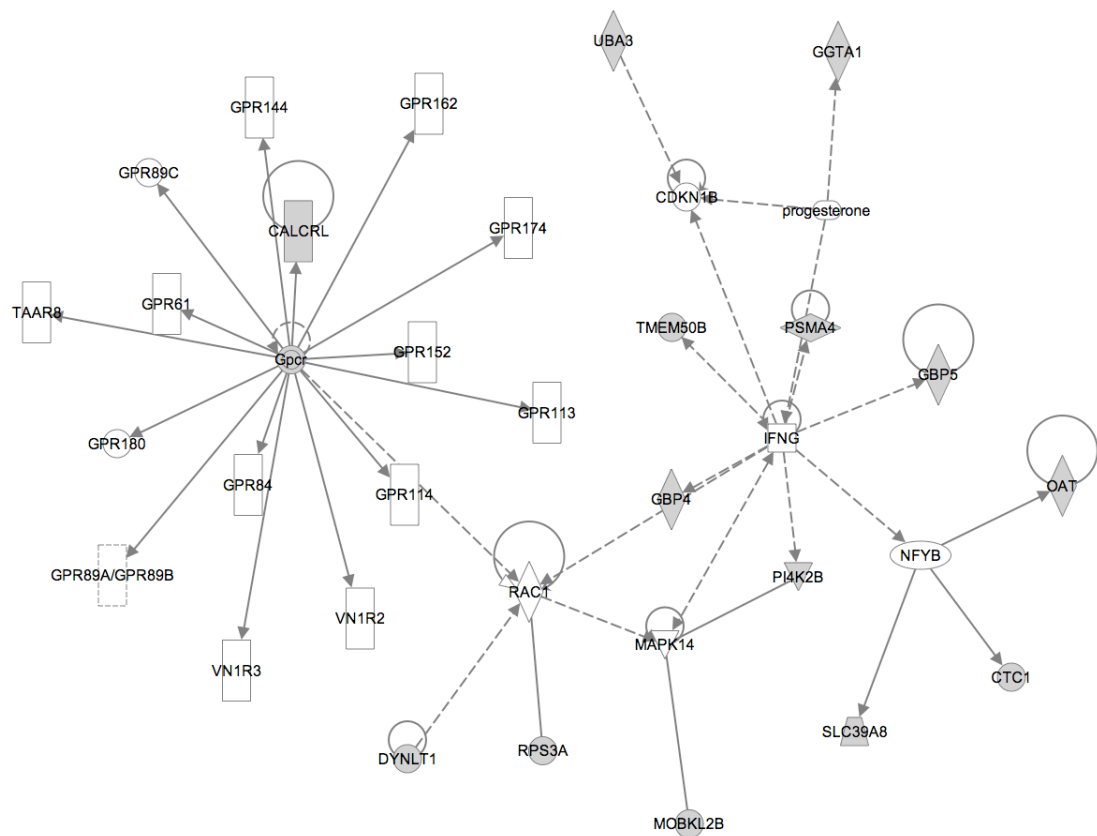


Figure 6.4: Ingenuity network analysis of differentially regulated genes following miR-650 over expression: Network 2. Network 2 includes genes which are involved in cellular movement and cell morphology. Differentially regulated genes are highlighted in grey.

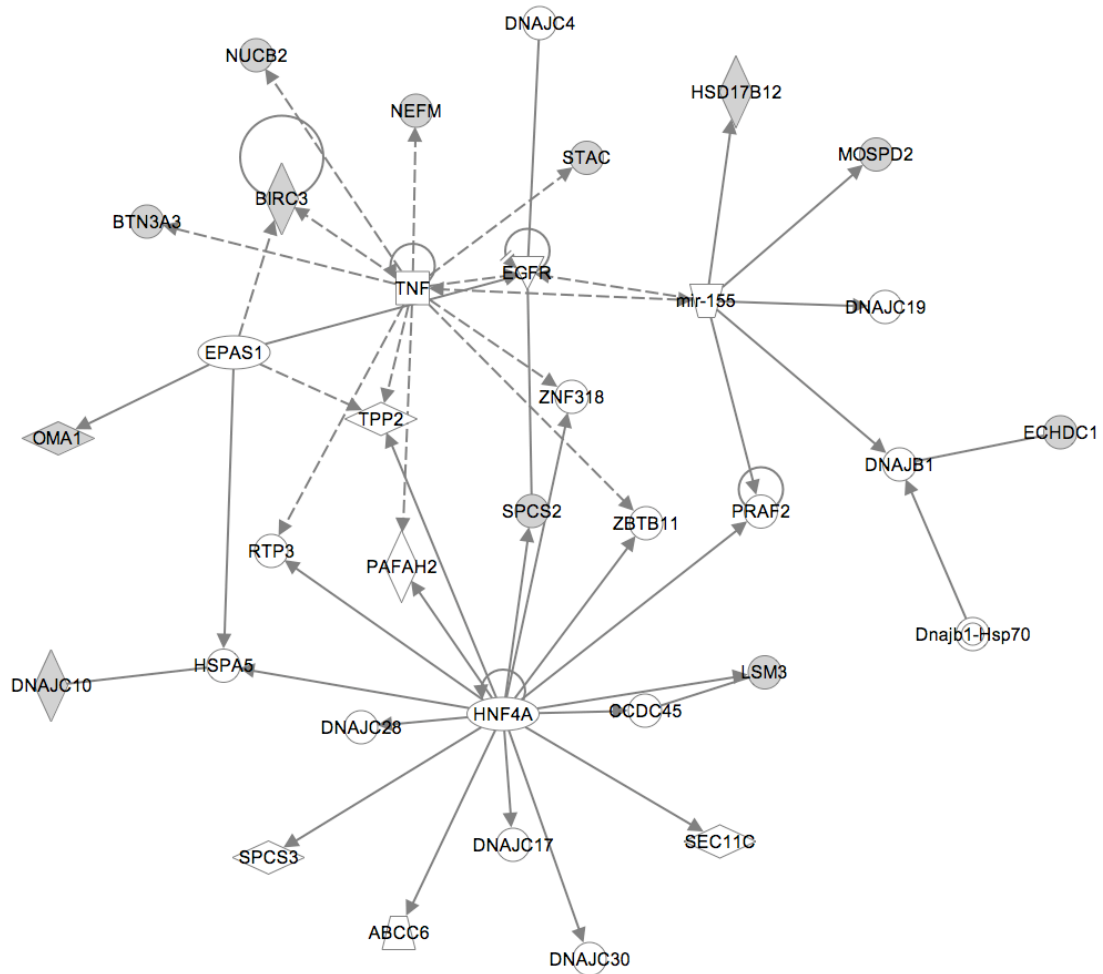


Figure 6.5: Ingenuity network analysis of differentially regulated genes following miR-650 over expression: Network 3. Network 3 includes genes which are involved in general cellular function and maintenance as well as tumour morphology. Differentially regulated genes are highlighted in grey.

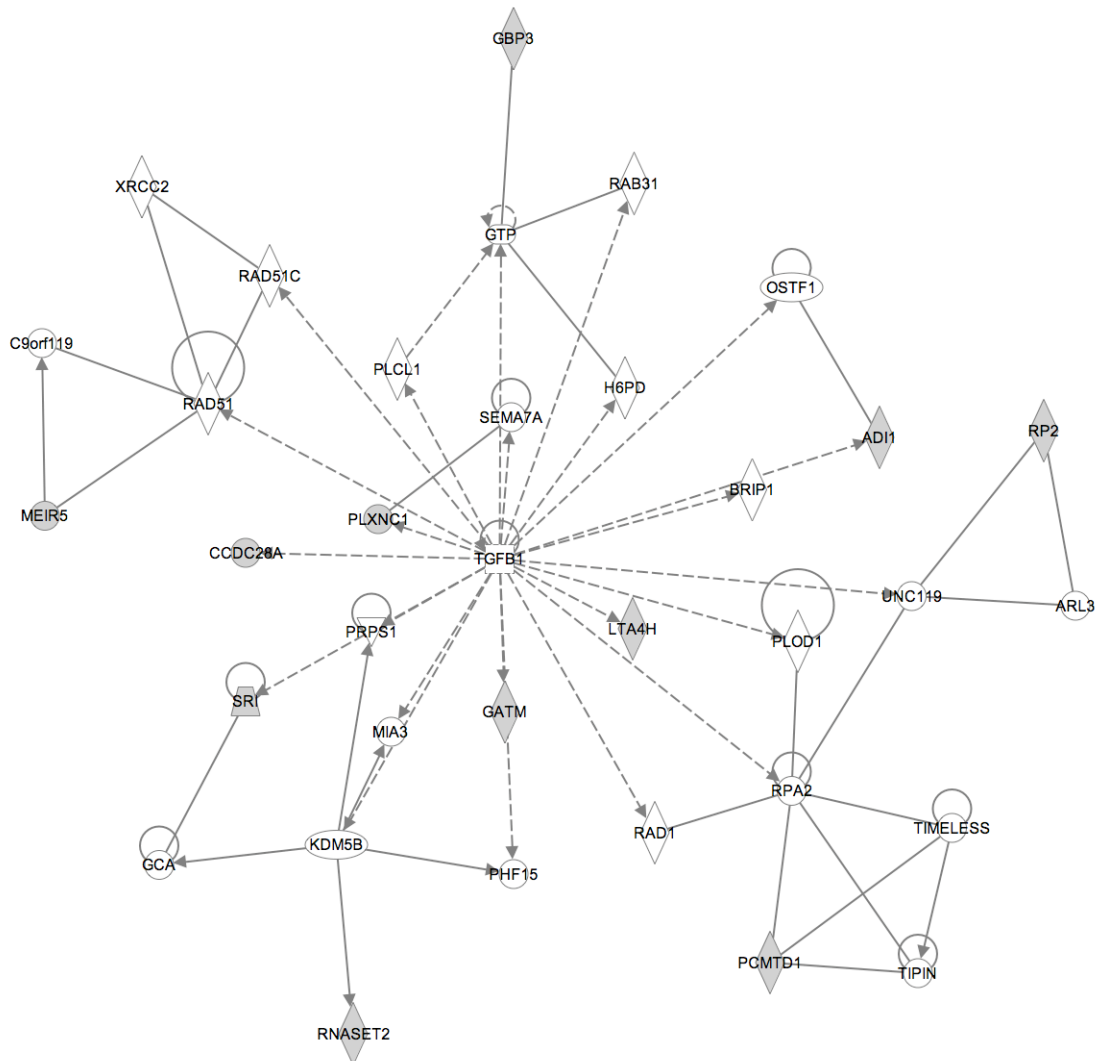


Figure 6.6: Ingenuity network analysis of differentially regulated genes following miR-650 over expression: Network 4. Network 4 includes genes which are involved in general cell cycle, DNA replication as well as recombination and repair. Differentially regulated genes are highlighted in grey.

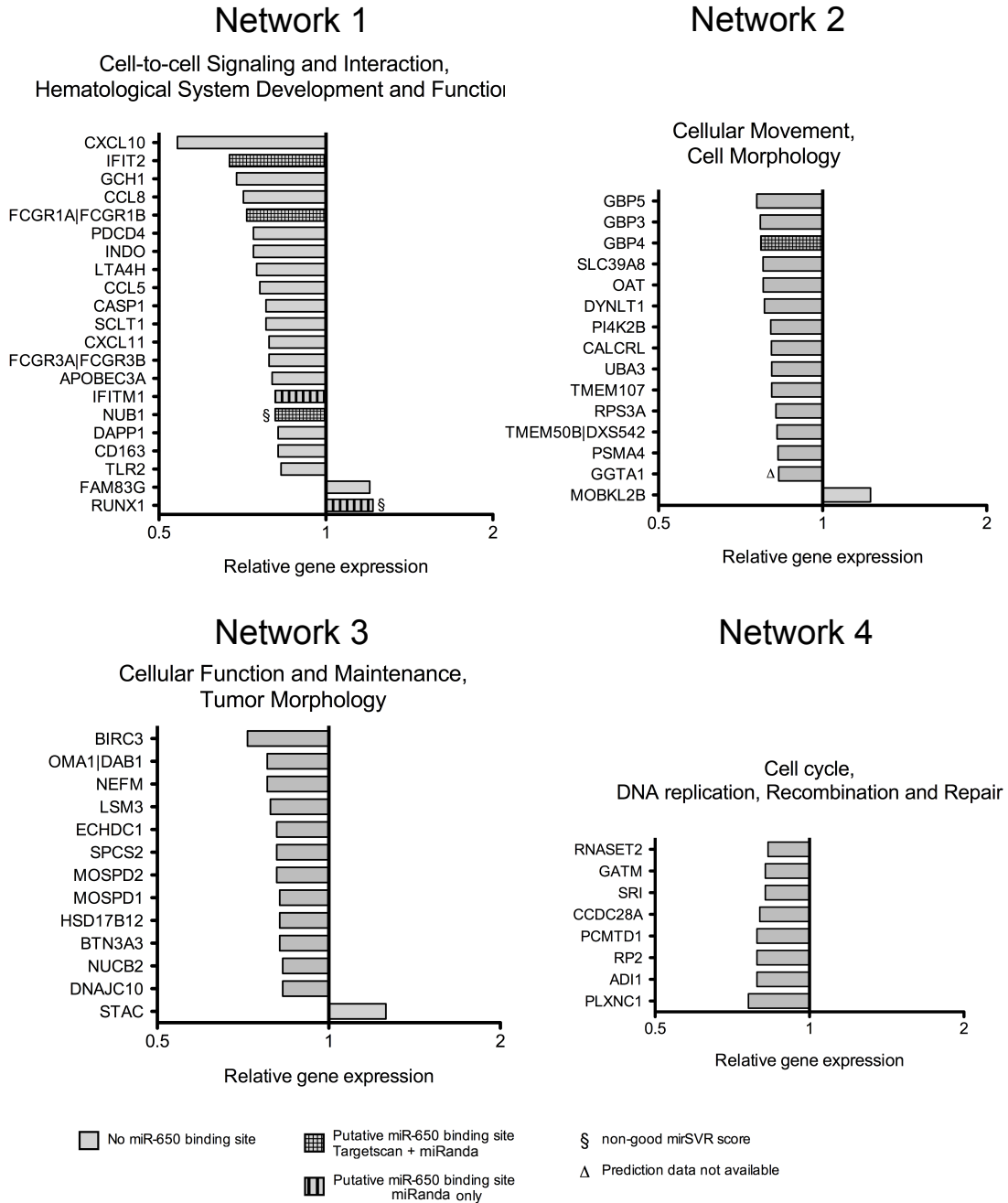
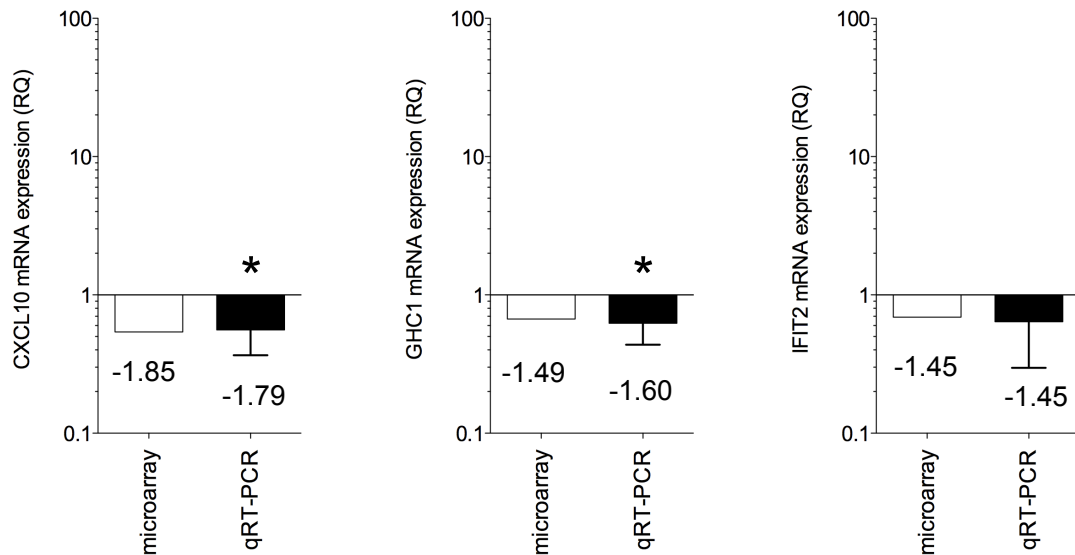


Figure 6.7: Computational analysis identifying putative miR-650 binding sites in genes differentially regulated in response to miR-650 over expression. Genes differentially regulated in DCs transfected with miR-650 mimic, measured by microarray and grouped according to the top four functional networks identified by Ingenuity analysis. Genes with putative miR-650 binding sites in the 3'UTR sequence are highlighted. Target prediction was performed using TargetScan and the miRanda algorithm.

(A) miR-650 mimic vs.control



(B) miR-155 mimic vs.control

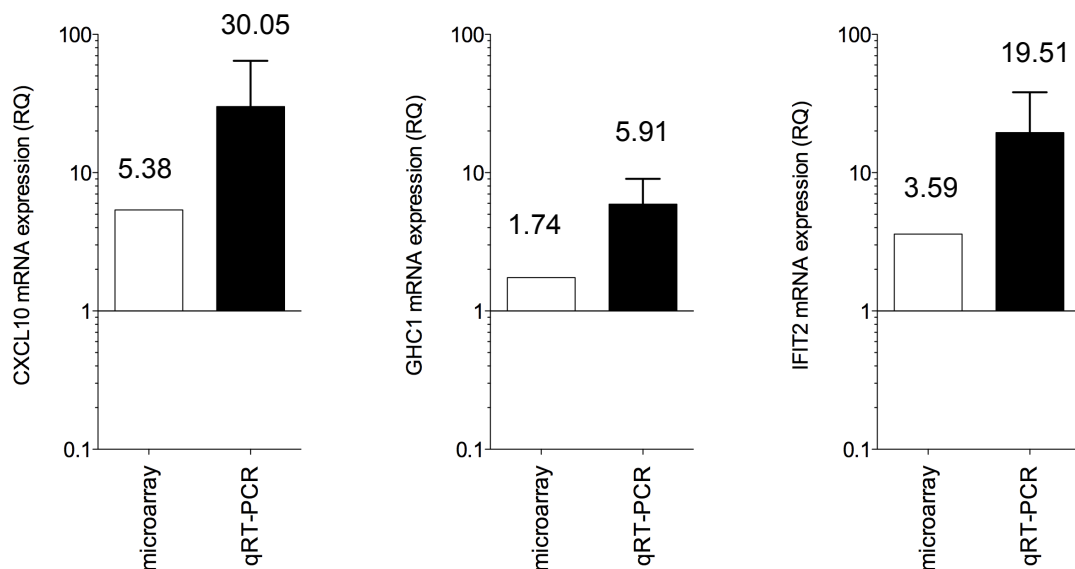
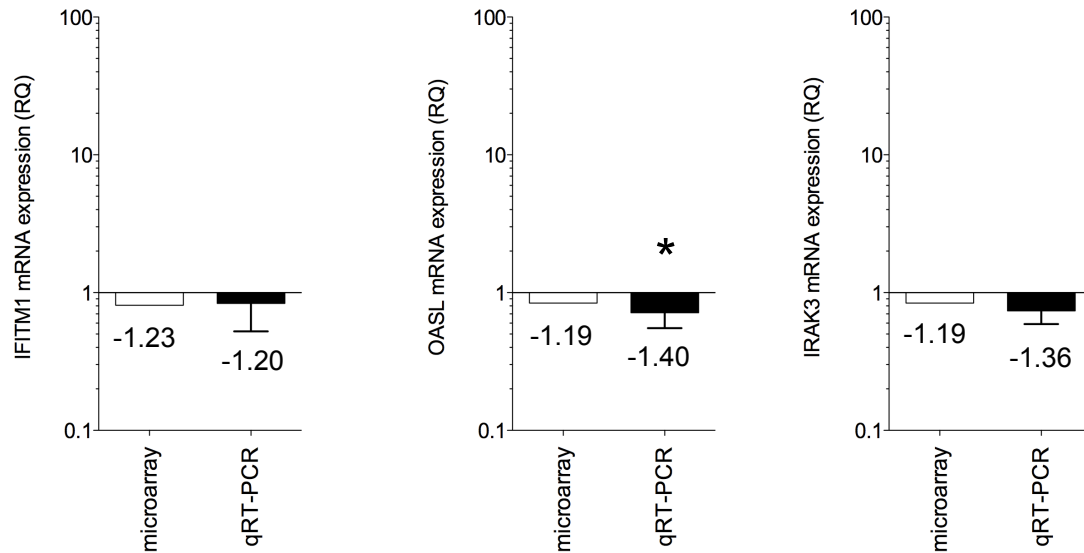


Figure 6.8: Validation of the three most significant array findings for miR-650 by qRT-PCR. Measuring the expression of CXCL10, GCH1 and IFIT2 in response to (A) miR-650 or (B) miR-155 over expression by qRT-PCR. Expression is normalised against GAPDH and is expressed relative to the nonsense control. Data is from three different donors and is shown as mean $\pm$ SEM. Each graph also shows the relative expression obtained from array analysis. Statistical significance of the qPCR expression data was tested using a paired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(A) miR-650 mimic vs.control



(B) miR-155 mimic vs.control

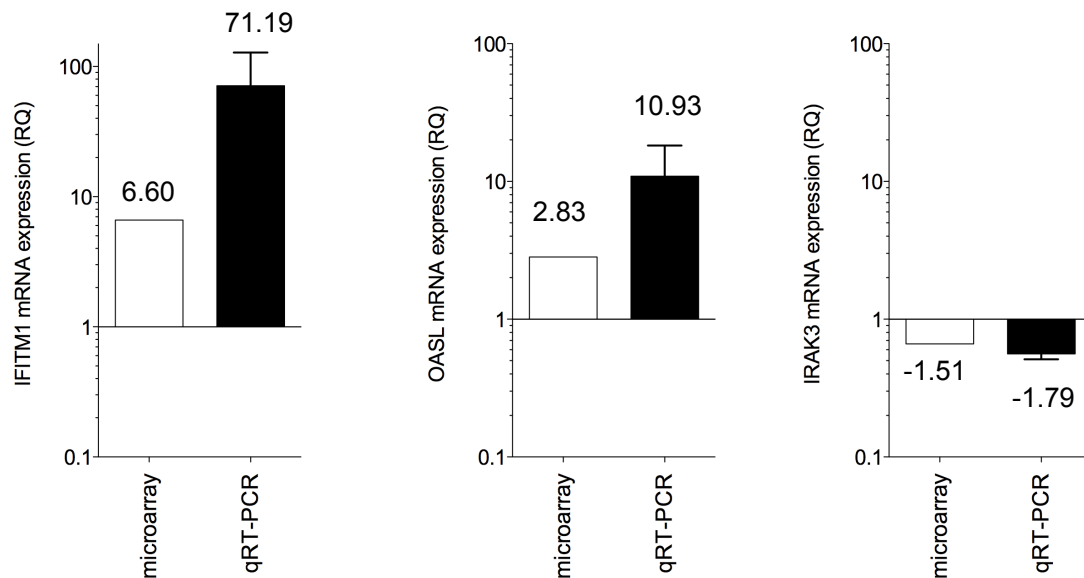


Figure 6.9: Validation of additional array findings by qRT-PCR. Measuring the expression of IFITM1, OASL and IRAK3 in response to (A) miR-650 or (B) miR-155 over expression by qRT-PCR. Expression is normalised against GAPDH and is expressed relative to the nonsense control. Data is from three different donors and is shown as mean $\pm$ SEM. Each graph also shows the relative expression obtained from array analysis. Statistical significance of the qPCR expression data was tested using a paired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

regulates a range of genes known to be involved in the innate immune response.

An interesting observation is that many of the genes regulated by miR-650 are also altered in response to miR-155 over expression. While CXCL10, GCH1, IFIT2, IFITM1 and OASL were regulated in opposite direction, IRAK3 was down regulated following up regulation of miR-155 as well as miR-650.

6.3.4 Overlap between genes regulated by miR-155 and miR-650

Although in this study miR-155 was initially only chosen as a positive control in order to confirm that the experimental setup was functional, analysis revealed that a significant fraction of the transcripts regulated by miR-650 had also responded to miR-155. To highlight the overlap between the two data sets, a comparative study was performed. Therefore, overlapping transcripts were divided into two main groups according to the direction of regulation (Table 6.3):

- Group 1: transcripts up regulated by miR-155 and down regulated by miR-650, or vice versa.
- Group 2: transcripts either both up or both down regulated by miR-650 and miR-155.

It is important to bear in mind that upon PRR signalling miR-650 and miR-155 are reciprocally regulated, i.e. miR-650 expression is decreased and miR-155 expression is increased. Thus, in response to PRR signalling, all genes in Group 1 are expected to be both up or both down regulated by miR-650 and miR-155; this will be referred to as the cooperative case. In contrast, genes in Group 2 are expected to be regulated in opposite directions; this will be referred to as the antagonizing case. As showed in Table 6.3, an approximately equal proportion of genes is regulated in a cooperative and antagonising manner by the two miRNAs.

6.3.5 Exploring potential mechanisms of cooperation between miR-650 and miR-155

Cooperativity between miRNAs is a very interesting concept and several studies have shown that co-regulated miRNAs may target the 3'UTR of a selected target gene and therefore work together. However, given that miR-650 and miR-155 are reciprocally regulated in response to

Table 6.3: Fold changes of overlapping targets regulated by both miR-650 and miR-155. Regulation of targets by these two miRNAs can result in cooperative (left table) or antagonising effects (right table).

Gene ID	miR-650 mimic	miR-155 mimic
IRF7	1.21 up	1.82 up
OR52K3P	1.20 up	1.89 up
DOT1L	1.17 up	1.53 up
IL3RA	1.16 up	1.97 up
IL3RA	1.16 up	1.97 up
SLC39A8	1.32 down	1.64 down
PDCD4	1.28 down	1.90 down
FCGR1A FCGR1B	1.25 down	2.49 down
LTA4H	1.25 down	2.18 down
ADH	1.23 down	1.58 down
FCGR3A FCGR3B	1.22 down	1.75 down
PLXNC1	1.22 down	1.53 down
OMA1 DAB1	1.21 down	1.56 down
ECHDC1	1.21 down	1.72 down
RNASET2	1.18 down	1.53 down
BRP44L	1.17 down	1.60 down
IRAK3	1.17 down	1.51 down
GATM	1.17 down	2.75 down
SLC35A1	1.16 down	1.51 down
OAT	1.16 down	1.88 down
NUCB2	1.16 down	1.60 down
ATG4C	1.16 down	1.52 down
ABCA6	1.15 down	1.62 down
HSD17B12	1.15 down	1.55 down

Gene ID	miR-650 mimic	miR-155 mimic
CXCL10	1.64 down	5.38 up
GCH1	1.41 down	1.74 up
IFIT2	1.37 down	3.59 up
CXCL11	1.35 down	4.03 up
CCL5	1.33 down	1.87 up
BIRC3	1.31 down	1.59 up
CALCRL	1.29 down	1.70 up
CCL8	1.29 down	6.77 up
GBP5	1.27 down	3.05 up
INDO	1.26 down	3.25 up
GBP4	1.26 down	3.03 up
MYO1G	1.20 down	1.66 up
APOBEC3A	1.20 down	6.15 up
DYNLT1	1.20 down	1.71 up
GBP3	1.20 down	1.73 up
PI4K2B	1.19 down	1.58 up
IFITM1	1.18 down	6.60 up
NUB1	1.16 down	1.50 up
OASL	1.16 down	2.83 up
CASP1	1.16 down	1.52 up

the same stimuli, it is not immediately clear how these two miRNAs function in a cooperative manner.

Interestingly, all transcripts belonging to Group 1, i.e. the cooperative case, were down regulated by miR-650 and up regulated by miR-155. In order to further dissect the potential cooperation between miR-650 and miR-155, target site information was retrieved from the miRanda and TargetScan databases (shown in Figure 6.10). This analysis reveals that cooperatively regulated genes can be further divided into four different subtypes according to whether their 3'UTR:

- (A) lacks binding sites for miR-650 and miR-155,
- (B) contains putative binding sites for miR-650 but lacks binding sites for miR-155,
- (C) contains putative binding sites for miR-155 but lacks binding sites for miR-650,
- (D) contains putative binding sites for both miR-650 and miR-155.

This analysis suggests four hypothetical mechanisms by which miR-650 and miR-155 could act together to regulate the expression of these genes (Figure 6.11).

The simplest example of cooperativity would be when neither miR-650 nor miR-155 contain binding sites in the 3'UTR of commonly regulated genes (Figure 6.11(A)). In this case regulation of the gene would occur via an indirect mechanism, for example through targeting a positive or negative regulator of gene expression by either miR-650 or miR-155, respectively. Alternatively, expression of targets that contain a putative binding site for miR-650 could also be down regulated by miR-650 via a direct mechanism (Figure 6.11(B)). However, indirect enhancing effects cannot be excluded in this case. In addition to these straight forward cases, more cryptic cases may exist. One group of genes lacks binding sites for miR-650 but contains putative sites for miR-155 (Figure 6.11(C)), but since target gene expression is increased in response to miR-155 over expression, an additional overriding indirect regulatory mechanism exerted by miR-155 must exist. Finally, one gene containing putative binding sites for both miR-650 and miR-155 was identified and could therefore be regulated by a complex interaction of direct and indirect mechanisms (Figure 6.11(D)).

6.3.6 Exploring direct interactions between miR-650 and the 3'UTR of selected genes

In order to investigate whether any of these three differentially regulated genes are direct targets of miR-650, 3'UTR reporter assays were performed. *In silico* analysis revealed that

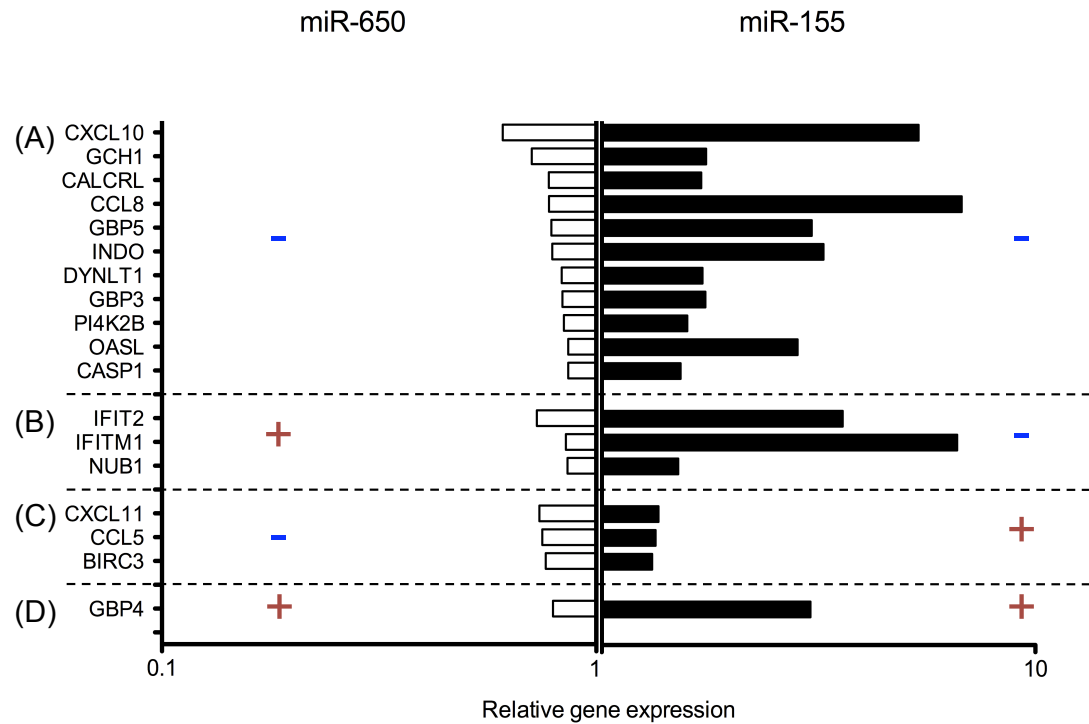


Figure 6.10: The cooperative case: genes down regulated by miR-650 and up regulated by miR-155. These genes belong to the cooperative case, because miR-650 and miR-155 are reciprocally regulated during the inflammatory response see Table 6.3). Members of this group can be further divided into four subgroups depending on the presence (+) or absence (-) of putative miR-650 or miR-155 binding sites: (A) no target site for either miR-650 or miR-155, (B) target site for miR-650 only, (C) target site for miR-155 only and (D) binding site for both miRNAs. Target sites are defined as being present, if predicted by either TargetScan or the miRanda algorithm.

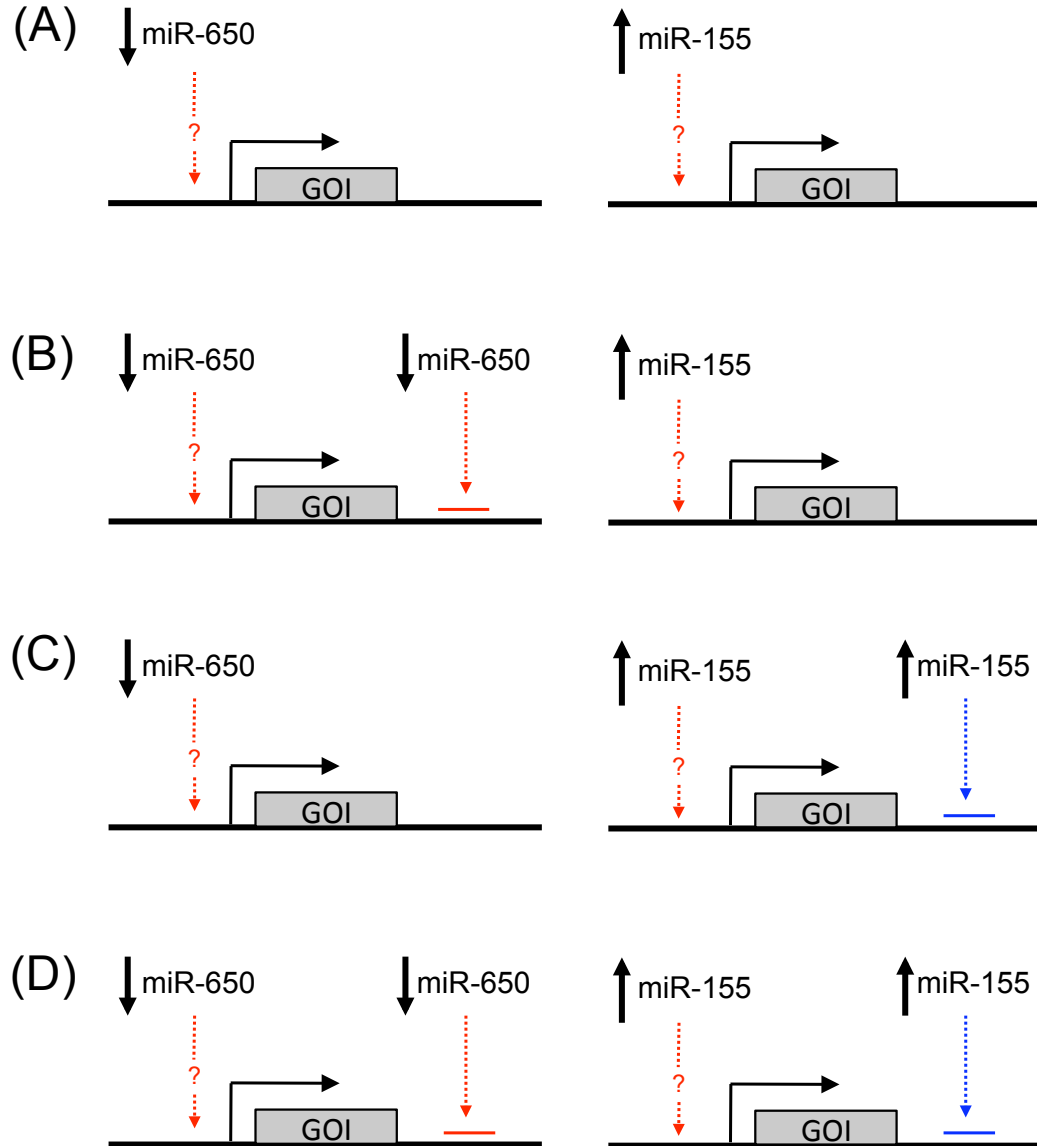


Figure 6.11: Schematic diagram showing the hypothetical mechanisms by which miR-650 and miR-155 could cooperatively regulate identified targets. Indirect effects (indicated by '?') may affect target transcription via an unknown intermediate while direct miRNA binding to sites in the 3'UTR (indicated by a solid line) decreases gene expression. Targets either have (A) no target site for either miR-650 or miR-155, (B) target site for miR-650 only, (C) target site for miR-155 only and (D) binding site for both miRNAs. Indirect effects may occur in all cases.

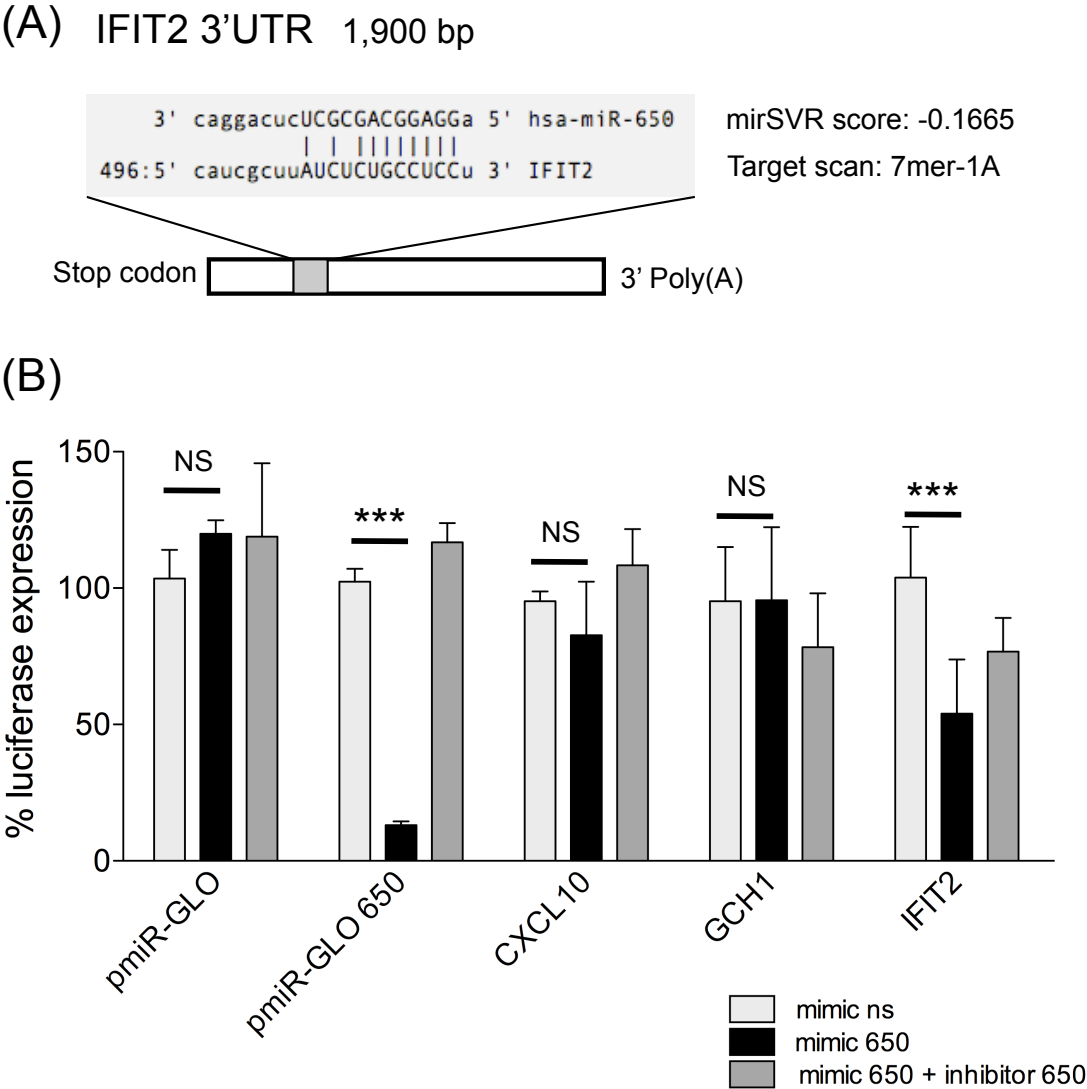


Figure 6.12: Investigating the direct effect of miR-650 on the regulation of CXCL10, GCH1 and IFIT2 by 3'UTR reporter assay. (A) This figure highlights the miR-650 binding sites in the 3'UTR of IFIT2 with TargetScan and miRanda scores. (B) Luciferase reporter assays were carried out to confirm that compared to CXCL10 and GCH1, IFIT2 is a direct target for miR-650. Firefly activity was normalised against renilla expression. Experiments were performed in quadruplicates and data is shown as mean $\pm$ SD. Statistical significance was tested using an unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ NS not significant.

only IFIT2 contains a single potential 7mer-1A miR-650 binding site (Figure 6.12(A)). In order to confirm whether miR-650 regulates the expression of IFIT2 in a direct manner, the sequence was cloned into the pmiR-GLO vector. As control, the 3'UTRs of GCH1 and CXCL10, both lacking a predicted miR-650 binding site, were also cloned. Luciferase assays were performed in HEK293T cells as described in Section 5.3.2. As shown in Figure 6.12, luciferase activity was reduced in cells co-transfected with miR-650 mimic as well as with the pmiR-GLO vector containing the 3'UTR of IFIT2. Reduced luciferase activity was partially recovered by addition of the miR-650 inhibitor. In contrast, transfection of cells with pmiR-GLO vector carrying the 3'UTR sequence of GCH1 showed no susceptibility to miR-650 over expression. Interestingly, addition of the 3'UTR of CXCL10 conferred a small degree of susceptibility to reporter gene expression. This was recovered by the addition of miR-650 antisense inhibitor. However, this effect was not considered statistically significant and even the use of a more extensive range of various software programs, including TargetScan, miRanda, MicroCosm and Diana-microT, failed to find potential miR-650 target sites in the 3'UTR sequence of CXCL10.

In summary, this data shows that miR-650 targets a number of interferon-inducible genes, including CXCL10, IFIT2, GCH1 and IFITM1. However, 3'UTR reporter assays show that only IFIT2 is targeted directly. Together this data indicates that miR-650, together with miR-155, may contribute to the establishment of an antiviral state following viral infection.

6.3.7 Exploring the effect of miR-650 on the expression of the interferon-inducible factor MxA

In light of the potential role of miR-650 in regulating the interferon response to virus infection, I decided to investigate its effect on a well-described interferon induced antiviral protein MxA. The human MxA protein belongs to the family of dynamin like GTPases and is efficiently up regulated by type I interferons [Haller & Kochs, 2011]. It exhibits antiviral activity against a number of different RNA viruses [Haller *et al.*, 1998] [Haller & Kochs, 2002] including Influenza A virus and Thogoto virus. The expression of MxA has been shown to be regulated by miR-155. The study of Selbach *et al.* shows that over expression of miR-155 in HeLa cells results in 1.5-fold increase in MxA mRNA levels [Selbach *et al.*, 2008]. Considering the cooperative action of miR-155 and miR-650, I sought to investigate whether miR-650 also has an effect on MxA expression. Unfortunately, neither TargetScan, nor miRanda identified a putative miR-650 binding site in the 3'UTR of MxA. However, due to the aforementioned high false-negative rate of *in silico* target prediction tools, I decided, for

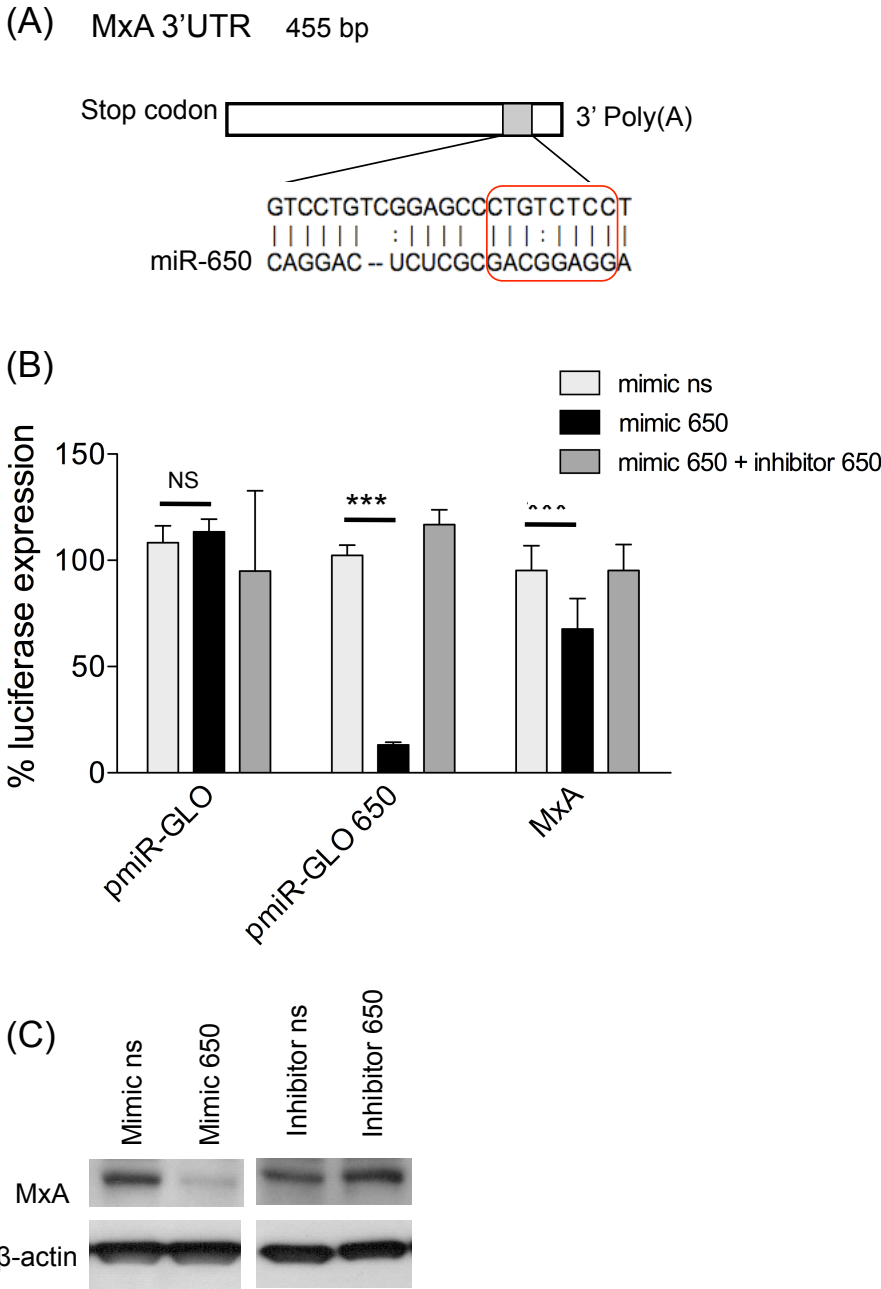


Figure 6.13: Exploring the direct effect of miR-650 on the expression of MxA. (A) This figure highlights the miR-650 binding sites in the 3'UTR of MxA as predicted by MicroCosm. (B) Luciferase reporter assays were carried out to confirm that MxA is a direct target for miR-650. Firefly activity was normalised against renilla expression. Experiments were performed in quadruplicates and data is shown as mean $\pm$ SD. Statistical significance was tested using an unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ NS not significant. (C) Exploring the effect of modulating the expression of miR-650 on MxA levels in primary DCs. DCs were transfected with miR-650 mimic or antisense inhibitor and MxA expression was measured after 24 hrs by immunoblotting. One representative donor out of three shown.

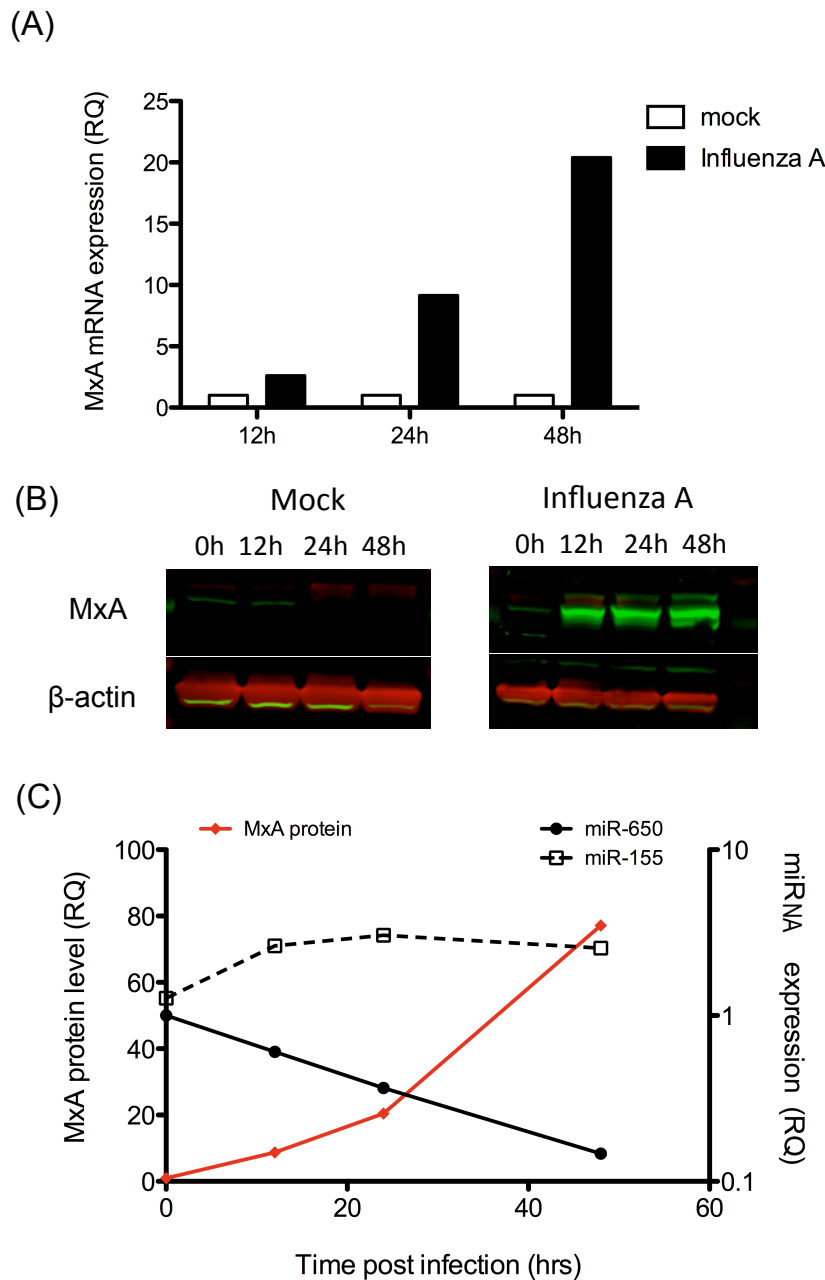


Figure 6.14: Expression of MxA during Influenza A virus infection of primary DCs. DCs were infected with Influenza A (40 HAU/ml) and cells were harvested at various time points post-infection. **(A)** RNA was extracted and MxA mRNA levels were measured by qRT-PCR. Expression was normalised against GAPDH and is shown relative to an uninfected control. **(B)** Cells were lysed and MxA protein was measured by fluorescent immunoblotting (green = MxA, red = β -actin). **(C)** Quantification of MxA protein levels normalised against β -actin expression plotted against the relative expression of miR-650 and miR-155 in response to the same condition (see Figure 4.9). The graph suggests reciprocal regulation of MxA and miR-650 expression during Influenza A virus infection in DCs. One representative donor shown.

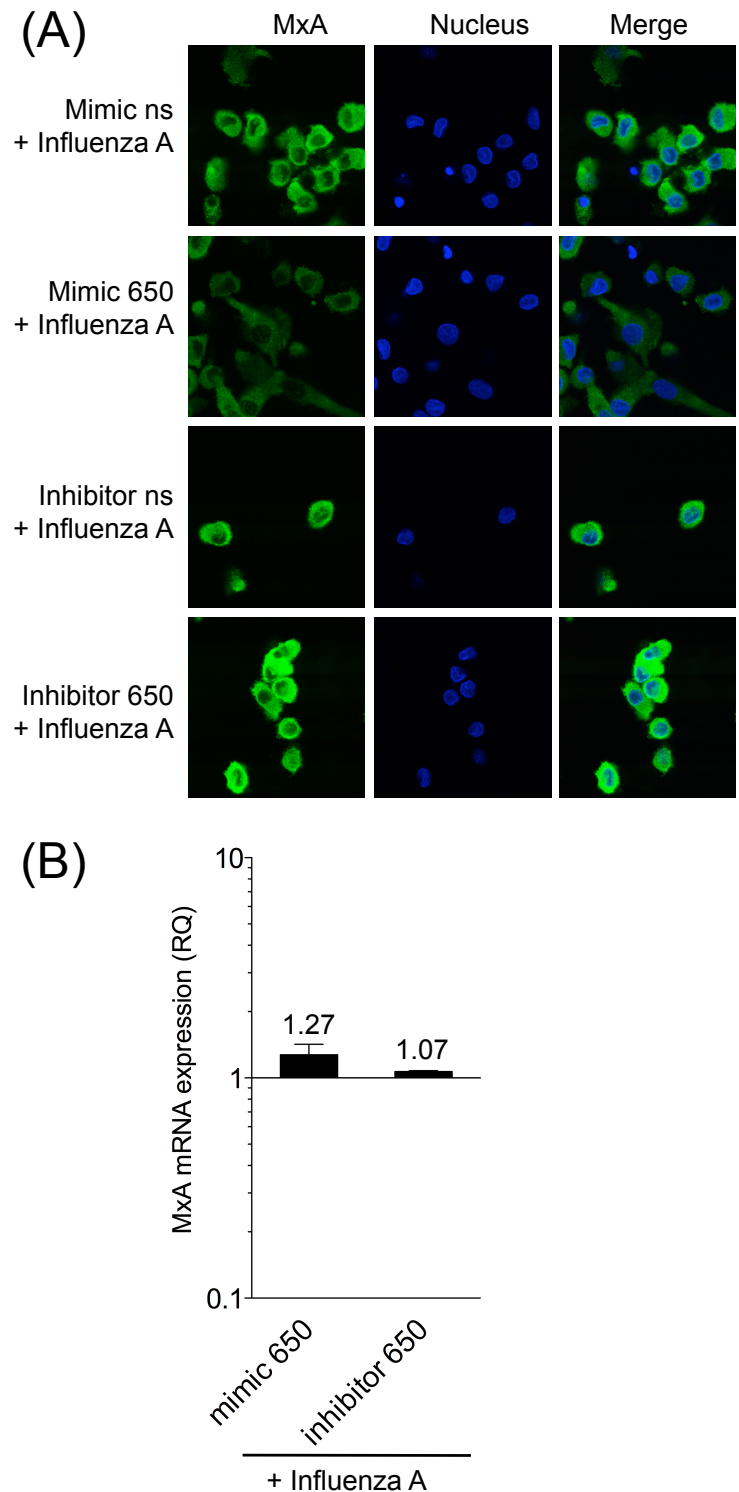


Figure 6.15: Investigating the role of miR-650 in the role of Influenza-induced MxA expression during infection of DCs. DCs were transfected with miR-650 mimic or antisense inhibitor prior to infection with Influenza A (40 HAU/ml). At 6 hrs post-infection DCs were harvested and (A) MxA protein levels were visualised by confocal microscopy. One representative donor shown. (B) RNA was extracted to measure MxA mRNA levels by qRT-PCR. Expression is normalised against GAPDH and is expressed relative to the nonsense control. Data is from two different donors and is shown as mean $\pm$ SEM.

this special case, to use a range of different programs which use a different set of prediction rules. In this analysis, I came across the MicroCosm tool which uses a modified version of the miRanda algorithm and is available on the miRBase website (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>) [Griffiths-Jones *et al.*, 2008] [Griffiths-Jones *et al.*, 2006] [Enright *et al.*, 2003].

Interestingly, this tool identifies a potential binding site for miR-650 in the 3'UTR of the MxA transcript. Not only does it predict a potential site, it ranks MxA among the top 50 scoring target genes for miR-650. Additionally, miR-650 is the highest scoring among all miRNAs predicted to target 455 bp long MxA 3'UTR. As shown in Figure 6.13(A), the miR-650:MxA duplex is characterised by imperfect seed complementarity with a G:U wobble at position 6 of the miRNA. In addition, extensive base pairing can be observed at the 3' end of the miRNA. Many experimental studies have found that experimentally validated miRNA targets are enriched for exact miRNA seeds, but imperfect seed matches with G:U wobbles have been shown to be tolerated in several miRNA:target interactions [Reinhart *et al.*, 2000] [Didiano & Hobert, 2006]. However, TargetScan does not predict this interaction, as this program requires a perfect match at positions 2-7 followed by an A. Although MicroCosm weights seed pairing more strongly than pairing elsewhere, it allows for G:U wobbles and mismatches in the seed region if compensated for by increased binding at the 3'UTR end.

In order to provide additional evidence for the direct regulation of MxA by miR-650 the 3'UTR sequence was cloned into the pmiR-GLO vector and luciferase reporter assay was performed as set up in Section 5.3.2. Figure 6.13(B) shows that luciferase expression is reduced following co-transfection of miR-650 mimic and the pmiR-GLO reporter vector containing the MxA 3'UTR. Co-transfection of the miR-650 antisense inhibitor fully rescues luciferase activity. This suggests that, despite imperfect seed matching, the putative miR-650 binding site is indeed functional. However, additional site-directed mutagenesis is required to map the exact MRE.

After establishing that the MxA 3'UTR carries a functional miR-650 binding site, I sought to investigate whether manipulation of miR-650 levels alters the expression of MxA in monocyte-derived DCs. For this purpose, DCs were transfected with either miR-650 mimic, inhibitor or their respective nonsense control oligonucleotides using the Amaxa nucleofector. After 24 hrs, cells were harvested and cell lysates were assayed for MxA expression by immunoblotting; a typical blot is shown in Figure 6.13(C). While over expression of miR-650 reduces the expression of MxA in DCs, inhibition of miR-650 using antisense oligonucleotide increases the levels of MxA, even though the effect of the inhibitor appears rather more subtle. This result indicates that miR-650 may regulate the expression of MxA in primary

DCs.

6.3.8 The effect of miR-650 on MxA expression during Influenza A infection

I next sought to investigate the impact of miR-650 on the induction of MxA expression in the context of Influenza A virus infection. The first aim was to assess the expression kinetics of MxA during Influenza A virus infection. Therefore, immature DCs were infected with Influenza A (400 HAU/ml) virus or left uninfected. DCs were harvested at various time points post stimulation and RNA was extracted for quantification of MxA mRNA levels by qRT-PCR. Similar to CXCL10 and IFIT2, the transcription of MxA is rapidly induced in response to Influenza A virus infection (Figure 6.14)(A)). In addition, cells were lysed in order to monitor the levels of MxA protein by fluorescence immunoblotting using the Odyssey Infrared Imaging System (LI-COR). Figure 6.14(A) shows that the levels of MxA protein mimics mRNA expression in their induction response. The induction of MxA protein expression over time was plotted together with the expression of miR-650 and miR-155 (measured previously in Figure 4.9), all in response to Influenza A virus infection. Figure 6.14(C) clearly shows that both MxA and the two miRNAs follow similar expression kinetics in a physiological setting; while miR-155 and MxA are both up regulated, miR-650 is down regulated.

In order to investigate whether down regulation of miR-650 expression enhances the expression of MxA in DCs infected with Influenza A virus, DCs were transfected with miR-650 mimic or inhibitor 24 hrs prior to infection with the virus. At 6 hrs post-infection, MxA expression was monitored by immunofluorescence. Briefly, DCs were allowed to adhere to poly-L lysine coated tissue culture slides for 10 min, washed, fixed, permeabilised and stained for endogenous MxA. In addition nuclei were visualised using TO-PRO stain. In agreement with previous studies which had shown that MxA is associated with the smooth ER [Stertz *et al.*, 2006], confocal microscopy revealed that MxA is localised in the cytoplasm (Figure 6.15(A)). Similar to the results obtained by immunoblotting, MxA expression was reduced in cells transfected with miR-650 mimic prior to infection. Conversely, cells in which miR-650 was inhibited displayed more prominent staining for MxA. Simultaneous analysis of the mRNA levels of MxA in these samples revealed that, in agreement with the array findings, the levels of MxA fail to show reciprocal regulation in response to miR-650 manipulation (Figure 6.15(B)). Together these findings suggest that miR-650 regulates MxA expression on a post-transcriptional level.

This work is the first to show that MxA expression is enhanced on a post-transcriptional

level by miR-650 during viral infection. However, it is important to note that the level of miR-650 expression achieved by transfection of artificial mimic and inhibitor may not be physiologically relevant. Furthermore, it is also interesting that transfection of the miR-650 inhibitor could further enhance the expression of MxA despite already reduced expression of miR-650 following infection of DCs with Influenza A virus.

6.3.9 Investigation into the effect of miR-650 on type I IFN secretion of DCs

CXCL10, IFIT2, GCH1 and MxA are all interferon-stimulated genes that are produced during viral infection. Type I IFNs act through the JAK/STAT signalling pathway to activate the ISG factor 3 (ISGF3) which, in turn, binds to interferon-stimulated response elements (ISREs) present in the promoter region of ISGs [Darnell *et al.*, 1994]. In addition, some ISGs have also been shown, in the absence of IFN production, to be induced directly by virus infection via an alternative pathway. For example, dsRNA induces the expression of IFIT1 (also known as ISG56) via binding of IRF3 to the ISRE site of the IFIT1 promoter [Guo *et al.*, 2000] [Wathelet *et al.*, 1987].

In the case of MxA, the mechanism of induction remains controversial. Studies using cell lines defective for IFN production or signalling suggest that MxA is exclusively induced by type I IFNs [Holzinger *et al.*, 2007]. MxA contains three ISREs but only IRSE2 has shown the ability to be recognised by IRF3. However, binding of IRF3 to IRSE2 is thought to have only an enhancing effect [Holzinger *et al.*, 2007]. This is supported by the observation that IFN-neutralising antibodies inhibit induction of MxA in response to PR8 infection in monocyte-derived DCs [Cella *et al.*, 1999]. However, IFN blocking only partially inhibits MxA induction in response to LPS or Poly(I:C) [Cella *et al.*, 1999]. In conclusion, these data suggest that whilst IFN signalling is, at least partially, responsible for MxA induction, a direct mechanism cannot be excluded.

My next step was therefore to exclude the possibility that miR-650 regulates the expression of IFNs and therefore indirectly affects the expression of the above-mentioned ISGs. To do so, RNA was isolated from DCs transfected with miR-650 mimic or inhibitor for 24 hrs and infected with Influenza A virus for 6 hrs, whereupon expression of IFN- β was analysed by qRT-PCR (Figure 6.16). This experiment indicates that manipulation of miR-650 levels has no effect on the induction of IFN- β . However, in order to conclusively show that miR-650 has no effect on the overall IFN production, these results requires further confirmation on the protein level and need to be extended to include IFN α and its various subtypes. Further-

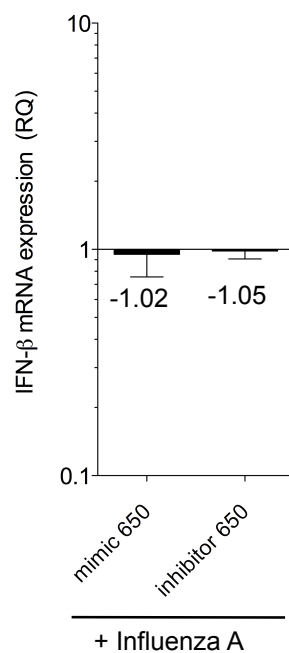


Figure 6.16: Investigating the effect of miR-650 on IFN β induction during Influenza A virus infection. DCs were transfected with miR-650 mimic or antisense inhibitor prior to infection with Influenza A (40 HAU/ml). At 6 hrs post-infection DCs were harvested and RNA was extracted to measure IFN β mRNA levels by qRT-PCR. Expression is normalised against GAPDH and is expressed relative to the nonsense control. Data is from two different donors and is shown as mean $\pm$ SEM.

more, this finding requires further confirmation by monitoring the level of secreted IFNs in supernatants.

Taken together, these data suggest that miR-650 is able to directly modulate the expression of ISGs without affecting the levels of IFNs.

6.4 Discussion

This chapter describes the first attempt to assess the effect of miR-650 on a genome-wide level. Gene expression profiling shows that miR-650 has a relatively modest effect on the mRNA level, despite being over expressed at least as well, if not better, than miR-155. Furthermore, the lack of putative binding sites in the group of down regulated targets suggests that many of these effects may not be caused by direct miR-650 binding. It is therefore tempting to speculate that miR-650 exerts most of its effects through translational repression. Over expression of miR-155 resulted in differential regulation of a number of genes. However, most transcripts were up regulated suggesting that these changes are again not due to direct regulation. Instead, this data suggests that miR-155 has profound secondary effects. Together, these findings show that although the direct effect of miRNAs on the mRNA level may be small, if the target is an important regulator, the secondary effects can be greatly amplified. This highlights the need to explore both direct and indirect effects of miRNAs.

IFIT2 and MxA are novel targets of miR-650

The work in this chapter demonstrates that miR-650 targets two interferon-inducible proteins, IFIT2 and MxA, with antiviral activity. Neither of these proteins has been previously shown to be regulated by miRNAs.

Gene expression analysis initially showed that miR-650 is able to regulate IFIT2 on the level of transcription. The direct interaction between miR-650 and the IFIT2 was further confirmed by 3'UTR luciferase reporter assay, but further work is required to confirm this interaction in DCs, especially in the course of an infection.

IFIT2 (also known as ISG54) belongs to the ISG56/IFIT1 gene family [Levy *et al.*, 1986]. Both IFIT1 and IFIT2 are induced in response to infection by a range of different DNA and RNA viruses such as Influenza A, lymphocytic choriomeningitis virus (LCMV), West Nile virus, Sendai virus or Herpes Simplex virus [Nicholl *et al.*, 2000] [Terenzi *et al.*, 2006] [Baas *et al.*, 2006] [Kawada *et al.*, 2006] [Wacher *et al.*, 2007]. Induction of IFIT2 is dependent on type I IFNs but has also been shown to be directly regulated by a range of different

PAMPs including LPS and Poly(I:C) via an IRF-dependent pathway [Wathelet *et al.*, 1988] [Grandvaux *et al.*, 2002] [Terenzi *et al.*, 2006]. Despite being among the first ISGs identified, little is known about the function of IFIT2. It has been reported that IFIT2 interacts with the translation initiation factor eIF-3, resulting in inhibition of translation initiation and thus protein synthesis [Terenzi *et al.*, 2006]. Furthermore, a recent study suggests that expression of IFIT2 elicits apoptotic cell death via a mitochondrial pathway [Stawowczyk *et al.*, 2011]. Finally IFIT2 has been shown to interfere with innate immune signalling [Li *et al.*, 2009b] and selectively inhibit LPS-induced TNF α expression, probably on a post-transcriptional level [Berchtold *et al.*, 2008]. These studies suggest that IFIT2 not only has an antiviral effect but may also function in a negative feedback loop to inhibit the inflammatory response. This supports the findings of the previous chapters which suggest a potential role for miR-650 in controlling the innate immune response.

MxA is a key regulator of IFN-induced antiviral response against a wide range of different viruses irrespective of their site of replication [Haller & Kochs, 2011]. It is a dynamin-like GTPase which comprises a GTP-binding domain, a middle oligomerisation domain and a GTPase effector domain [Gao *et al.*, 2010]. Its GTPase activity is dependent on oligomerisation [Martens & Howard, 2006] [Haller *et al.*, 2010]. It is at present not clear how MxA confers such a wide range of specificities, as a common denominator has not yet been identified. Studies focussing on negative strand RNA viruses, especially Influenza A virus and LaCrosse virus, have shown that oligomerisation is required for antiviral activity [Haller & Kochs, 2011]. In general, it is thought that MxA blocks the early steps in viral replication by binding to the viral nucleocapsid protein and directing it away from sites of viral replication.

The work presented in this chapter suggests that targeting of MxA by miR-650 does not lead to decreased mRNA levels. As a consequence, this interaction was not detected by microarray analysis. More intriguingly, this interaction was not predicted by any of the standard algorithms due to a single mismatch in the seed region. Nevertheless, 3'UTR reporter assay confirmed MxA as a target for miR-650 and, more importantly, this interaction was subsequently confirmed in DCs infected with Influenza A virus. It is surprising that despite the combination of several different approaches for target identification, only one of the tools used here predicted this interaction. This clearly highlights that there is still much room for improvement for *in silico* target prediction, and any conclusions drawn from such analysis must be considered with this caveat in mind.

Additional genes which may be regulated by miR-650 expression

Array analysis provides evidence that miR-650 may control the expression of a number of other genes. However, these interactions remain to be validated in primary DCs and further work is required to establish whether they are regulated by miR-650 directly and, if not, what the linking element is.

In addition to MxA, array analysis revealed that miR-650 may regulate the transcription of additional members belonging to the superfamily of IFN-induced dynamin-related GTP binding proteins [Martens & Howard, 2006]: Gbp3, Gbp 4 and Gbp5. Of these proteins, only Gbp5 contains putative binding sites for miR-650 and further studies are required to investigate the principles underlying this regulation. Several members of the Gbp family have been shown to have immunoprotective properties against a range of different viruses and bacteria [Martens & Howard, 2006]. A loss of function study in RAW264.7 macrophages showed that out of Gbp3, 4 and 5, only the latter has a mildly protective effect against *Listeria monocytogenes* and *Mycobacterium bovis* BCG [Kim *et al.*, 2011]. In this study, all protective Gbps translocated to bacteria-containing vacuoles and were involved in the delivery of engulfed bacteria to the lysosome. In addition, Gbp5 has been shown to regulate pyroptosis in Salmonella-infected RAW296.7 macrophages [Rupper & Cardelli, 2008].

Another gene identified by microarray is NUB1, which is induced by IFNs and interacts with NEDD8 to recruit it to the proteasome and promote its degradation [Kito *et al.*, 2001] [Kamitani *et al.*, 2001]. NEDD8 is a ubiquitin-like protein that conjugates the proteins of the cullin family and is involved in the regulation of many important biological functions such as NF- κ B signalling and cell cycle regulation. Over expression of NUB1 inhibits cell growth [Hosono *et al.*, 2010], but its role in inflammation is currently still unknown.

Down regulation of miR-650 may also induce the expression of IFITM1. A genome-wide siRNA screen revealed that the interferon-inducible transmembrane proteins IFITM1, 2 and 3 inhibit replication of a range of different viruses including Influenza A, West Nile and Dengue flaviviruses and Marburg and Ebola by interfering with early steps in their viral life cycle [Brass *et al.*, 2009] [Huang *et al.*, 2011]. Microarray analysis also revealed a number of soluble factors which may be regulated by miR-650 in an indirect manner. More specifically, a variety of Th1 associated chemokines, CXCL10, CXCL11, CCL5 and CCL8, were down regulated in response to miR-650 over expression.

Cooperation between miR-650 and miR-155

Although miR-155 was initially chosen to serve as a positive control with the purpose of confirming the functionality of the microarray, analysis reveals that many of the above-described genes regulated by miR-650, including MxA, IFIT2 and CXCL10, are also controlled by miR-155 expression. This may not be surprising considering that miR-155 decreases the expression of SOCS1, a negative regulator of IFN signalling, which has been shown to control the expression of various chemokines. While this work was ongoing, Wang *et al.* showed that this is indeed the case. Induction of miR-155 in VSV-infected murine macrophages down regulates the expression of SOCS1 which, in turn, promotes the secretion of IFN-associated inflammatory mediators such as CXCL10 [Wang *et al.*, 2010].

In order to fully confirm the cooperative action of miR-650 and miR-155 it is essential to confirm that the artificial up-regulation of one of the two has no effect on the expression level of the other. It could be, for example, possible that transfection of artificial miR-650 mimics leads to saturation of the RNAi machinery and therefore inhibits the processing of endogenous miRNAs, including miR-155. In order to exclude these secondary effects, miR-155 levels have to be monitored in the samples in which miR-650 was up-regulated.

In theory, however, this data suggests a potentially complex interaction network between miR-650 and miR-155. A number of studies have tried to model complex synergistic relationships between miRNAs [Shalgi *et al.*, 2007] [Boross *et al.*, 2009] [An *et al.*, 2010] [Xu *et al.*, 2011]. Co-regulation of miRNAs which bind to the same 3'UTR has been shown to work synergistically. For example, miR-375, miR-124 and let-7b jointly regulate Mtpn [Krek *et al.*, 2005] and the expression of Waf1 is efficiently regulated by 28 different miRNAs [Wu *et al.*, 2010b]. However, this study gives insights into a novel layer of interaction between two miRNAs which are reciprocally regulated and, by a variety of direct and indirect mechanisms, seem to cooperate in a synergistic as well as antagonistic manner.

6.5 Conclusion and future work

Several miRNAs, including miR-155 and miR-132, have been shown to modulate the antiviral IFN response by targeting central regulators of gene transcription such as SOCS1 and p300, respectively. Supporting the role of miRNAs in the antiviral response, work in this chapter shows that miRNAs can fine-tune the expression of ISGs in a direct manner, thereby potentially enhancing the antiviral state of the cell. Together, the data in this chapter suggests a possible dual mechanism of miR-650 by which it enhances the antiviral state, while

simultaneously promoting a negative feedback loop, thereby regulating the pro-inflammatory response.

However, considerably more work is required to consolidate the findings presented in this chapter. Direct miR-650:target interactions need to be confirmed by manipulating endogenous miR-650 levels and monitoring target expression. Furthermore, it would be interesting to map the exact MRE for direct interactions or identify the linking element for indirect effects. Finally, these data provide a good starting point for further investigation into the overall function of miR-650 in the immune response of DCs and further work is ongoing to investigate the significance of miR-650 during infection.

Chapter 7

General discussion

MicroRNAs are post-transcriptional regulators of gene expression which are emerging as key players in a variety of biological processes. Recent publications have provided compelling evidence that a variety of miRNAs are involved in fine-tuning innate immune responses [O'Neill *et al.*, 2011]. The study of miRNAs not only gives further insight into the complex regulation of the innate immune response and host-pathogen interaction but may also provide a basis for novel therapeutic strategies [Lanford *et al.*, 2010].

The work in this thesis describes the discovery of miR-650 as a novel PRR-responsive miRNA in monocyte-derived dendritic cells. While Chapter 4 investigates the expression pattern of miR-650 in response to PRR signalling, the remaining chapters described a multi pronged approach including bioinformatic analyses, target confirmation by 3'UTR reporter assays (Chapter 5) and global transcriptional analysis (Chapter 6) to study the effect of miR-650 on the gene expression profile of primary human DCs.

PRR signalling down regulates miR-650 expression in DCs

PRR signalling has been shown to regulate the expression of various miRNAs including miR-155, miR-146a and miR-21 [O'Neill *et al.*, 2011]. Chapter 4 describes the regulation of miR-650 downstream of a variety of different PRR signalling pathways. Similar to miR-155, miR-146a and miR-21, the regulation of miR-650 is neither limited to a specific group of PRRs nor dependent on the origin of the specific PAMP (see Section 4.3.2). Instead, down regulation of miR-650 seems to be dependent on the stimulatory capacity of the PRR ligand; the more immune activation induced by the ligand the greater the down regulation of miR-650 observed. This finding was put into the context of a real infection scenario by showing that miR-650 is down regulated in DCs infected with Influenza A virus (see Section

4.3.4), a pathogen which has been shown to infect DCs and stimulate a variety of PRRs, both endosomal and intracellular.

In general, very little is known about the mechanisms regulating miRNA expression. Given that miR-650 seems to be regulated through a variety of different PRR signalling cascades, it is likely that its expression is controlled by a common mechanism. Preliminary work using an NF- κ B inhibitor suggests that TLR8-mediated down regulation of miR-650 requires NF- κ B activity (Section 4.3.3). This is in agreement with previous studies showing that NF- κ B is a central regulator for the up as well as down regulation of miRNA expression downstream of PRR signalling pathways [O'Neill *et al.*, 2011]. However, while NF- κ B has been shown to bind directly to miRNA promoter element inducing miRNA transcription [Bazzoni *et al.*, 2009], its role in miRNA repression is less well understood and may include a variety of indirect mechanisms. In the case of let-7, for example, NF- κ B induces LIN28B expression which in turn blocks processing of the miRNA [Iliopoulos *et al.*, 2009] [Viswanathan *et al.*, 2008]. Therefore inhibition of let-7 downstream of TLR4 occurs at a post-transcriptional level. A similar scenario could be envisaged for miR-650 and further work is required to elucidate the full mechanism.

As shown in Section 4.3.3, miR-650 down regulation was not completely abrogated by NF- κ B inhibition, either suggesting incomplete inhibition of NF- κ B or, alternatively, the existence of additional regulatory mechanisms. For example, AP-1 has also been shown to control the expression of PRR-responsive miRNAs [Yin *et al.*, 2008]. Assuming that regulation occurs on a transcriptional level, a promoter analysis could provide important clues regarding the range of different factors capable of regulating miR-650 expression. This would be especially interesting because miR-650 is the only miRNA described so far which is located in the leader exon of a protein coding gene, in this case the *IGLV* gene, and is suggested to share the promoter region [Das, 2009]. It would also be interesting to investigate the regulatory mechanisms that direct the decision as to whether transcription leads to the production of protein or miR-650.

The potential role of miR-650 in controlling innate immune responses

Unfortunately, a canonical workflow for investigating the regulatory network of individual miRNAs is currently missing. Therefore, in Chapters 5 and 6 a multi pronged approach was taken which combined several different techniques including bioinformatic target prediction, genome wide expression profiling, and 3'UTR assays. Each technique has its limitations and can therefore only provide a partial picture. However, the data generated show that, when

combined, they are able to elucidate both indirect as well as secondary effects mediated by miR-650 on both the mRNA and protein level.

The work in Chapters 5 and 6 confirm the already known target ING4, and identified a number of novel targets for miR-650. While some of these targets, for example MxA, were regulated exclusively on a post-transcriptional level (see Section 6.3.7), the expression of others, for example IFIT2, was also affected on an mRNA level (Section 6.3.3).

Even though the overall effect of miR-650 in the innate immune response has not yet been shown, the targets identified in Chapters 5 and 6 provide a good basis for further studies. A summary of the function of miR-650 based on the main findings presented in this thesis is shown in Figure 7.1.

The work presented in Chapter 6 suggests that miR-650 regulates a range of interferon-stimulated genes by both direct and indirect mechanisms without affecting the levels of type I IFNs. Hence, PRR-mediated down regulation of miR-650 may contribute to the establishment of an antiviral state in the absence of interferon signalling. Of the proteins identified, MxA and IFIT2 were validated as direct targets (see Section 6.3.6), and MxA was shown to be regulated by miR-650 in the context of Influenza A infection (Section 6.3.7). MxA is a classical IFN-inducible protein that has antiviral effects against a range of RNA viruses including Influenza [Haller & Kochs, 2011]. In contrast, the role of IFIT2 is less well understood. It has been shown to form an antiviral complex with IFIT1 and IFIT3 [Schoggins *et al.*, 2011] [Pichlmair *et al.*, 2011] but also negatively regulates cytokine secretion in response to LPS activation [Berchtold *et al.*, 2008].

In addition to the finding that miR-650 targets IFIT2, a potential negative regulator, this thesis provides a number of indicators for the involvement of miR-650 in dampening the innate immune response. Down regulation of miR-650 mildly reduced the levels of IL-12 and IL-10 (Section 4.3.5) and the 3'UTR reporter screen in Section 5.3.3 validates SIRP β and ING4 as direct targets for miR-650. Both proteins would therefore be up regulated during the inflammatory response. SIRP β is a transmembrane protein expressed on a variety of monocytic cells. Upon cross-linking of SIRP β , it associates with the ITAM bearing protein DAP12 resulting in a signalling cascade via the adaptor protein Syk [Tomasello *et al.*, 2000]. Even though it is thought to be an activating receptor [Dietrich *et al.*, 2000], work in primary mouse microglial cells shows that activation of SIRP β dampens the induction of TNF- α and NOS2 in response to LPS signalling [Gaikwad *et al.*, 2009]. ING4, on the other hand, has been shown to inhibit NF- κ B-induced production of IL-6 and IL-8 during the immune response by associating with p65, thereby possibly preventing it from binding to its DNA target sequence [Nozell *et al.*, 2008]. Unfortunately, both IL-6 and IL-8 were above the detection limit in the

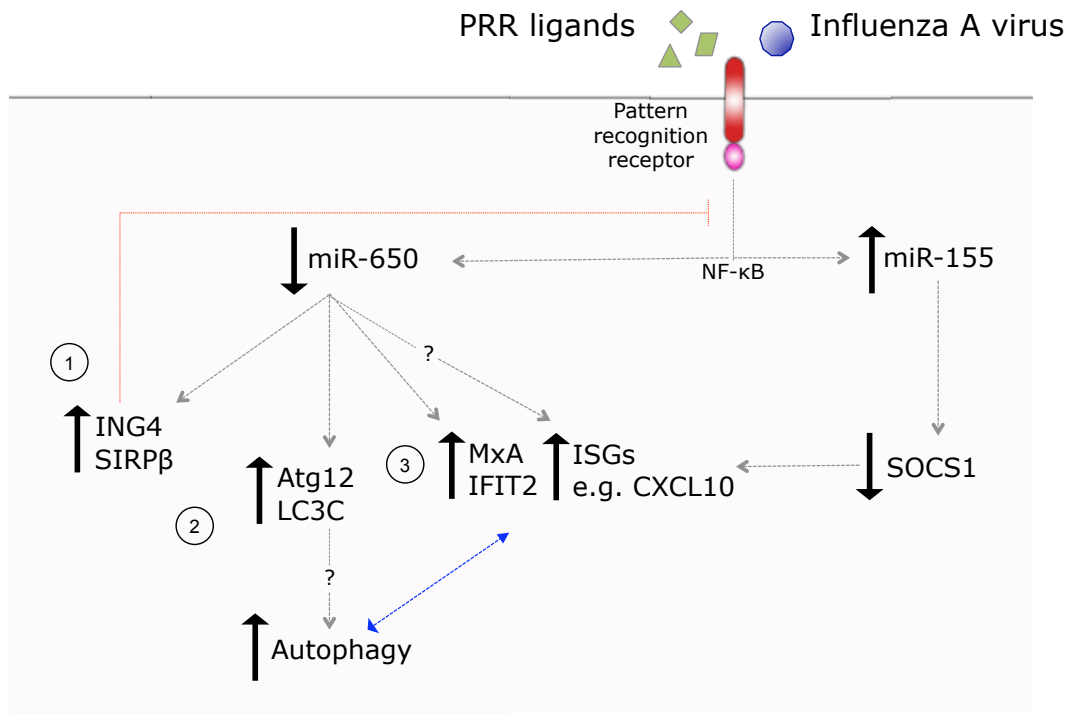


Figure 7.1: The potential role of miR-650 in fine-tuning innate immune responses in DCs. Stimulation of monocyte-derived DCs with a range of different PAMPs, or by infection with Influenza A virus, leads to down regulation of miR-650 expression and induction of miR-155. In the case of TLR8, this regulation is, at least partially dependent on the activation of NF- κ B. Data presented in this thesis provides evidence for different roles of miR-650 in the innate immune response. Firstly, miR-650 targets potential negative regulators ING4 and SIRP β and may therefore function as a negative feedback regulator helping to control innate immune responses. Secondly, miR-650 targets Atg12 and LC3C, both components of the autophagosome. Consistent with this, down regulation of miR-650 was found to induce autophagy. However, whether this is due to targeting of the above mentioned components remains to be shown. Lastly, miR-650 was found to regulate a number of ISGs via direct as well as potential indirect mechanisms, although it does not itself induce interferon production. It may therefore function to enhance the antiviral state of the cell in the absence of these cytokines. The expression of many of these ISGs is also controlled by miR-155 via regulation of SOCS1. This suggests a potential cooperation between the two reciprocally regulated miRNAs, miR-650 and miR155, in the innate immune response.

Luminex assay (Section 4.3.5), and additional work would be required to define this negative feedback loop in DCs. In all cases, the down-regulation of miR-650 observed upon PPR stimulation may lead to enhanced expression of these inhibitory molecules which, in turn, might serve to limit inflammation and prevent immune-mediated pathology.

In summary, similar to miR-155 which has been assigned both pro- as well as anti-inflammatory roles, the work in this thesis suggests a dual role for miR-650 as it both supports

the establishment of an anti-viral state but also controls innate responses by providing a negative feedback loop. However, these two effects do not necessarily contradict each other. On the contrary, miRNAs could adopt different roles depending on the timing, thus enabling miR-650 to strengthen initial responses while helping to dampen them at later time points. Alternatively, it may be beneficial for a miRNA to induce a certain effect while, at the same time, ensuring that it remains controlled. The work in this thesis suggests that miR-650 may break this paradox by limiting the pro-inflammatory response and thereby preventing immunopathology, while simultaneously inducing the expression of antiviral genes induced by the same cytokines. This provides a perfect example of the subtle and extremely sophisticated way in which miRNAs may be able to fine-tune innate immune responses.

The role of miR-650 in the regulation of autophagy

The work presented in Section 5.3.6 shows that down regulation of miR-650 induces autophagosome formation. This is in agreement with several recent studies which show that PRR signalling as well as infection with live pathogens induces autophagy in a variety of immune cells [Romao & Münz, 2011]. However, it is yet to be determined how and to what extent miR-650 controls this cellular process. The 3'UTR screen validated Atg12 and LC3C as direct targets, both components of the autophagosome, but it remains unclear whether increased expression of these two proteins is sufficient to induce autophagy. Furthermore, LC3 has three different isoforms and it is not known whether regulation of only one of the three has an effect on autophagosome formation. Computational analysis in combination with 3'UTR assays may point to other autophagy related proteins, as was the case for FKBP1A (see Section 5.3.3). Finally, since autophagy is a ubiquitous cellular function that is induced by a variety of stimuli, it would be interesting to apply this finding to other cell types as well as different stimulatory conditions. This would be of great interest given that only a single miRNA has as yet been shown to have an effect on autophagosome formation [Zhu *et al.*, 2009].

In addition to Atg12 and LC3C, gene expression profiling revealed that miR-650 may be involved in regulating the expression of members of the family of dynamin-like GTPases (Gbp3, 4 and 5) (Section 6.3.2). These IFN inducible proteins have been shown to be involved in the intracellular trafficking of organelles as well as viral components. Although they have as yet not been associated with autophagosome formation, this nevertheless underlines the potential role for miR-650 in intracellular organelle trafficking and could therefore provide an interesting finding in the context of innate immunity.

Influenza A virus infection is known to induce autophagy, however its exact role in viral

replication remains to be elucidated. Interestingly, a recent study has shown that infection with the pandemic H9N2 virus results in higher levels of autophagy in comparison to the H1N1 viruses [Law *et al.*, 2010]. Increased levels of autophagy, in turn, promote cytokine hyperinduction, which is central to the pathogenesis of a range of pandemic Influenza viruses including H2N9 and H5N1 and is linked to a fatal outcome [Li *et al.*, 2011] [de Jong *et al.*, 2006].

This effect is attributed to increased delivery of viral PAMPs to the endosomal compartment by autophagy leading to enhanced immune activation. This is especially interesting because the main inflammatory mediator to be up regulated by autophagy induction in the study by Li *et al.* was CXCL10 which is also regulated by miR-650 (Section 6.3.3). This does not only suggest that miR-650 may control CXCL10 production by enhancing autophagy but may also provide evidence for a feed forward mechanism by which miR-650 contributes to the cytokine storm observed in H2N9 infection. Hypothetically, increased viral replication could lead to the production of large amount of PAMPs which induce strong PRR triggering resulting in miR-650 down regulation. Lower miR-650 levels promote a strong induction of autophagosome formation, which in turn increases delivery of PAMPs to endosomal compartments and therefore promotes a cytokine storm that contributes to the virulence of pandemic H2N9 virus.

This highlights that the role of miR-650 may be more complex than described above. Therefore, further work is needed to establish the exact role of miR-650 in the innate immune response against Influenza A virus.

Cross-talk between different miRNA regulatory networks

A further aspect highlighted by the work in this thesis is the interaction between miR-650 and miR-155. These two miRNAs are reciprocally regulated in response to the same stimuli (Section 4.3.4) and, despite completely different seed sequences, and therefore different sets of targets, show overlapping functions (Section 6.3.4). Several studies have highlighted the fact that different miRNAs may directly target the same transcript and therefore synergistically regulate the same target. For example, simultaneously knocking down five anti-HIV-1 miRNAs with different seed sequences in latently infected cells, leads to a greater recovery of viral replication compared to knocking down each miRNA individually [Huang *et al.*, 2007b]. However, the findings in this thesis demonstrate that another, more complex level of interaction exists which takes into account secondary effects. The work in Chapter 6 demonstrates that miR-650 and miR-155 commonly regulate a number of IFN inducible genes, such as CXCL10, MxA and IFIT2. MiR-650 seems to, at least in part, regulate their expression

through direct targeting. MiR-155, on the other hand, has been shown to target SOCS1 [Lu *et al.*, 2009a], a negative regulator of gene expression which, in the steady state, has been shown to suppress the expression of a variety of antiviral genes [Yasukawa *et al.*, 2000]. Therefore miR-155 potentially induces the expression of these genes on a transcriptional level, whereas miR-650 fine-tunes expression on a post-transcriptional level. This shows that even miRNAs that are reciprocally regulated and contain different seed sequences may be able to cooperate by using direct as well as indirect mechanisms (Figures 6.11 and 6.12).

As shown in the case of DC-SIGN (Section 5.3.5), this can also result in antagonising effects. However, the antagonism may also be a form of cooperation. While increased levels of miR-155 decrease DC-SIGN transcription, decreased expression of miR-650 may ensure constant protein levels. This suggests that the exact nature of the cooperation depends on the relative contributions of miR-155 and miR-650 as well as on the target itself.

In summary, the work in this thesis not only underlines the importance of secondary effects mediated by miRNAs, but also highlights the need for development of systems biology approaches which can incorporate all the information, including target expression and target site analysis. Furthermore, complex combinatorial knockdown or over expression experiments could be used to investigate overlapping regulatory networks of different miRNAs.

Future work

Given the finding that miRNAs are subtle regulators of gene expression and that over expression of miR-650 does not lead to many significant changes in gene expression, is it important to investigate whether miR-650 has an impact on the way invading pathogens are handled by DCs. Furthermore, as described above, miR-650 may adopt a dual role by supporting antiviral responses while simultaneously controlling innate immune responses.

The main question arising from the findings presented here is whether miR-650 has an effect on viral replication or, more generally, on the innate immune response of DCs to invading pathogens. As shown in Chapter 4, DCs infected with Influenza A virus express lower levels of miR-650. This *in vitro* infection system would provide a good model for further studies, and further work is ongoing to elucidate whether modulating the level of miR-650 expression plays a role in Influenza A infection. However, given that siRNA screens have shown that different viruses are targeted by unique sets of ISGs [Schoggins *et al.*, 2011], it may be necessary to investigate the role of miR-650 in the context of a range of viruses. Furthermore, it would be interesting to explore whether escape from miR-650 down regulation, as observed for HIV-1, may provide a benefit for selected viruses and therefore contribute to

their immune evasion strategy.

Finally, this work was performed using monocyte-derived dendritic cells as an *in vitro* model system and it would be interesting to explore these findings in primary mucosal CD103⁺ DCs or even extended investigations to lung epithelial cells, which have been shown to be important mediators in the antiviral innate immune response to Influenza A virus.

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