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A role for eIF4A1 in IL-10 induction by LPS

Thesis submitted to the

University of Dublin

For the

Degree of Doctor of Philosophy

By

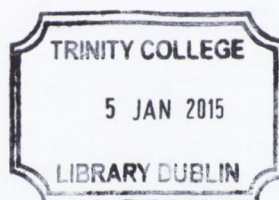
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June 2014

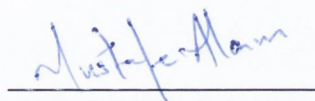


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Mustafa Alam

Acknowledgments

As I submit this thesis, poignant sense of fulfillment and purpose comes to me. PhD projects are never easy, with many pitfalls and much disheartenment along the way. Yet I had never imagined to learn so much in the space of three and a half years.

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Abbreviations

Ago	Argonaute
AID	activation-induced cytidine deaminase
AP-1	activating protein-1
APC	Antigen presenting cell
ARE	Adenine:Uridine-rich elements
ATP	Adenosine triphosphate
bp	base pair
BMDM	bone marrow derived macrophage
BSA	bovine serum albumin
Btk	Bruton’s tyrosine kinase
cAMP	cyclic adenosine monophosphate
CARD	Caspase activation and recruitment domain
CLR	C-type lectin domain-like containing receptor
CpG	methylated cytosine-guanine DNA
Ct	Threshold cycle
DAMP	Damage or danger associated molecular pattern
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ds	Double stranded
DSS	Disuccinimydyl suberate
EAE	Experimental autoimmune encephalitis
DC	dendritic cell
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetracetic acid
eIF	eukaryotic initiation factor
ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular signal regulated kinase

FCS	Foetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G-protein coupled receptor
GRP94	Glucose regulated protein of 94 kDa
h	hour(s)
HCV	Hepatitis-C virus
HEK	Human embryonic kidney
HSV	Herpes-Simplex virus
IFN	Interferon
Ig	Immunoglobulin
I κ B	Inhibitor of NF κ B
IKK	I κ B kinase
IL	Interleukin
IL-1R I	IL-1 receptor type I
iNOS	inducible nitric oxide synthase
IRAK	Interleukin 1 receptor associated kinase
IRF	Interferon Regulatory Factor
ISRE	Interferon stimulated response element
JNK	c-jun-N-terminal kinase
kDa	Kilodalton
KSHV	Kaposi's sarcoma herpes virus
KSRP	KH-type splicing regulatory protein factor
LMP	latent membrane protein
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
Malp-2	Macrophage-activating lipoprotein-2kDa
MAPK	Mitogen activated protein kinase
Mal	MyD88 adaptor like
MDA5	Melanoma differentiation associated gene 5

min	minute(s)
miRNA	microRNA
miR	miRNA
mRNA	messenger RNA
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor kappa B
NLR	Nod-like receptor
nt	nucleotide
PAGE	Polyacrylamide gel electrophoresis
pam3CSK4	pam ₃ CysSerLys ₄
PAMP	Pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
P-body	Processing body
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDCD4	programmed cell death 4
PKA	Protein kinase A
PKR	RNA-dependent protein kinase
p(I:C)	polyinosinic:polycytidilic acid
PRR	Pattern recognition receptor
PVDF	Polyvinylidene difluoride
RA	rheumatoid arthritis
RA-SF	rheumatoid arthritis synovial fibroblast
RISC	RNA-induced silencing complex
RANTES	Regulated on Activation, Normal T Expressed and
Secreted	
RLC	RISC loading complex
RLR	RIG-I like receptor
RIG-I	Retinoic acid inducible gene I
RIP	Receptor interacting protein
RNA Pol	RNA polymerase

ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcription PCR
SARM	SAM and ARM-containing protein
SDS	Sodium Dodecyl Sulphate
SHIP-1	Src homology-2 domain-containing inositol 5-phosphatase 1
SHP-1	Tyrosine-protein phosphatase non-receptor type 6 (a.k.a Src homology region 2 domain-containing phosphatase-1)
siRNA	small interfering RNA
SOCS	Suppressor of Cytokine Signalling
STAT-3	signal transducer and activator of transcription-3
TAB	TAK1 binding protein
TAK	TGF β -activated kinase
TBK-1	Tank binding kinase -1
TEMED	N, N, N', N', - Tetramethylethylenediamine
TGF- β	tumour growth factor- β
TIA-1	T-cell intracellular antigen-1
TIR	Toll-IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll like receptor
TMEM49	transmembrane protein 49
TNF	Tumour necrosis factor
TPM	tropomyosin
TRIF	TIR domain-containing adaptor inducing IFN-beta
TRAF	TNF-receptor associated factor
tRNA	transfer RNA
TTP	tristetraprolin
TRAM	TRIF-related adaptor protein
UTR	untranslated region

Abstract

microRNAs are a family of short non-coding RNAs that act as fine tuners of gene expression by controlling mRNAs. They have been implicated in numerous diseases including inflammatory diseases. A number of studies have looked at differential expression in response to stimulators of innate immunity, notably the gram negative bacterial product lipopolysaccharide (LPS) which acts via toll-like receptor 4. This study initially focused on LPS-induced miRNA repression. A number of miRNAs that were LPS-repressed were identified and an analysis was performed to identify their targets and determine their role in innate immunity. Let-7c-1-3p was identified as an LPS-repressed miRNA, and was predicted to target the eukaryotic translation initiation factor eIF4A2.

Consistent with the decrease in let-7c-1-3p, LPS induced eIF4A2 and the related isoform eIF4A1 at 8 and 24 hours respectively. Promoter analysis of eIF4A1 and eIF4A2 revealed that the promoters contain binding sites for transcription factors including NF κ B and AP1. Paradoxically LPS caused a decrease in mRNA for eIF4A1 and eIF4A2, indicating eIF4A2 was not being regulated by let-7c-1-3p.

eIF4A has been implicated in the translation of cytokine genes. eIF4A1 was shown to interact directly with mRNA encoding for IL-10. The small molecule inhibitors, Silvestrol and Hippuristanol, have been identified to block eIF4A activity. They blocked IL-10 induction and had no inhibitory effect on TNF α expression. IL-6 mRNA was strongly induced in response to Hippuristnaol however this did not

translate into protein. Additionally the 5'UTR of IL-10 was shown to act as a repressor of translation and was targeted by eIF4A1. These findings demonstrate that in LPS signaling, the eIF4A1 isoform regulates IL-10 translation and that the delayed production of IL-10 is potentially due to its complex 5'UTR, which is targeted by eIF4A1.

Chapter 1

Introduction

Chapter 1: Introduction

Over the last two decades our understanding of the innate immune system has improved. We now understand the critical role and complexity of innate immunity in host defence against pathogens. Great progress has been made in our understanding of different signaling pathways that are involved in pathogen recognition and defence.

The Innate immune system acts as the first line of defence and alerts the body about an integrity breach. It recognizes pathogens through generic markers using pattern recognition receptors (PRRs). On the other hand adaptive immune system involves a more specific response. It recognizes pathogens using antigen specific receptors. Adaptive immune cells, such as T- and B- cells have memory. This allows the response to be more rigorous and directed when the same pathogen is encountered for the second time (Janeway, 1989).

The innate immune system recognizes pathogen-associated-molecular-patterns (PAMPs). The best understood example of a PAMP is lipopolysaccharide (LPS), which is found on the cell wall of gram-negative bacteria (Janeway, 1989). Innate immune cells recognize a broad range of pathogens and often engulf them to present their antigens to cells of adaptive immune system. Its task is to limit infection, signal and recruit other cells to the site of infection, by producing cytokines and chemokines. Immune cells also limit self-damage by an exaggerated inflammatory response through regulatory mechanisms. The activation of the innate immune response involves a cascade of events that most often result in activation

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and/or translocation of transcription factors such as NF κ B that transcribe genes encoding inflammatory mediators. Regulatory mechanisms for the resolution of inflammation are activated later on in the time course of infection that limit the signals and transcription factors.

A number of cells participate in innate immunity, including: Natural killer (NK) cells, mast cells, eosinophils, basophils, macrophages, neutrophils and dendritic cells. Different cells are involved with dealing with different pathogens, for instance macrophages and dendritic cells are primarily phagocytic and antigen-presenting cells. NK cells kill host cells that are infected with microbes, whereas mast cells are resident in connective tissues and mucous membranes and are involved in wound healing and the allergic response.

1.1 PRRs

PRRs allow for the recognition of pathogens by cells. These receptors are found either on cell membranes or in the cytoplasm to alert the innate system of pathogens present either extracellularly or intracellularly. PRRs are classified on the basis of their location, PAMP specificity, function, response and evolutionary relationships (Janeway, 1989).

The first PRRs to be recognized were the scavenger receptors, SC-A1 and SC-A2 (Peiser et al., 2002, Taylor et al., 2005). They are found on macrophages and are

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involved in recognition of gram-positive and gram-negative bacteria. Their activation leads to engulfing of bacteria into the cell, where they are killed. However the most widely studied family of PRRs is the Toll-like receptor (TLR) family, most of which are found on the cell membrane, and also on endosomal membranes. These receptors share homology with the type 1 interleukin 1 receptor (IL-1R1) in their cytosolic domain and together they form a receptor superfamily known as “IL1R/TLR superfamily” (O'Neill et al., 2013, Gay and Keith, 1991). They all share the Toll-IL-1 receptor-resistance (TIR) domain and recognize a wide variety of PAMPs such as LPS, flagellin and single-stranded RNA.

Another group of PRRs, found in cytoplasm, are RIG-I-like helicases or RIG-I like receptors (RLRs). They mostly recognize RNA from viruses. There are three types of RLRs, RIG-I, MDA-5 and LGP-2. RIG-I and MDA-5 act by inducing a cellular response to double-stranded RNA (produced in viral replication) through the CARD domain. LGP-2 lacks a CARD domain and therefore it cannot act alone (Satoh et al., 2010).

Another group of cytoplasmic receptors are NOD-like receptors (NLRs). NLRs are involved in inflammation and apoptosis (Coll and O'Neill, 2010). All NLRs share some common features: they all have leucine rich repeats (LRRs); a nucleotide binding domain (NBD), and either a CARD or PYRIN domain. Some NLRs (e.g. NLRP3) are involved in activation of caspases, e.g. caspase-1, which is involved in the cleavage and activation of the inflammatory cytokine IL-1 (Coll and O'Neill, 2010).

Myeloid C-type lectin receptors (CLRs) form another group of PRRs. These receptors contain a carbohydrate-binding motif. An example of a CLR is Dectin-1, which recognizes zymosan from fungal infections. It also leads to NF κ B activation (Robinson et al., 2006).

1.2 The discovery and repertoire of TLRs

This project largely concerns TLR4 which recognizes the gram negative bacterial product lipopolysaccharide (LPS). TLRs are named after Toll protein, which was first identified in *Drosophila melanogaster*, whose first function was shown to be in development. In 1996 Hoffmann and colleagues identified the role of Toll in immunity in *D. melanogaster* (Lemaitre et al., 1996). Two years later in 1998 the genes encoding several human TLRs were cloned (Rock et al., 1998).

As stated above TLRs can be present on the cell surface or on cytosolic endosomes. TLR1, TLR2, TLR4, TLR5, and TLR6 are found on the cell surface, whereas TLR3, TLR7, TLR8, TLR9, TLR11 and TLR13 are in endosomes. Some TLRs are better defined and functionally understood than others. TLRs 11, 12 and 13 are murine-specific whilst TLR10 is human specific. Different TLRs recognize different PAMPs. Most TLRs function by forming homodimers, however TLR2 heterodimerizes with TLR1 or TLR6 (Farhat et al., 2008).

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TLRs recognize a wide range of microbial molecules from bacteria, viruses, fungi and protozoan parasites. TLR1 in a dimer with TLR2 recognizes triacyl lipopeptides, the TLR2/6 dimer recognizes diacyl lipopeptides from *Escherichia coli* and *Mycoplasma* species. It is also involved in recognition of peptidoglycan from Gram-positive bacteria and the polysaccharide zymosan from yeast (Zahringer et al., 2008). In addition a role for TLR2 in viral recognition and type-1 interferon induction in inflammatory monocytes has also been described (Barbalat et al., 2009). TLR-3 recognizes double stranded RNA, whilst TLR4 recognizes LPS. Human but not murine TLR4 can also be activated by nickel in allergic reactions (Kumar et al., 2009, Schmidt et al., 2010). TLR5 recognizes flagellin from various bacteria such as *Salmonella typhimurium*, TLR7 and TLR8 recognize single stranded RNA, while TLR9 recognizes unmethylated 2'-deoxyribo(cytidine-phosphate-guanosine) (CpG) motifs from bacteria and viruses and the malarial infection by-product crystal hemozoin (Coban et al., 2010). The ligand for TLR10 is unknown however it has been shown to form a heterodimer with either TLR1 or TLR2 (Hasan et al., 2005). TLR11 and TLR12 recognize Profilin and TLR13 recognizes a specific bacterial RNA sequence "CGGAAAGACC".

1.3 TLR accessory proteins

TLR ligand recognition and function involves a number of accessory molecules. A number of such accessory proteins have been identified to date. Cluster of differentiation 14 (CD-14) regulates endocytosis of TLR4 (Zanoni et al., 2011). In addition it has been identified that CD-36 mediates formation of TLR4/6

heterodimer that is involved in recognition of oxidized low density lipoprotein (LDL) and fibrillar amyloid- β (Stewart et al., 2010). Furthermore, CD-36 also promotes the formation of TLR2/6 heterodimer that is involved in recognition of microbial diacylglycerides (Hoebe et al., 2005). The TLR4 interactor with leucine rich repeats (TRIL) protein interacts with TLR4 and LPS and it is required for LPS-induced cytokine production, furthermore TRIL has also been shown to interact with TLR-3 (Carpenter et al., 2011, Carpenter et al., 2009). Endoplasmic reticulum (ER) chaperone proteins glucose regulated protein of 94 kDa (GRP94) and protein associated with TLR4a (PRAT4a) are critically important for TLR1, 2, 4, 7 and 9 functionality because they mediate correct protein folding. The trafficking from ER to the endosomes of endosomal TLRs (3, 7, 8 and 9) is mediated by the glycoprotein uncoordinated 93 homology B1 (UNC93B1) (Lee et al., 2012).

1.4 TLR signaling

TLR signaling is very similar to IL-1R signaling. Once the receptor is triggered, which occurs as a result of dimerization, the dimerised TIR domains associate with an adaptor protein or proteins. Six cytoplasmic TIR domain containing adaptor proteins exist: (1) MyD88, which contains a TIR domain and death domain (Medzhitov et al., 1998), (2) MyD88 adaptor like (MAL) (Fitzgerald et al., 2001), (3) TIR domain containing adaptor inducing interferon- β (TRIF) (Yamamoto et al., 2002, Oshiumi et al., 2003), (4) TRIF related adapter molecule (TRAM) (Yamamoto et al., 2003, Fitzgerald et al., 2003b), (5) sterile alpha- and HEAT/armadillo-motif-containing protein (SARM) (O'Neill and Bowie, 2007) and (6) B-cell adaptor for

PI3K (BCAP) (Troutman et al., 2012). All the adapter proteins are positive regulators, except for SARM and BCAP, which are negative regulators. All the TLRs with the exception of TLR-3 can signal through MyD88. TLR-3 signals through TRIF, whereas TLR4 can use TRIF and MyD88. TLR4 and to a lesser extent TLR2 use a second adaptor 'MAL' to interact with MyD88 (Kenny et al., 2009). TLR4 uses TRAM as a bridging adapter to interact with TRIF. Figure 1.1 describes the main signaling pathways activated by TLRs.

1.5 MyD88 dependent signaling

In MyD88 dependent signaling, MyD88 interacts with IL-1 receptor associated kinase (IRAK)-4. IRAK-4 is a serine/threonine kinase and phosphorylates IRAK-1 and IRAK-2. Following phosphorylation tumour-necrosis factor receptor-associated factor-6 (TRAF-6) is recruited and self-polyubiquitinated (Flannery and Bowie, 2010).

Transforming growth-factor β -activated kinase-1 (TAK-1) is then recruited with the help of two TAK binding proteins, TAB-2 and TAB-3. TAK-1 kinase activates the I κ B kinase (IKK) complex. Following this, IKK phosphorylates inhibitor of κ B (I κ B) that results in release of NF κ B. Once NF κ B is free of its inhibitor, its nuclear-localisation signals allow it to translocate into the nucleus where it participates in the transcription of inflammatory cytokines. The MAP Kinases Jun N-terminal kinase (JNK), and p38 are also activated by TAK1.

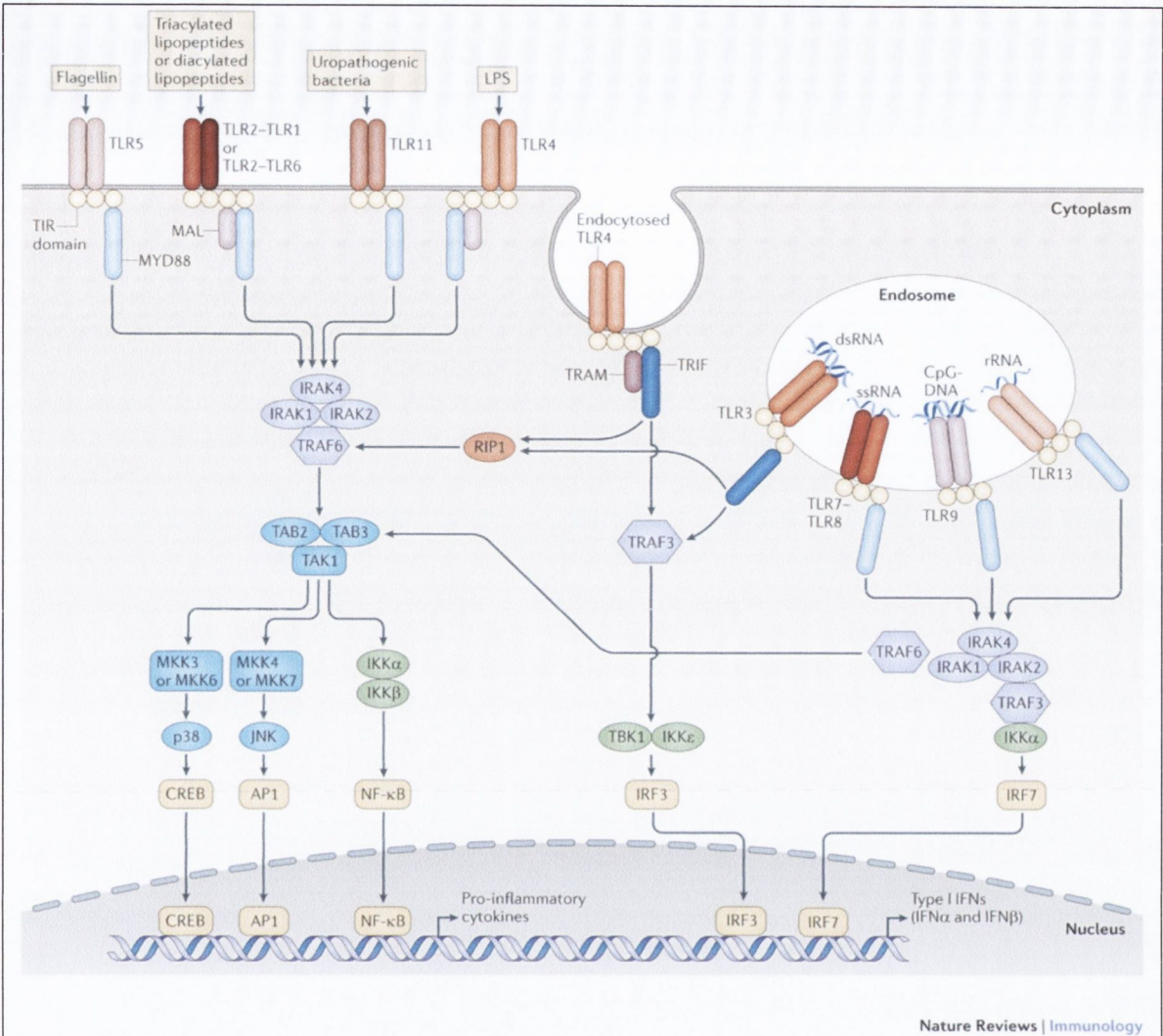


Figure 1.1 TLR signaling pathway taken from (O'Neill et al., 2013)

TLR4, 5, 11 and heterodimers TLR2/1 and TLR2/6 are found on cell membrane whereas TLR-3, 7,8,9 and 13 are found in endosomes. TLR4 can be on cell membrane as well as in endosomes. TLR signaling involves recruitment of different adaptor proteins, MyD88, MAL, TRIF and TRAM. As shown in case of TLR4, it can use MyD88 when present on plasma membrane or TRIF when present in endosome. Once TLRs are stimulated, adaptors are recruited and cascade of signaling events start that result in the activation of MAPKs, JNKs, and p38 through IRAKs and TRAFs. Following this, transcription factors, NFκB, IRFs, CREB and AP1 are activated and translocated into nucleus. These transcription factors then facilitate the transcription of pro-inflammatory cytokines.

1.6 NFκB

NFκB is a key signal activated by TLRs. It is a pro-inflammatory transcription factor that gets activated upon pathogen encounter. It responds to pathogens and also to a number of other stimuli, such as cytokines, stress, free-radicals, UV-radiation and oxidized LDL. There are five family members of NFκB, which are divided into two classes. Class I has two pre-cursor members NFκB1 (p105) and NFκB2 (p100), which are processed into p50 and p52 respectively. Class II has three members RelA (p65), RelB and c-Rel. NFκB1 and NFκB2 form heterodimers with class II members to be functionally active. The most abundant heterodimer is p50/p65. All NFκB family members have a Rel homology domain in their N-terminus, whilst only class II members (RelA, RelB and c-Rel) have an additional transactivation domain in their C-termini.

IκB contains ankyrin repeats that keep the nuclear localization signals masked and hence NFκB stays inactive in the cytoplasm of the cell. IKK phosphorylates two serine residues on the regulatory domain of IκB. Once IκB is degraded, NFκB nuclear localization signals are naked and active. This allows it to translocate into the nucleus, bind to DNA and turn on the expression of inflammatory genes. Specifically NFκB binds to upstream regions of target promoters at a particular nucleotide sequence 5'-GGGACTTCC-3'. This sequence is found in promoter regions of genes encoding pro-inflammatory cytokines such as IL-2, IL-6, TNFα, IL-1β (Karin and Ben-Neriah, 2000) and nitric oxide synthase (iNOS).

1.7 TRIF dependent signaling

TRIF-dependent signaling, is an alternate to MyD88-dependent signaling. To date, TLR4 and TLR-3 have been shown to use the TRIF-dependent signaling pathway. TRIF-dependent signaling can lead to the activation of NF κ B, IRF-3 and IRF-7. TRIF binds to TRAF-6 via its binding domain located at the N-terminal and binds to receptor interacting protein-1 (RIP-1) via its C-terminal RIP homotypic interaction motif (RHIM) (Meylan et al., 2004). This facilitates activation of NF κ B. On the other hand interaction of RIP-3 at RHIM inhibits NF κ B (Kawai and Akira, 2006). TRIF dependent signaling also activates IRF-3 and IRF-7. Two IKK-related proteins, TRAF family member associated NF κ B activator (TANK) -binding kinase-1 (TBK-1) and inducible IKK (IKK- ϵ) mediate the activation of IRF-3. IRF-3 forms a dimer with IRF-7 and translocates into the nucleus and binds the interferon response elements (ISREs). The TRIF pathway is mostly involved and crucial for viral infections (Sato et al., 2003) and results in induction of type-1 interferons.

1.8 IRFs

Interferon regulatory factors (IRFs) are a family of transcription factors that also participate as immuno-regulatory factors. There are nine members in the IRF family, IRF-1 – 9 (Taniguchi et al., 2001, Lohoff and Mak, 2005). All members share a 120 amino acid DNA binding domain, which recognizes the IFN-stimulated response element (ISRE) (Taniguchi et al., 2001). ISREs are found in the promoter region of a number of genes, including genes that encode type I IFNs. The IRF response is

directed towards viral pathogens. Certain TLRs such as TLR-3, TLR7, 8 and 9 are triggered by viral pathogens and induce an anti-viral response leading to activation of interferons and chemokines e.g. RANTES (Honda et al., 2006, Honda and Taniguchi, 2006). As stated above, TLR-3 acts via TRIF, which interacts with TBK1. TBK1 phosphorylates IRF-3 leading to its activation and homodimerisation. Once it forms a homodimer it enters the nucleus and binds to ISRE and induces IFN- β and RANTES (McWhirter et al., 2004, Fitzgerald et al., 2003a). TLR4 activation causes IRF-3 homo-dimerisation, and also IRF3-p65 heterodimerization. The homodimer/heterodimer then activates a distinct set of genes (Wietek et al., 2003, Ogawa et al., 2005). TLR7/8 and 9 activate IRF-5 and IRF-7. They act through IRAK-1 and TRAF-6 and lead to production of IFN α (Uematsu et al., 2005, Kawai et al., 2004). Other members of the IRF family do not act directly in response to TLR activation. They are activated by interferons produced via viral induced TLR activation. IRFs induced indirectly and not through TLRs are involved in numerous inflammatory processes e.g. IRF-1 regulates caspase-1 and NOS-2, while IRF-4 is involved in the development of T, B and Dendritic cells whilst IRF-8 is involved in T-helper cell response (Honda and Taniguchi, 2006). IRFs are also activated through RIG-I helicases via RIP-1 kinase (Yoneyama et al., 2004, Kawai et al., 2005).

1.9 MAP Kinases

MAP Kinases are a family of serine/threonine kinases that are implicated in TLR signaling. MAPK-3 (ERK-1) is the first family member that was discovered in

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mammals and has a role in growth factor signaling. Since then, several MAP Kinases have been discovered and their role in immunology identified. All MAP Kinase family members have shared characteristics, for instance most require two phosphorylation events for activation. MAP Kinases are grouped into three families, 1) extracellular-signal-regulated kinases (ERKs), 2) Jun amino-terminal kinases (JNKs), and 3) stress-activated protein kinases (p38/SAPKs). MAPKs are activated via phosphorylation carried out by MAPK Kinases (MAPKK) at tyrosine and threonine (Kyriakis and Avruch, 2001). JNK family and p38 family participate in inflammation amongst other roles, whilst the ERK family is involved in cellular growth and differentiation and responds primarily to growth factors and mitogens (Morrison, 2012). The JNK family contains the TPY motif and includes JNK-1, JNK-2 and JNK-3. Signaling through JNK usually involves the Rho family GTPases Cdc-42 and Rac (Johnson and Nakamura, 2007). On the other hand the p38 family contains the TGY motif and comprise p38- α , p38- β , p38- γ , and p38- δ .

LPS activates all three members of the MAP Kinase family. ERK-1/2 proteins are activated via the Raf-1/MEK pathway (van der Bruggen et al., 1999), JNK proteins are activated via the MEKK-1/MEK-4 pathway (Delgado and Ganea, 2000) and p38 MAPKs are activated via MEK-3 (Prickett and Brautigan, 2007). MAP Kinases play diverse role in enhancing the immune response. For instance p38 is involved in stabilizing cytokine mRNAs that contain AU rich sequences and thereby enhancing their expression (Winzen et al., 1999). It also activates the nuclear factor activated of T-cells (NFAT) (Rao et al., 1997). JNK is involved in the activation of the

transcription factor activation protein (AP)-1, the main form of which comprises c-jun and c-fos (Karin et al., 1997).

1.10 Negative regulation of TLR signaling

Prolonged inflammation is detrimental and therefore it is essential to have mechanisms to bring the system back to homeostasis once the intruding pathogens have been dealt with. There are numerous feedback loops and regulatory mechanisms that participate in maintaining the system. Control processes occur at different parts of the inflammatory loop. The control could occur at the ligand-binding step, dimerization of TLRs, adapter binding and also active negative regulation of TLRs and their signaling components (Liew et al., 2005).

One example of a negative regulatory mechanism involves phosphorylation of Mal by Bruton's tyrosine kinase (Btk) (Gray et al., 2006, Piao et al., 2008). This leads to recruitment of suppressor of cytokine signaling-1 (SOCS-1), which mediates polyubiquitination of Mal that results in its degradation (Mansell et al., 2006). SH2-containing protein tyrosine phosphatase-1 (SHP-1) has been shown to bind to IRAK-1 and thereby inhibits its activity and blocks NF κ B and MAPK activation, however it also mediates type-1 interferon production (An et al., 2008).

Other negative regulatory mechanisms include molecules such as soluble TLR2 (sTLR2), which compete with extracellular TLR2 ligands (LeBouder et al., 2003).

Membrane bound proteins such as ST-2 and SIGIRR may also block TLR activity by binding TIR domains of MyD88 and MAL (Brint et al., 2004) and the TIR domain of TLR4 (Wald et al., 2003). A few intracellular proteins are also involved in negative regulation of the Toll pathway; examples include SARM and TRAM adaptor with GOLD domain (TAG), that block TRIF signaling (Carty et al., 2006, Palsson-McDermott et al., 2009), and IRAK-M that blocks IRAK-1 activity (Kobayashi et al., 2002). A number of the TLR signaling pathway components have splice variants that have been implicated as negative regulators. A short form of MyD88 exists, MyD88s, that does not contain the interdomain and it can bind to MyD88 and IRAK-1 but it is incapable of phosphorylating IRAK-1 and thereby the downstream NF κ B activation is inhibited (Burns et al., 2003). IRAK-2 has four splice variants (a,b,c and d) in mice, however these splice variants do not exist in humans (Hardy and O'Neill, 2004). One of the splice variants, IRAK-2c has been implicated in hyper responsiveness to TLR stimulation (Conner et al., 2009).

Additionally anti-inflammatory cytokines, such as IL-10, which will be a major focus of this thesis, are also produced by cells in response to TLR4 activation, which has an inhibiting effect on signaling such as NF κ B (Murray, 2006). The inflammatory response is therefore a very tightly regulated signaling process, where many cellular responses may have negative repercussions on the cell if not controlled correctly. Additionally NF κ B, mediates its negative regulation through self-mediated re-synthesis of its inhibitor I κ B (Scott et al., 1993) Figure 1.2 illustrates negative regulation of the TLR4 pathway.

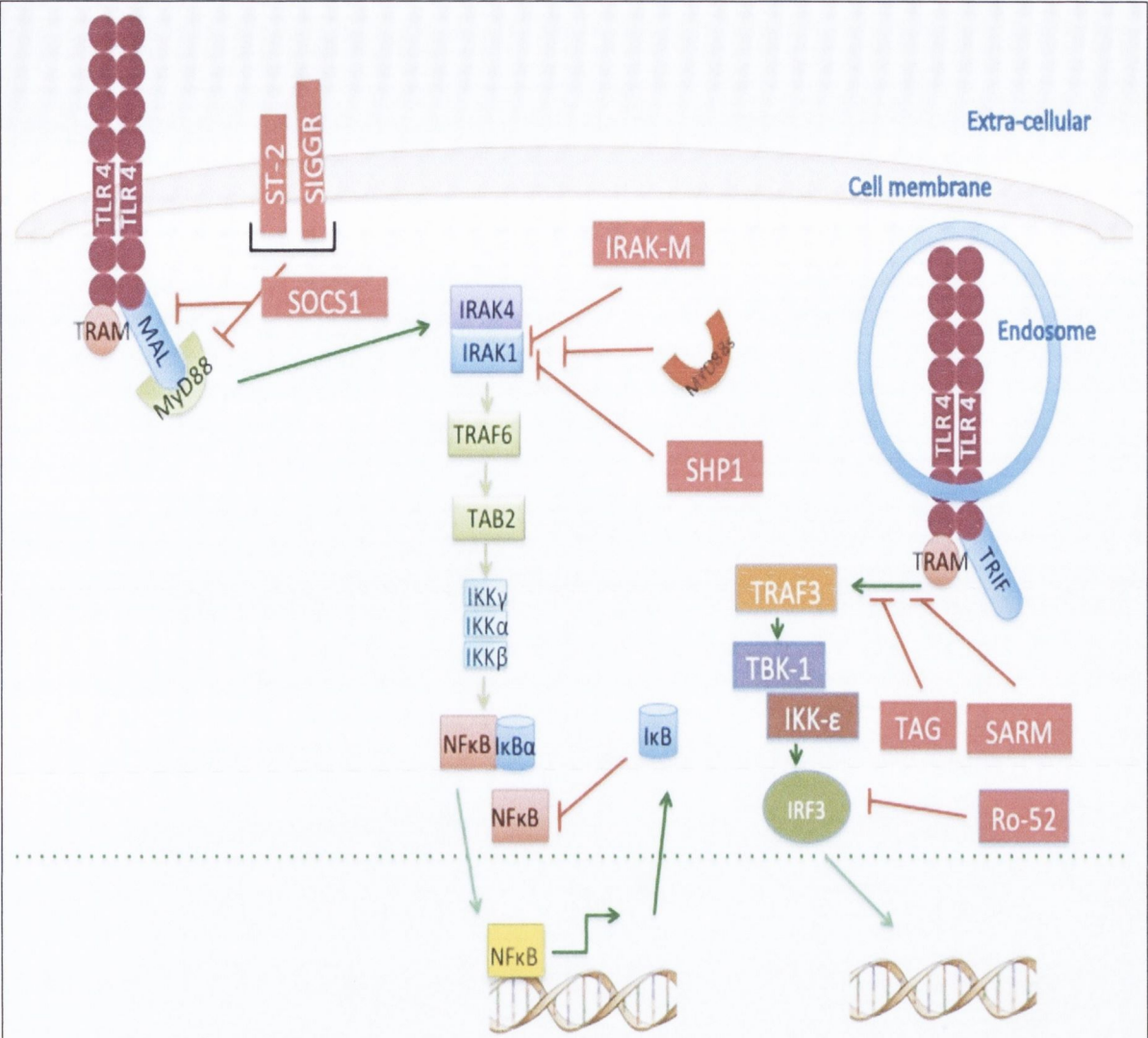


Figure 1.2 Negative regulation of TLR4 pathway

The TLR4 pathway activated in response to LPS leads to the activation of IRF-3 and NFκB. The pathway leading to activation is negatively regulated at number of stages, with some steps targeted by multiple repressors, whilst some interactors are not targeted at all. SOCS1, ST2 and SIGGR repress MyD88 and MAL dependent TLR4 signalling, whilst TAG and SARM repress TRIF-TRAM dependent signaling pathway. IRAK-M, SHP1 and MyD88s repress IRAK1 and thereby limit NFκB activation. NFκB negatively regulates its own activation by transcribing IκB that inhibits NFκB.

1.11 microRNA

microRNA (miRNA) have emerged as critical regulators of TLR signaling and are a focus of the current study. miRNA are small RNAs that are 21-22 nucleotides long non-coding sequences that are involved mostly in repression and degradation of target mRNAs. However, some miRNA participate in mRNA stabilization.

1.11.1 Discovery of miRNA

The first miRNA was discovered in *C. elegans* during a forward genetic screen. Lin-4 was shown to have a role in larva development and was observed to regulate the expression of LIN-14 gene (Lee et al., 1993). An up-regulation of lin-4 resulted in downregulation of LIN-14 protein (Lee et al., 1993, Wightman et al., 1993). Loss of function of lin-4 prevented larval development progression from stage 1 to stage 2. Analysis of the LIN-14 gene revealed multiple complimentary sites to the mature lin-4 miRNA at the 3'UTR of LIN-14. Lin-4 was concluded to be a small temporal RNA that was present in *C. elegans* and controlled developmental timing (Wightman et al., 1993, Lee et al., 1993).

Let-7, another small non-coding RNA, was identified a few years later in *C. elegans*. It also had a role in development and it controlled progression from larval stage to adult stage. It regulated expression of the LIN-41 transcription factor (Reinhart et al., 2000, Slack et al., 2000)

Identification of let-7 led to exploratory studies to identify small non-coding RNA in other species. Soon it was realized that mammals also had miRNAs (Pasquinelli et al., 2000). A number of studies were then conducted in *C. elegans*, *D. melanogaster*, *M. musculus*, and *H. sapiens* that identified hundreds of small 20-22 nt RNAs, termed miRNA (Lau et al., 2001, Lee and Ambros, 2001, Lagos-Quintana et al., 2001, Lagos-Quintana et al., 2002). These 21-22nt sequences were processed from 60-70nt precursor miRNA (pre-miR) that contained a hairpin loop. To date, more than a thousand miRs have been identified, which impact on all biological process.

1.11.2 miRNA processing

Mature miRNA are produced in four steps. The first step is transcription of a primary miRNA transcript, the second step is processing of this primary transcript to precursor transcript, the third step is export of pre-miR from nucleus to cytoplasm and the last step is precursor to mature miRNA processing in the cytosol. miRNA processing is illustrated in Figure 1.3 and explained below.

1.11.3 Transcription of primary miRNA transcript

Genes encoding miRNA can be found either within protein encoding genes or they can be present in their own independent islands within the genome. The miRNA produced from within genes are co-expressed with the gene present either in introns of mRNA or are produced via splicing of mRNA. Such miRNAs are under

control of promoter and regulatory elements controlling gene expression. However certain miRNAs located within genes can have their own regulatory elements.

miRNA can be transcribed from their own genes, for instance miR-155 which is found in the BIC gene (O'connell et al., 2007). Certain miRNA genes encode a whole cluster of miRNA, for instance the miR-25-95-100b cluster (Rodriguez et al., 2004).

There is evidence suggesting that miRNA, like mRNA, contain a 5' cap and a 3' poly-A tail (Cai et al., 2004). The majority of primary miRNA (pri-miRNA) transcripts are transcribed by RNA Pol II (Lee et al., 2004), however a few are also transcribed by RNA pol III e.g. miR-19 (Borchert et al., 2006).

1.11.4 Processing of primary miRNA transcript to precursor

The pri-miRNA contains a characteristic stem-loop structure. This stem-loop structure contains the precursor miRNA (pre-miR) and needs to be cleaved. A multi-protein complex called the Microprocessor is involved in causing this cleavage (Gregory et al., 2004). The Microprocessor contains two main proteins 1) Drosha – an RNase enzyme and 2) DiGeorge syndrome critical region-8 (DGCR8) – an RNA binding protein (Gregory et al., 2004, Denli et al., 2004, Han et al., 2004, Landthaler et al., 2004). DGCR8 recognizes the stem-loop structure along with its 5' and 3' flanking sequences in the pri-miRNA and thereby assists Drosha in cleavage at the exact sites. The two proteins coordinate the whole cleavage process by recognizing the structure and size rather than any particular sequences, and the cleavage occurs

at 11 base pairs from the single/double strand junction (Han et al., 2006). Following the production of pre-miRNA it is targeted for export.

1.11.5 Export of precursor miRNA: nucleus to cytosol

miRNAs, like any other cellular content are quality controlled. For the export from the nucleus to be successful, the length of the stem-loop and the 3' overhang are essential (Lund et al., 2004, Zeng and Cullen, 2004). The export is carried out by exportin-5 Ran-GTPase (Yi et al., 2003, Bohnsack et al., 2004). Exportin-5 is essential not only for nuclear export but also for miRNA stability in the nucleus (Zeng and Cullen, 2004, Yi et al., 2003). Once the pre-miR is in cytosol, it undergoes further processing to become mature miRNA.

1.11.6 Processing of precursor to mature miRNA

In the cytosol Pre-miR associates with RNA Induced Silencing Complex (RISC) loading complex (RLC). RLC is a multi-protein complex that contains a number of proteins that include: an RNase enzyme- Dicer (Bernstein et al., 2001, Hutvagner et al., 2001), TAR RNA binding protein (TRBP)(Haase et al., 2005), protein activator of PKR (PACT)(Lee et al., 2006), and Argonaute-2 (Ago-2) – the RNA silencing protein (Chendrimada et al., 2005).

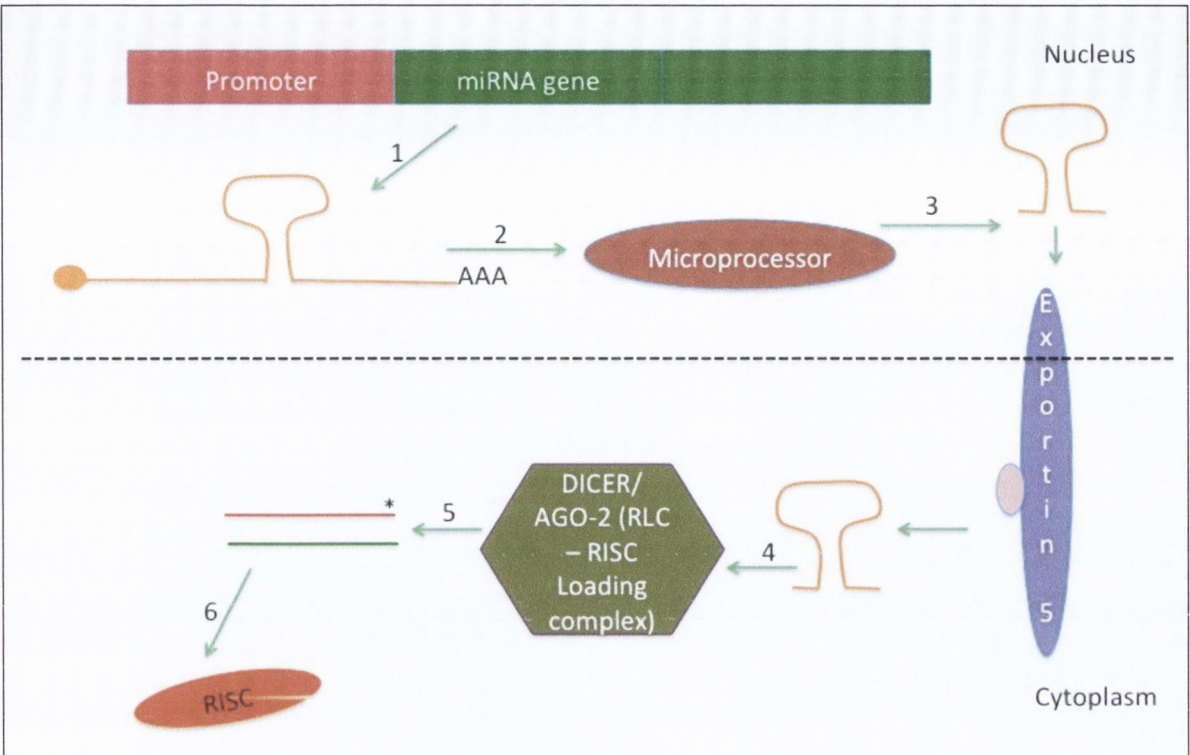


Figure 1.3 miRNA biogenesis There are 7 stages in the generation of mature miRNA. 1) RNA polymerase II is involved (although RNA polymerase III can also be involved in some cases) in transcription of primary-miRNA (pri-miRNA) transcript. miRNA can be transcribed from their own genes or they can be transcribed as part of another gene. The resulting transcript forms a stem loop structure because of the complementary sequences present. 2) The microprocessor is a nuclear riboendonuclease complex with Drosha and Pasha enzymes. This multiprotein complex recognizes hairpin structure present in pri-miRNA and cleaves out the hairpin structure from the long transcript. The hair-pin contain the mature miRNA. This results in the formation of premature-miRNA (pre-miRNA). 3) The pre-miRNA is then exported from nucleus to the cytoplasm with the help of Ran-GTP coupled exporter protein exportin-5. 4 & 5) In the cytoplasm the pre-miRNA is cleaved by the riboendonuclease Dicer, as part of a larger complex – the RLC – RISC Loading Complex. This cleavage event releases the mature miRNA plus its complementary anti-sense sequence, called the miRNA* sequence, as a miRNA dimer. 6) The mature miRNA sequence enters a pre-existing ribonucleoprotein complex, RNA interference silencing complex (RISC), which contains Argonaute protein. The RISC complex scans mRNA sequences to target their repression and degradation.

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RLC forms in an ATP-independent manner and following the formation of the complex, pre-miR is recruited and cleaved by Dicer (Maniataki and Mourelatos, 2005). Dicer cleaves the stem-loop, and forms a small double-stranded RNA complex that contains a mature miRNA strand and a complementary strand that is referred to as miR*. The miR* sequence is 2nt shorter at the 5' end and 2nt longer at 3' end, compared to mature miR sequence (Schwarz et al., 2003). The RNA duplex is unwound by RNA helicase; following this the single stranded RNA, that binds stronger at the 5' end to RISC, is loaded (Schwarz et al., 2003, Khvorova et al., 2003).

All proteins except for Ago-2 dissociate from the RLC, leaving mature miR and Ago-2 associated in the RISC complex (MacRae et al., 2008). The RISC complex comprising Ago-2 and mature miRNA can now target mRNA, which contain complementary sequences to the miRNA seed sequence.

1.11.7 miRNA mediated mRNA targeting

miRNAs contain a seed sequence that contains the complementary or close to complementary sequence to its target mRNA. The seed sequence is nt 2-7 from the 5' end of mature miRNA. miRNA binds to its target mRNA, mostly, at the 3'Untranslated region (UTR) although it can also bind at other parts of the mRNA. It has been suggested that complete complementarity leads to degradation whilst, partial complementarity leads to repression of the target mRNA (Zeng et al., 2003, Hutvagner and Zamore, 2002, Lai, 2002).

The RISC complex contains a number of proteins besides Ago-2. Proteins also found in the RISC complex include: Fragile-X related protein (FXRP), VIG and a nuclease Tudor SN (Caudy et al., 2002, Caudy et al., 2003, Ishizuka et al., 2002).

mRNA translation is very tightly regulated. Most cap-dependent gene translation is dependent on eukaryotic initiation factor 4 (eIF4) family members. These members associate at the 5'UTR of mRNA and recruit message to the ribosome. Ago-2 has been seen to compete with eIF4E and thereby prevent translation (Kiriakidou et al., 2007). Some studies have described miRNA-based mRNA decay and degradation. It involves removing the polyA tail of the mRNA with the help of the deadenylase complex CCR-4-NOT and the decapping enzymes DCP-1 and DCP-2. These are recruited to destabilize the mRNA, following which it is degraded (Bagga et al., 2005, Behm-Ansmant et al., 2006). Recently it was shown that miRNA-mediated repression is required first, following which eIF4A2 is recruited to 5'UTR and it interacts with CCR-4-NOT complex (Meijer et al., 2013). The presence of eIF4A2 at the 5'UTR prevents translation initiation factors eIF4A1 and eIF4G from binding, thereby preventing translation (Meijer et al., 2013).

1.11.8 miRNA target prediction

miRNA-mRNA targeting is based on the complementarity between the miRNA seed sequence and mRNA sequence. A number of databases (PicTar, TargetScan, miRBase, miRANDA etc) have emerged that predict targets for miRNA. These

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predicted targets are based on the seed sequence complementarity, number of seed sequences present (greater number of seed sequence = higher chances of being a target) and conservation of target site between different species. The databases, however, don't take into account the expression profiles of different miRNA, nor do they take into account cellular specificity of different miRNA. Therefore, although these predicted targets are a good starting point, they are usually very unreliable. Thus far it has been hard to predict miRNA targets using computational analysis only. The targets vary between different cell types, and under different conditions. A single miRNA can target the mRNA of numerous genes, and at the same time a number of miRNA can target the same mRNA (Reinhart et al., 2000, Lin et al., 2003). miRNA seem to regulate various different mRNA in different ways, for instance in some cases mRNA is only repressed and not degraded whilst in other cases mRNA are degraded.

A good starting point to confirm a miRNA-mediated gene repression is to look for an inverse relationship between increased level of miRNA followed decreased level of protein and following confirmed target prediction using seed-sequence complementarity. Once this observation has been made, a luciferase assay needs to be performed where a target miRNA needs to target the corresponding mRNA. In luciferase-based assays, luciferase activity is measured. The 3'UTR of a gene of interest is placed downstream of the luciferase construct. Following this a miRNA mimic is introduced, the miRNA mimic would bind the 3'UTR and block the gene expression and thereby decrease the luciferase activity. Furthermore through

miRNA mimic and miRNA knockdown an inverse relationship of miRNA needs to be demonstrated for the target protein. An increase in a miRNA through transfection with a miRNA mimic represses the putative gene, whilst a decrease in miRNA through knockdown will induces the putative gene.

1.11.9 miRNA in immunity and disease

miRNA have been implicated in inflammatory diseases, neurological disorders, genetic diseases and cancer. Our laboratory was one of the first, alongside others, that screened and functionally analyzed LPS-induced miRNA in macrophages. A number of studies have demonstrated the role of miRNA in the regulation of the NF κ B pathway e.g. miR-155, miR-21 and miR-146a (Alam and O'Neill, 2011, O'Neill et al., 2011).

miRNA have diverse roles in innate immunity, ranging from pro-inflammatory mediators to anti-inflammatory moderators and participants in anti-microbial activity. One of the most highly up-regulated miRNA in response to LPS in BMDM, miR-155, has been investigated in over 800 studies. miR-155 has turned out to be an important regulatory participant in numerous cellular processes.

miR-155 blocks the translation of SHIP-1, a negative regulator of TLR4, thereby allowing the TLR4-induced inflammatory response to occur, as evidenced by increased stabilization and production of TNF α (O'connell et al., 2009, Bala et al.,

2011). miR-155 in essence acts as a pro-inflammatory miRNA in LPS-treated BMDM. Another LPS inducible miRNA, miR-21 targets PDCD4 (Sheedy et al., 2010). PDCD4 sequesters eIF4A, preventing eIF4A from participating in the eIF4F complex (Yang et al., 2003a). eIF4A is a RNA helicase and it is involved in resolving the complex 5' untranslated region (UTR) structures found in mRNAs. Thus, the PDCD4-eIF4A interaction blocks cap-dependent translation. miR-21 induction in response to LPS decreases PDCD4 levels thus allowing eIF4A to enter the eIF4F complex thus lifting the brake on cap-dependent translation. miR-146a is also induced in response to LPS (Taganov et al., 2006). In this case the miRNA, miR-146a, acts as an anti-inflammatory mediator by reducing the levels of IRAK1 and TRAF6, two components of the NF κ B activation pathway. These three miRNA highlight the divergent roles of miRNA in inflammation. Certain miRNA, such as miR-155 induce inflammation, whereas miR-146a prevents inflammation. At the same time other miRNA, like miR-21, have more diverse roles, where their induction allows activation of translation machinery to produce proteins that can be either pro- or anti-inflammatory mediators.

Most studies on the role of miRNA in innate immunity have focused on LPS-inducible miRNA. The role of miRNA that are repressed in response to LPS has been less well-characterised. Even though a few studies have identified miRNA that are repressed in response to LPS, this has not been the main focus of these studies. miR-107, miR-125b and let-7i have been described as LPS-repressed miRNA. These studies have uncovered critical roles that these miRNA play in BMDM biology. miR-

107 blocks CDK6 translation and thereby prevents macrophage adhesion, this repression is lifted in LPS-stimulated macrophage. Let-7i blocks TLR4 protein synthesis, and miR-125b blocks TNF α . These brakes are lifted in response to LPS. Figure 1.4 illustrates the miRNA-mediated responses in LPS-induced macrophage.

1.12 Translational regulation in TLR signalling

Another area that has received attention in the mechanism of action of TLRs has been translational control. The observation that miR-21 can promote PDCD4 depletion in response to TLR4 action and thereby release eIF4A to participate in translation is an example. Mammalian cells have evolved elaborate mechanisms for translational control most of which are sensitive to nutrient availability, cellular energy, stress, hormones and growth factor stimuli. Translational control is an important mechanism for protein abundance and numerous studies have found that mRNA is sometimes a poor indicator of the corresponding protein levels and hence functionally irrelevant (Anderson and Seilhamer, 1997, Futcher et al., 1999, Gygi et al., 1999, Tew et al., 1996, Zong et al., 1999). Two different transcripts, present in cytoplasm in identical quantities are at times translated at very different rates (Sonenberg N et al., 2000).

Translation of mRNA into its cognate protein is accomplished in three successive steps controlled by a wide range of regulatory factors. Translation in eukaryotes involves three stages, 1) initiation, 2) elongation and 3) termination. The translation

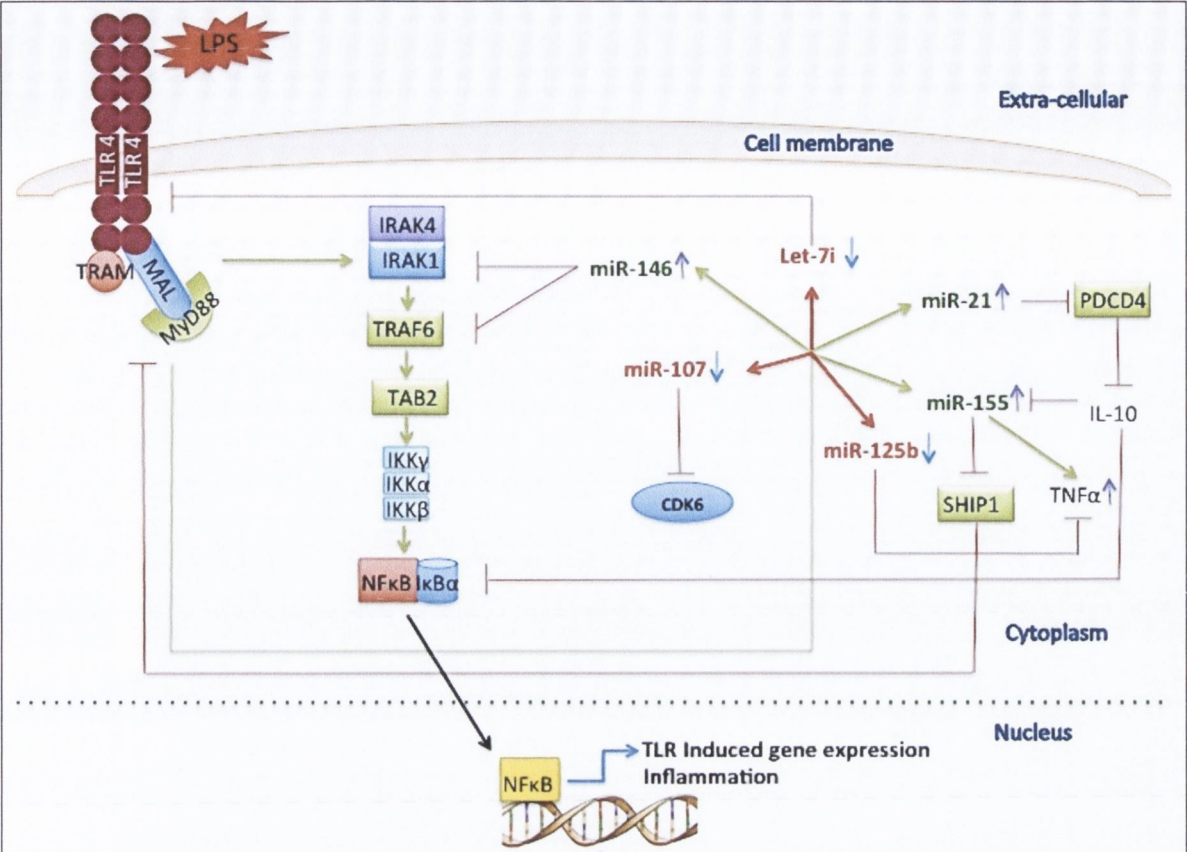


Figure 1.4. Diverse role of miRNA in macrophages.

LPS activates TLR4 which initiates a cascade of events resulting in the activation of NFκB. Upon LPS stimulation various miRNA are also induced (and some are repressed) that participate along the NFκB activation pathway. miR-146 blocks IRAK1 and TRAF6, miR-21 blocks PDCD4 whilst, miR-155, a pro-inflammatory miRNA, induces TNFα (by mRNA stabalization) which further activates macrophages and blocks SHIP1. SHIP1 negatively regulates TLR-induced responses. IL-10 is an anti-inflammatory cytokine, blocked by PDCD4. Upon miR-21 induction PDCD4 is blocked and IL-10 blocks miR-155 and NFκB. Hence a negative regulatory loop forms. LPS-repressed miRNA, miR-125b, blocks TNFα, miR-107 blocks CDK6 and let-7i repress TLR4 protein translation. Overall the figure illustrates how miRNA work together to induce inflammation and bring system back to homeostasis- post inflammation.

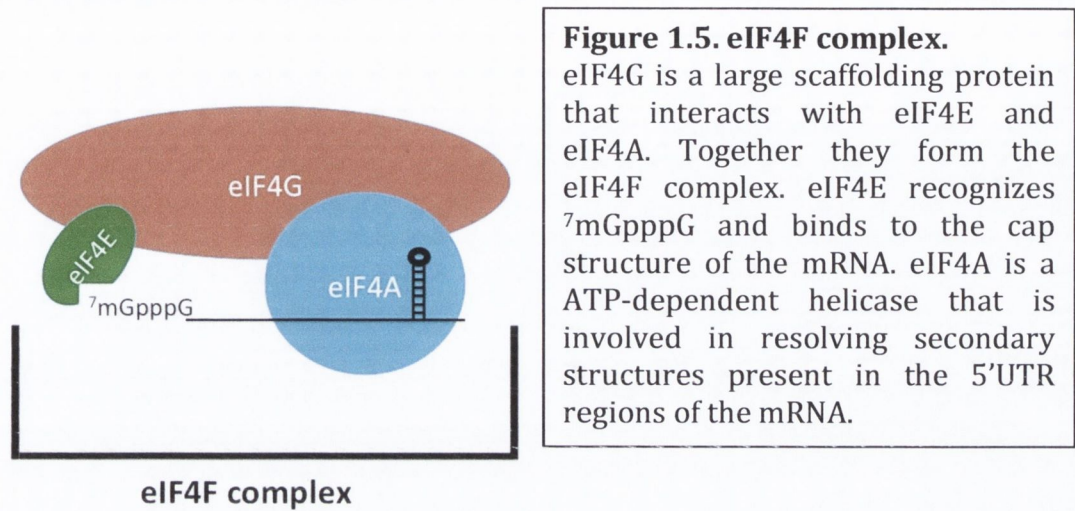
Key: → Repressed; → Induced; - Blocked

initiation step is highly regulated, especially because it is the rate-limiting step (Sonenberg and Hinnebusch, 2009). Broadly speaking, translation starts when large (60S) and small (40S) ribosomal subunits come together into an 80S elongation-competent ribosome where the methionyl-tRNA (Met-tRNA_i) is correctly positioned over AUG in the mRNA to initiate translation. The entire process involves 12 initiation factors (eIFs) (Jackson et al., 2010a, Hinnebusch, 2011). The process starts with the formation of the 43S pre-initiation complex (PIC) involving Met-tRNA_i, eIF2 and GTP along with eIF-1, 1A, 3, 5 and the 40S subunit. Following this the PIC is translocated to the 7-methylguanosine cap structure of mRNA. This translocation is facilitated by a number of factors: eIF3, poly(A)-binding protein (PABP), eIF4B and the eIF4F complex.

1.12.1 Cap-dependent translation

As mentioned above, the rate-limiting step of protein synthesis is translation initiation, during which the small ribosomal subunit is recruited to the 5' end of mRNA and scanning for start codon begins (Jackson et al., 2010b). For the recruitment of the small ribosomal subunit with various different factors of PIC to mRNA, the assembly of the eukaryotic translation initiation factor 4F (eIF4F) complex is required in the mRNA cap (Jackson et al., 2010b). eIF4F consists of a number of proteins that are involved in mRNA recruitment, ribosomal binding, RNA secondary structure unwinding and recognition of the start codon (Jackson et al.,

2010b). Principally eIF4F is comprised of a large subunit protein, eIF4G along with eIF4E and eIF4A. This is shown in Figure 1.5.



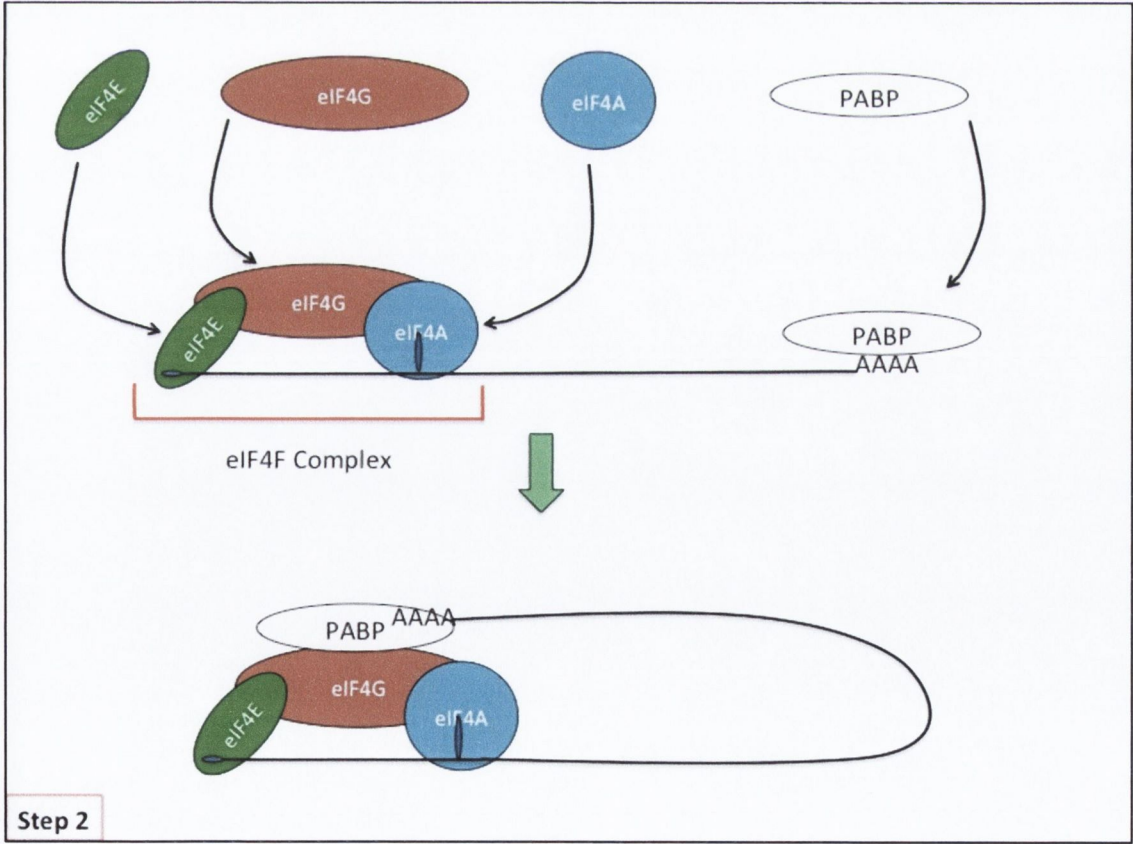
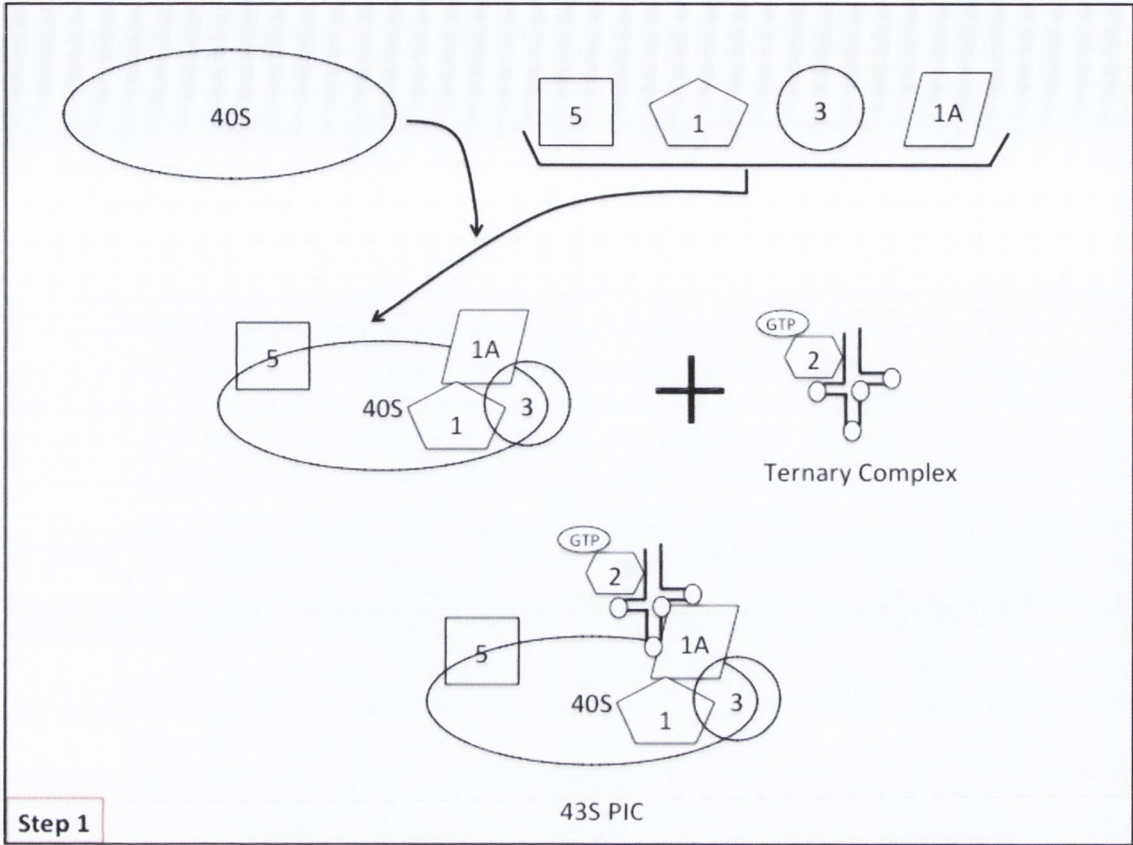
This complex is involved in recruitment of mRNA and its translocation to the ribosome. During mRNA recruitment, eIF4E recognizes and binds to the mRNA cap-structure, and following this eIF4G and eIF4E interact (Jackson et al., 2010b). This interaction allows the eIF4F complex to come in association with PIC (Jackson et al., 2010b). Two RNA binding proteins, eIF4B and eIF4H work in a mutually exclusive manner to interact with eIF4A (Jackson et al., 2010b). This interaction enhances eIF4A activity and together the proteins join the PIC complex at this stage. Poly-A-tail binding proteins (PABP) binds to the poly A tail of mRNA, which allows the mRNA to be circularized by interactions between eIF4G and PABP (Jackson et al., 2010b).

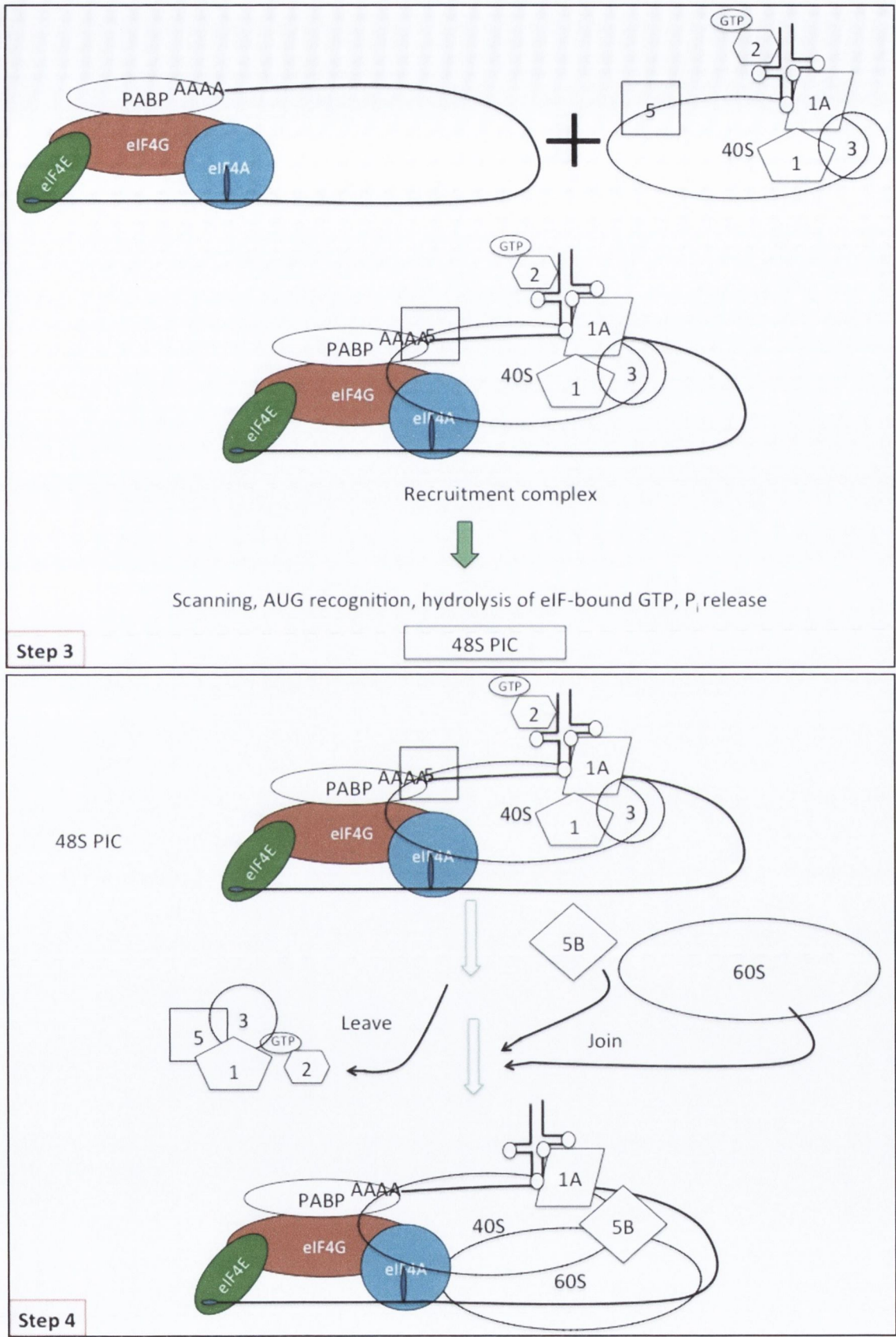
The PIC complex is involved in scanning the mRNA in the 5' to 3' direction (Jackson et al., 2010b). It stops wherever it encounters a secondary structure, following

which eIF4A and its accessory proteins unwind the secondary structures in mRNA (Jackson et al., 2010b). The scanning continues until the start codon, AUG, is reached. Once the start codon is reached the PIC complex forms the 48S PIC (Jackson et al., 2010b). As soon as the 48S complex is formed, the 60S ribosomal subunit is recruited with the help of eIF5B-GTP and this leads to the formation of the 80S complex. While 80S is being formed, eIF2-GDP, eIF1, eIF3, eIF4A, eIF4B, eIF4G, eIF4H and eIF5 leave the complex. Following this, GTP is hydrolyzed by eIF5B; causing eIF1A and eIF5B to leave the 80S complex. Translation initiation thereby begins and now the complex is ready for elongation factors to enter the complex. This entire process is depicted in Figure 1.6.

1.12.2 eIF4F complex

eIF4F complex is involved in the recruitment of mRNA recruitment to the 43S PIC to initiate translation. A number of eIF4F complex members are basally present however they are kept in complexes preventing them from initiating mRNA recruitment. eIF4E-BP binds to eIF4E whilst PDCD4 binds to eIF4A, thereby the two participants of eIF4F complex are blocked from interacting with eIF4G and hence the active eIF4F complex does not form. The activation of the complex is mTOR-dependent, which regulates cap-dependent translation, discussed later in section 1.13.





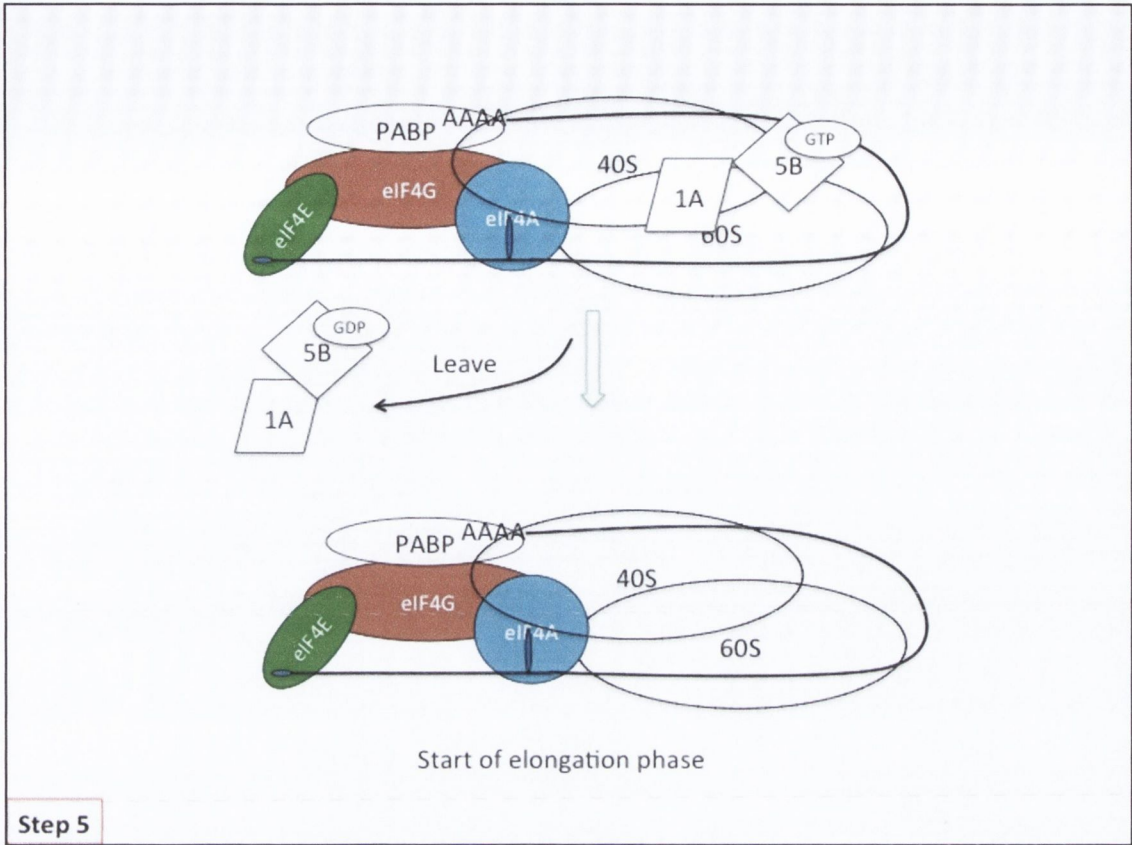


Figure 1.6 Translation initiation.
Step1: The small ribosomal subunit, 40S, and eIF-1, 1A, 3 and 5 become associated. Following this the ternary complex is formed, which is composed of the initiator Met-tRNA_i, eIF-2, and GTP. The two complexes interact. Together they form the 43S pre-initiation complex (PIC).
Step2: The mRNA is recruited with the help of eIF4F complex and PABP proteins. eIF4E binds to the cap and interacts with eIF4G, following which eIF4A is recruited and the eIF4F complex forms. PABP and eIF4G interact to circularize the mRNA.
Step3: mRNA is brought to the 43S PIC through interaction between PABP, eIF4G and 40S-bound eIF-3. Following mRNA recruitment by the recruitment complex scanning for the start site begins from 5' to 3' until a start site is identified. In the process GTP is hydrolysed and this results in the formation of 48S PIC.
Step4: Next, the large ribosomal subunit 60S is recruited to the 48S PIC. The recruitment of 60S involved eIF5B and the release of eIF-1, 2-GTP, 3, and 5.
Step5: Once the 60S subunit joins the complex, eIF1A and eIF5B-GDP are released. The translation initiation step is now complete and elongation factors now join in to start the elongation phase of translation.

KEY: 1:EIF1, 1A:EIF1A, 3: EIF3, 5: EIF5, 2:EIF2, 40S: Small ribosomal subunit, PABP: PolyA-binding-protein, 5B: eIF5B, 60S: Large ribosomal subunit.

1.12.3 eIF4G

eIF4G is a large scaffolding protein of the cap-binding complex eIF4F (Figure 1.7). It has binding sites for eIF4E, eIF4A and PABP. Mammalian eIF4G has two sites for eIF4A binding and each of the two sites consists of five anti-parallel hairpin repeats (HEAT repeats). Furthermore the C terminus of eIF4G binds to the MNK1 protein kinase. MNK1 is involved in eIF4E phosphorylation. Mammalian eIF4G also binds to eIF3 (Lamphear et al., 1995). eIF3 is a large complex containing a number of subunits that interact with the 40S ribosomal subunit (*Sonenberg et al., 2000*). eIF4G is therefore essential for the binding of mRNA to the small ribosomal subunit.

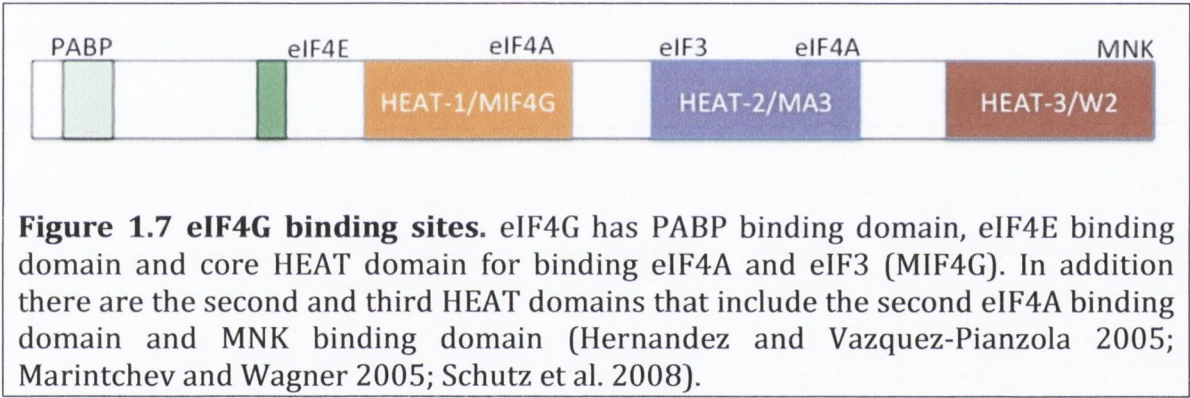


Figure 1.7 eIF4G binding sites. eIF4G has PABP binding domain, eIF4E binding domain and core HEAT domain for binding eIF4A and eIF3 (MIF4G). In addition there are the second and third HEAT domains that include the second eIF4A binding domain and MNK binding domain (Hernandez and Vazquez-Pianzola 2005; Marintchev and Wagner 2005; Schutz et al. 2008).

1.12.4 eIF4E

eIF4E binds to the mRNA cap and its role in cap-dependent translation is very crucial. PI3K/AKT/mTOR pathway regulates eIF4E in response a number of factors, including growth factors, mitogens, hormones, nutrients (amino acids, glucose). Signal transduction pathways activated by growth factors and stress result in activation of the Ras/Raf/ MAP kinase pathway. Activation of Ras and MEKK leads to a cascade of events such as JNKK and MAPK activation which culminates in Mnk activation. Mnk phosphorylates eIF4E within the eIF4F complex and permits the

recruitment of the ribosome to an mRNA at the initiation codon. This is shown in Figure 1.9 along with mTORC1 activation. Three proteins have been identified in mammals that repress eIF4E, eIF4E-BP1, BP2 and BP3 (Haghighat et al., 1995, Mader et al., 1995, Marcotrigiano et al., 1999). eIF4E interacts with eIF4E-BP via a site that can also be bound by eIF4G. Interaction with eIF4E-BP prevents eIF4E from interacting with eIF4G and thereby restricts active initiation complex formation. Hence 4E-BPs act as repressors for cap-dependent translation. The interaction between eIF4E and eIF4E-BP depends on the phosphorylation status of eIF4E-BP. eIF4E-BP1 has over half a dozen sites that can get phosphorylated, four of these sites are known to be regulated via the Akt/PKB and mTORC1 signaling pathway (Sonenberg and Gingras, 1998, Gingras et al., 2001a, Wang et al., 2005b). mTORC1 phosphorylates eIF4E inhibitor eIF4E-BP (Hay and Sonenberg, 2004a) (Gingras et al., 2001c). Hyperphosphorylated eIF4E-BP allows eIF4E-BP to dissociate from eIF4E and thereby allows eIF4E to interact with eIF4G. Following this interaction eIF4A is recruited to the 5' end of mRNA. Activation of mTORC1 resulting in eIF4E-BP dissociating from eIF4E will be discussed in detail in section 1.14.

1.12.5 eIF4A

eIF4A is the most abundant translation initiation factor present in cells and it is conserved from prokaryotes to eukaryotes (Duncan and Hershey, 1983, Conroy et al., 1990). As described above, eIF4A forms part of the eIF4F complex and it is an RNA-dependent ATPase DEAD-box helicase that functions to unwind secondary and

tertiary formations in the 5' end of mRNAs. RNA Helicases are a subset of a group of enzymes that have ATP hydrolysis activity and are central to all cellular processes involving RNA. Helicases are grouped into a set of superfamilies on the basis of conserved motifs (Gorbalenya and Koonin, 1993). The majority of helicases belong to either DEAD or DExH box families within the superfamily 2 of helicases. DEAD-box helicases form the largest sub-group of helicases (Gorbalenya et al., 1988, Hodgman, 1988). There are three large superfamilies termed SF1 – 3 and these are based on sequence analysis of conserved motifs (Gorbalenya et al., 1988, Hodgman, 1988).

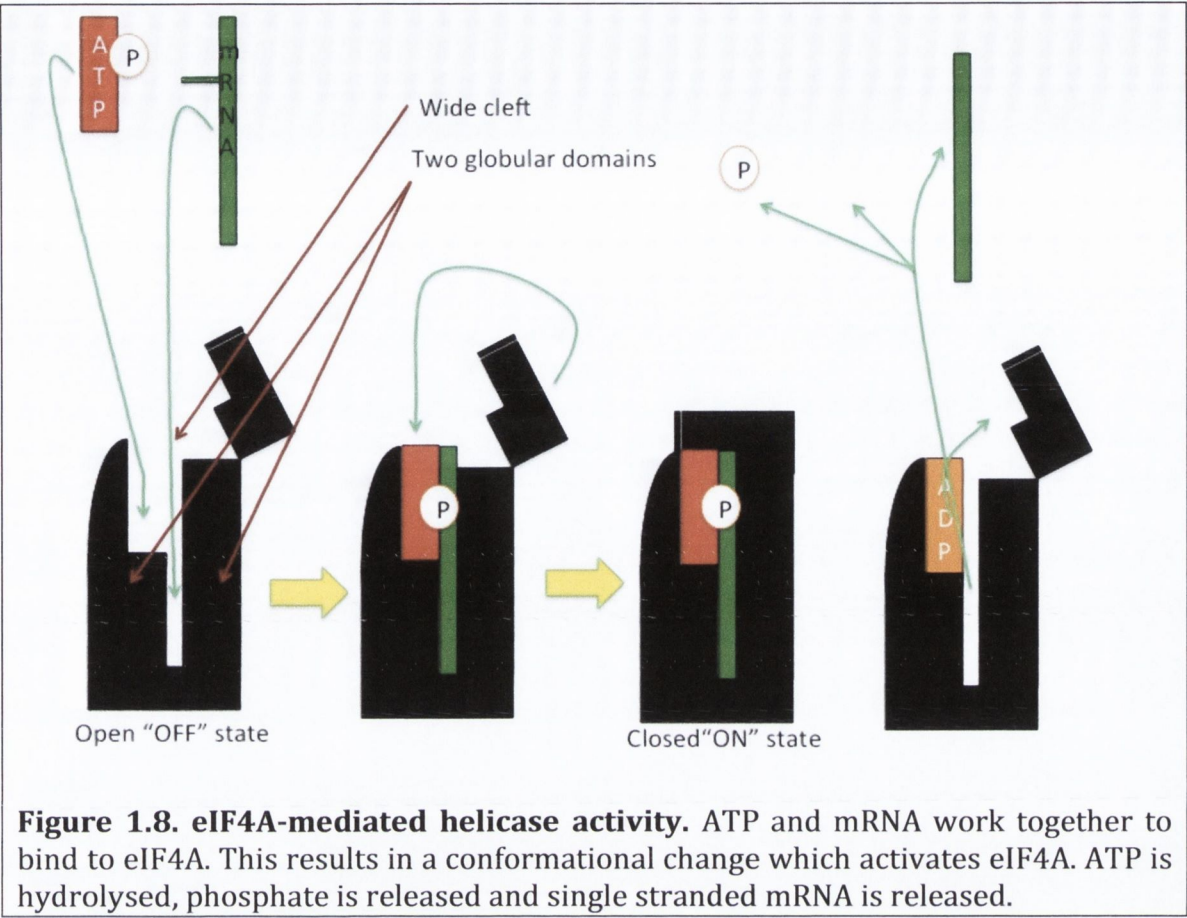
DEAD-box helicases are highly conserved within different species (Abdelhaleem et al., 2003) and their roles are critical in development, cellular differentiation, infections and disease. Helicases have been implicated in numerous cellular roles that include RNA metabolism, nuclear transcription, pre-mRNA splicing, ribosome biogenesis, nucleocytoplasmic transport, and RNA decay but most importantly in translational control with regards to eIF4A (Abdelhaleem et al., 2003, Abdelhaleem, 2005).

The exact mechanism by which DEAD-box helicases unwind RNA is still not fully understood. However the current understanding is that DEAD-box proteins are present in an open, “off” –state when RNA and ATP are not present. The “off” state is identified by the wide cleft found in between the two globular domains in the helicase core of DEAD-box helicases. ATP and mRNA work together to form a closed

structure in the inner-domain cleft that allows formation of the hydrolysis site for ATP and the attachment site for RNA (Sengoku et al., 2006, Theissen et al., 2008). Binding of RNA results in a bend that disrupts the canonical RNA A-form helix (Sengoku et al., 2006), thereby the local duplex gets destabilized. ATP hydrolysis occurs and after release of phosphate the helicase returns to an open state thereby releasing the RNA. This is depicted in Figure 1.8.

eIF4A works in association with eIF4B or eIF4H (RNA-binding protein), as demonstrated by *in-vitro* studies (Grifo et al., 1982, Rozen et al., 1990). eIF4A has a number of binding partners, some of which are activators whilst others have more repressive roles. These include PDCD4, eIF4G, eIF4H and eIF4A3-metastatic lymph node 51 (MLN51)-MAGOH-Y14 (Marintchev et al., 2009, Chang et al., 2009, Oberer et al., 2005, Andersen et al., 2006). As stated above basally PDCD4 acts as a repressor of eIF4A activity, PDCD4 is degraded by mTOR activation.

There are two RecA-like domains present in eIF4A proteins along with flexible central hinge regions that allow them to switch conformations from open to closed state via ATP hydrolysis. A total of nine conserved motifs are present in all eIF4A family members, Q, I, Ia, Ib, II, III, IV, V and VI (Cordin et al., 2006). Ia, Ib, IV and V are involved in RNA binding whilst I, II and III participate in RNA-dependent ATPase activity (Svitkin et al., 2001, Pause and Sonenberg, 1992). One motif, however, VI is essential for both RNA binding and ATP hydrolysis (Pause and Sonenberg, 1992, Pause et al., 1993). Functions of different motifs of eIF4A are illustrated in Table 1.1.



Binding of ATP to eIF4A-eIF4G complex allows eIF4A to adopt a closed conformation (Marintchev et al., 2009, Pause et al., 1993) following which ATP gets hydrolysed and eIF4A returns to open conformation that limits eIF4A affinity for RNA. This process repeats itself and thereby allows 5'UTR of an mRNA to be unwound.

One way to induce translational inhibition via eIF4A is to inactivate ATP binding or ATPase activity. This can be achieved via small-molecule inhibitors such as Hippuristanol, Silvestrol or Pateamine A or by bacterial toxin (*Burkholderia* lethal factor 1) (Cruz-Migoni et al., 2011, Bordeleau et al., 2006a, Bordeleau et al., 2006d)

There are three members of eIF4A family of proteins, eIF4A1, eIF4A2, and eIF4A3. Structurally eIF4A1 and eIF4A2 share 95% homology whilst eIF4A3 is only 67% related (Rogers et al., 2002, Chan et al., 2004b). The two isoforms, eIF4A1 and eIF4A2 appear to have the same biological activity *in-vitro* and are functionally indistinguishable (Rogers et al., 2002). However, recently it was shown that eIF4A2 activity is critically important for miRNA-based repression (Meijer et al., 2013). On the other hand eIF4A3 plays no role in protein translation (Li et al., 1999); it is involved in exon junction complex formation after mRNA splicing (Chan et al., 2004b).

Name	Function
Q	Involved in adenine base selection of ATP and required for ssRNA binding and conformational changes due to ATP binding and hydrolysis.
I	Walker A motif. Crucial for ATPase activity of the helicase.
Ia	Binds to RNA.
Ib	Binds to RNA.
II	Walker B motif. Crucial for ATP binding and hydrolysis.
III	Interdomain interaction with motif II and VI.
IV	Essential for the ATP-dependent cooperative binding of RNA.
V	Interacts to ATP and RNA.
VI	Interacts with α , β and γ phosphates of ATP and interdomain interaction with domain II

Table 1.1. Different motifs of eIF4A and their functional roles.

1.12.6 eIF4A1 and eIF4A2

Up until few years ago it was understood that eIF4A1 and eIF4A2 have indistinguishable roles in translation. However growing evidence is pointing towards differential roles for the two highly related proteins. *In vivo* experiments have showed that eIF4A1 and eIF4A2 are capable of binding eIF4G in 1:1 molar ratio however m⁷GTP Sepharose pull-down assays yielded more eIF4A1 than eIF4A2 suggesting that eIF4A1 is the preferred binding partner within the eIF4F complex (Li et al., 2001, Galicia-Vazquez et al., 2012). Furthermore, recently it was shown that eIF4A2 preferentially binds to cNOT7 (a member of CCR4-NOT complex-participates in miRNA based repression), whilst eIF4A1 binds to eIF4G (Galicia-Vazquez et al., 2012, Meijer et al., 2013). In addition siRNA-mediated knock-down of eIF4A1, but not eIF4A2, leads to altered global mRNA distribution in polysomes along with a decrease in [³⁵S]methionine incorporation (Galicia-Vazquez et al., 2012). In fact eIF4A1, but not eIF4A2 knockdown was detrimental to cell line growth (Galicia-Vazquez et al., 2012). Taken together this suggests that eIF4A1 and eIF4A2 might have some overlapping activities due to highly similar structures, but functionally they seem distinct (Bono et al., 2006).

1.12.7 eIF4A3

eIF4A3, unlike eIF4A1 and eIF4A2 is located in the nucleus and binds to the MAGOH-Y14 dimer and with MLN51 it participates as a key component of the Exon Junction Complex (Chan et al., 2004a). eIF4A3 binds to ssRNA in an ATP-dependent

manner and it has been proposed that eIF4A3 interaction with the MAGOH-Y14 dimer either locks eIF4A3 in a closed pre-ATP-hydrolysis conformation or serves to inhibit ATP hydrolysis which prevents eIF4A3 from dissociating from ssRNA (Ballut et al., 2005, Andersen et al., 2006). This results in formation of a tight Exon Junction Complex (EJC). It has been suggested that, upon dissociation of the MAGOH-Y14 complex from eIF4A3, ATP is hydrolysed which returns eIF4A3 into an open conformation and RNA is released (Bono et al., 2006).

1.12.8 Inhibitors of eIF4A

1.12.8.1 PDCD4

As stated above PDCD4 is a key negative regulator of eIF4A. PDCD4 was initially identified as having a role in apoptosis (Shibahara et al., 1995) and was seen mostly as a tumor suppressor (Yang et al., 2003b). Its role in translation inhibition, by repressing eIF4A activity, was subsequently defined (Goke et al., 2002, Yang et al., 2003a). In addition it has also been found that PDCD4 can bind to eIF4G (Kang et al., 2002) and thereby prevent translation by competing with eIF4G for binding eIF4A, or inhibiting eIF4A's helicase activity or both.

PDCD4 contains two MA3 domains, MA3-m which is in the middle and MA3-c which is in its C-terminus. It binds to eIF4A through these two domains and blocks the domain closure and RNA binding cleft of eIF4A (Goke et al., 2002, Chang et al., 2009).

PDCD4 expression is regulated through various different pathways, including mTORC1 that limits the amount of protein available through ubiquitination and proteasomal depletion (Dorrello et al., 2006b). S6K1 occurs downstream of mTORC1 and is directly involved in promoting the degradation of PDCD4. PDCD4 contains a canonical β TRCP-binding motif and has been shown to interact with proteolytic F-box protein β TRCP (β TRCP1 and β TRCP2) that mediates its proteolysis (Dorrello et al., 2006b).

The role of PDCD4 in inflammation is becoming clearer. In LPS-treated macrophage PDCD4 is degraded via miR-21 which allows translation of certain LPS-response genes, notably that encoding IL-10 (Sheedy et al., 2010). Furthermore, *PDCD4* knockout mice are resistant to the induction of inflammatory disease models and overexpress the anti-inflammatory cytokines IL-10 and IL-4 in a translation-dependent manner (Hilliard et al., 2006)(Sheedy *et al.*, 2010). Interestingly, the linoleic acid metabolite, ($\pm$)-13-hydroxy-10-oxo-trans-11-octadecenoic acid (13-HOA), suppresses the expression of pro-inflammatory genes but also induces the expression of *PDCD4* (Murakami et al., 2005, Yasuda et al., 2009). In addition PDCD4 represses c-jun activating MAP4K1 and hence AP-1 mediated transcription (Yang et al., 2006).

Degradation of PDCD4 is necessary for efficient protein translation, especially in mitogen-stimulated cells. It is a pre-requisite for cell proliferation and cell growth.

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Furthermore, PDCD4 is lost in certain aggressive human carcinomas and its expression inhibits transformation of cultured cells and in mouse tumorigenesis model (Palamarchuk et al., 2005, Jansen et al., 2005).

1.12.8.2 BC1

eIF4A has a specific neuronal regulator called BC1. BC1 is a long non-protein-coding dendritic RNA that is present in dendrites and enriched at the synapses (Muslimov et al., 1997, Muslimov et al., 2006, Tiedge et al., 1991, Chicurel et al., 1993). BC1 is has a homologue in mouse called BC200 (Tiedge et al., 1993). BC1/200 suppresses translation initiation by targeting the activity of eIF4AI and PABP (Wang et al., 2002, Kondrashov et al., 2005, Wang et al., 2005a). BC1/200 is able to bind to eIF4AI and prevent its helicase activity whilst stimulating ATPase activity, thereby uncoupling these two processes (Lin et al., 2008). In this manner BC1/200 is able to inhibit eIF4AI dependent translation initiation.

1.12.8.3 Pateamine A

There are three well-characterized inhibitors of eIF4A, Pateamine A, Silvestrol and Hippuristanol. Pateamine A is an anti-proliferative and pro-apoptotic marine product isolated from the marine sponge *Mycale* sp (Northcote et al., 1991, Hood et al., 2001). Pateamine A appears to exert its cellular effects by binding and stimulating eIF4A activity (Bordeleau et al., 2005, Low et al., 2005, Low et al., 2007, Bordeleau et al., 2006b). Pateamine A has been shown to be able to exert inhibition

on certain mRNAs whilst stimulate translation of others (Di Marco et al., 2012). Low *et al.* observed that Pateamine A inhibited the association of eIF4A with eIF4F but enhanced its binding to eIF4B and that both of these proteins isolated to stress granules after treatment (Low *et al.*, 2005). The increased interaction between eIF4AI and eIF4B was later found to be an indirect effect of Pateamine A inducing the stable binding of RNA by eIF4AI (Bordeleau *et al.*, 2006), which was proposed to sequester it from the initiation complex.

1.12.8.4 Silvesterol

Silvesterol comes from *Aglaia silvestris* and has anti-proliferative activity (Hwang et al., 2004). It inhibits translation by increasing eIF4A affinity for RNA and represses eIF4F complex formation thereby reducing ribosomal recruitment (Bordeleau et al., 2008, Cencic et al., 2009). Silvestrol has been shown to work in synergy with the standard cytotoxic reagents (daunorubicin, etoposide, cytarabine) used for the treatment of acute myeloid leukemia (AML) and could therefore be potentially used as an adjuvant therapy in the treatment of malignancies (Cencic et al., 2010).

1.12.8.4 Hippuristanol

Hippuristanol is a polyoxygenated steroid that comes from coral *Isis Hippuris* (Shen et al., 2001). It is a specific inhibitor of eIF4A and represses its activity by blocking ATPase activity, RNA binding and helicase activity in a dose dependent manner (Bordeleau et al., 2006c). Hippuristanol interacts with the CTD of eIF4AI and

inhibits its ATPase activity, its RNA binding activity and its helicase activity in a dose dependent manner

1.13 mTOR and translation

Mammalian target of rapamycin (mTOR) is a central player in regulating cellular protein translation, specifically cap-dependent translation. The mTOR-inhibitors repress cap-dependent translation. In mammals TOR occurs in at least two different complexes, mTORC1 and mTORC2. mTORC1 is rapamycin-sensitive and complexes with Raptor (regulatory associated protein of mTOR) and mTORC2 is rapamycin-insensitive and complexes with Rictor (rapamycin insensitive companion of mTOR) (Sarbasov et al., 2005). Both complexes bind to GβL. Raptor is the major component of mTORC1. It determines the specificity of mTORC1 by interacting with substrates containing the TOR signaling motif (Schalm and Blenis, 2002, Schaim et al., 2003). Raptor and GβL promote the activation of the ribosomal 70kDa protein S6 kinase (S6K).

mTORC1 is activated in response to a number of stimuli (Growth factors, mitogens, hormones and nutrients (amino acids and glucose)) and it is directly involved in the regulation of protein synthesis in mammals. (Jacinto and Hall, 2003). In addition a number of pathways are involved in activation of mTORC1. Phosphatidylinositide 3-kinase (PI3K), protein kinase B (PKB) and Ras/Raf/extracellular signal-regulated kinase (ERK) pathway are involved in activating mTORC1 complex (Corradetti and Guan, 2006).

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Growth factors and related hormones activated Tyrosine kinase-coupled (Tyr) and G protein-coupled receptors (GPCR), Tyr and GPCR activate PI3K-AKT and Ras-ERK pathways. Activation of PI3K-AKT and Ras-ERK pathways repress tuberous sclerosis complex (TSC) -1 and TSC-2 (Shaw and Cantley, 2006, Hay and Sonenberg, 2004b). TSC-1 and TSC-2 are negative regulators of mTORC1. TSC-2 has GTPase-activating protein (GAP) activity towards Ras homologue enriched in brain (RHEB) (a GTP-bound form of the small G protein). TSC-2 inactivates RHEB and therefore has an effect on mTORC1 (Manning and Cantley, 2003, Kwiatkowski and Manning, 2005). RHEB stimulates the phosphotransferase activity of mTORC1. In summary, activation of Tyr and GPCR lead to mTORC1 activation this is illustrated in Figure 1.9.

Activation of PI3K initiates a cascade of events which results in PDK1-mediated AKT activation. AKT phosphorylates TSC-2. TSC-2 phosphorylation makes the TSC-1/TSC-2 complex unstable and thereby inactivates it. So, RHEB is no longer inhibited by the GAP activity of TSC-2.

A number of other stimuli are also known to regulate mTORC1, for example LPS, insulin and cellular energy status (ATP:ADP ratio). LPS activates PI3K, which is involved in mTORC1 activation (Salh et al., 1998). Insulin can activate PKB/AKT, which inactivates TSC-2 by phosphorylation. In response to increased AMP or ADP levels, AMP-activated protein kinase (AMPK) phosphorylates TSC-2 at a different

site from PKB. This phosphorylation event activates TSC-2 and inactivates mTORC1 (Hardie et al., 2003). A few pathways that regulate mTORC1 are shown in Figure 1.9.

mTORC1 is involved in regulating cap-dependent protein translation. It directly regulates eIF4E-BPs (discussed in section 1.12.4), 40S ribosomal protein S6 kinases (S6Ks) and indirectly, PDCD4 (discussed in section 1.12.8) (Raught et al., 2001, Dorrello et al., 2006a).

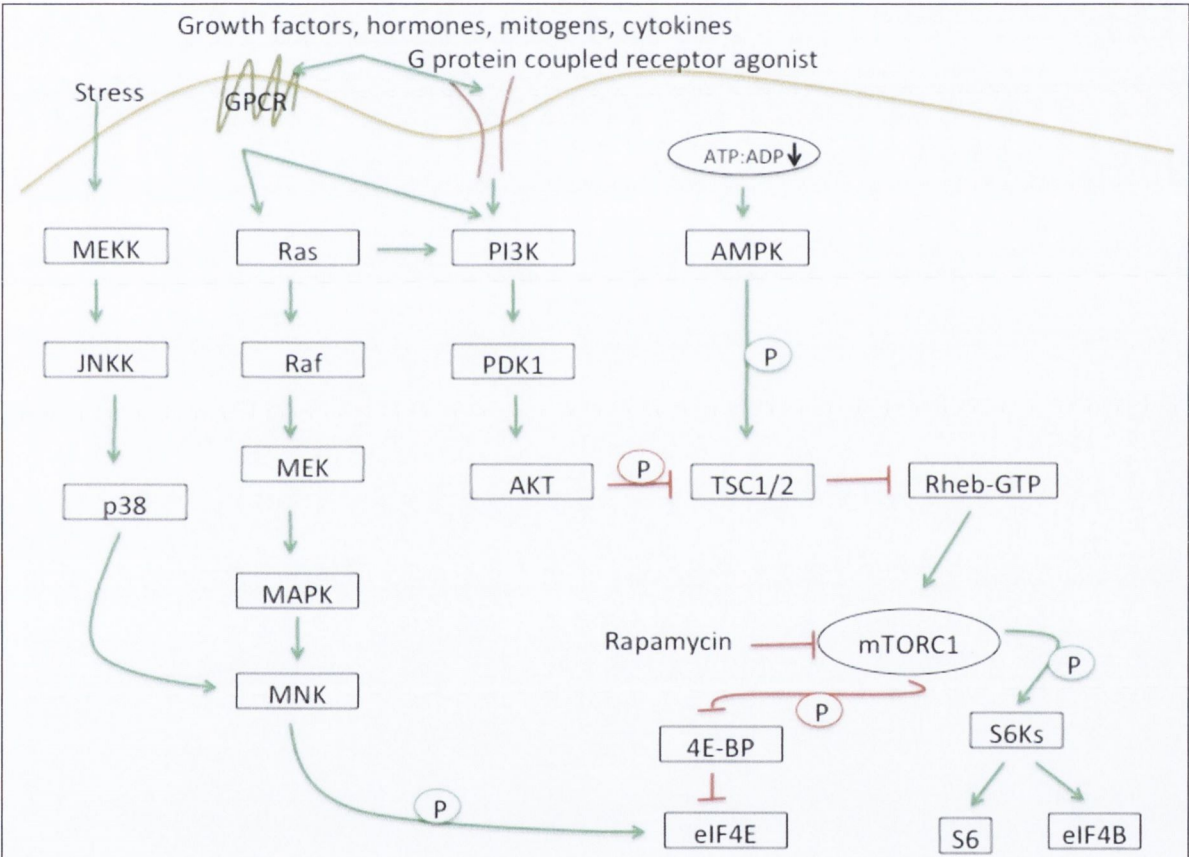


Figure 1.9 mTORC1 and eIF4E activation pathway
The MEKK/JNKK/p38/MNK and Ras/Raf/MEK/MAPK/MNK pathways lead to phosphorylation of eIF4E. RAS/PI3K/PDK1/AKT pathway activation leads to inactivation of the TSC-1/TSC-2 complex, which lifts the inhibition from RHEB-GTP. RHEB-GTP thereby leads to mTORC1 activation. mTORC1 inactivates eIF4E-BPs via phosphorylation event and activates S6 kinase via phosphorylation.

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eIF4E-BP1 has a TOR signaling (TOS) motif in its C-terminal that interacts with raptor. eIF4E-BP1 has a number of sites that are sequentially phosphorylated by mTORC1 (Gingras et al., 2001b). eIF4E-BP1 is phosphorylated at Thr37/46 first. This leads to phosphorylation of Ser65 and Thr70 of eIF4E-BP1 which results in eIF4E-BP1 inhibition (Gingras et al., 2001b). This allows eIF4E-BP1 to detach from eIF4E (Gingras et al., 2001b).

S6Ks are responsible for phosphorylation of S6 (a component of the 40S ribosomal subunit), eIF4B, and eIF3. This process triggers translation by promoting interaction of eIF4B with the eIF4F complex and eIF3 (Holz et al., 2005, Raught et al., 2004, Shahbazian et al., 2006). Furthermore, S6K1 mediates PDCD4 degradation, which allows release of eIF4A (Dorrello et al., 2006a). The whole process allows the formation of eIF4F complex and therefore promotes cap-dependent translation.

Specifically, the mTORC1 complex has been identified to be critically important for the translation of mRNAs with extensive secondary structure in their 5'UTR. Although inhibition of mTORC1 prevents cell proliferation and transformation, mTORC1 inhibition does not block general protein synthesis completely. In fact general translation inhibition is very weak, and one reason for this is thought to be that mTORC1 only strongly regulates the translation of certain mRNAs (Mamane et al., 2006). The role of mTORC1 in the activation of cap-dependent translation is illustrated in Figure 1.10.

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mTORC1 is the mechanistic regulator of cap-dependent translation and IL-10, as discussed in section 1.13 and 1.15. mTOR regulates a diverse set of components in cell, and more often than not it is found associated with tumours. Numerous mTOR regulated proteins e.g. eIF4A, eIF4E, and IL-10 amongst others have been found up-regulated in a number cancers. Many lines of evidence support the idea that disruption of translational control has oncogenic consequences. Transformed cell lines, for one, have higher rates of protein synthesis (Heys et al., 1991). Furthermore, overexpression of eIF4E (a component of eIF4F) has been associated with tumorigenesis in mouse models (Wendel et al., 2004, Ruggero et al., 2004). Consistent with this, eIF4E over-expression can suppress apoptosis and promote growth (Li et al., 2003, Li et al., 2004). Key tumour suppressor genes (e.g. *PTEN*), oncogenes (e.g. *Ras* and *Src*) and signalling pathways (e.g. PI3K and AKT) impinge on the translation apparatus – in particular on components of the eIF4F complex. Thus this results in changed gene expression at the translational level (Rajasekhar et al., 2003). Examples of mRNAs whose translation are responsive to eIF4F levels include c-Myc, ornithine decarboxylase, MMP-9; the growth factors FGF-2, VEGF and TGF β ; anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1. The immunosuppressive cytokine IL-10 is mTOR and eIF4F sensitive too.

As mentioned previously, mTORC1 can be activated by signaling via PI3K and AKT (also termed PKB) and by the Ras/Raf/ERK pathway (Corradetti and Guan, 2006). mTORC1 signaling is constitutively active in many tumor cells and their

proliferation can be inhibited by rapamycin (Easton and Houghton, 2006, Sabatini, 2006).

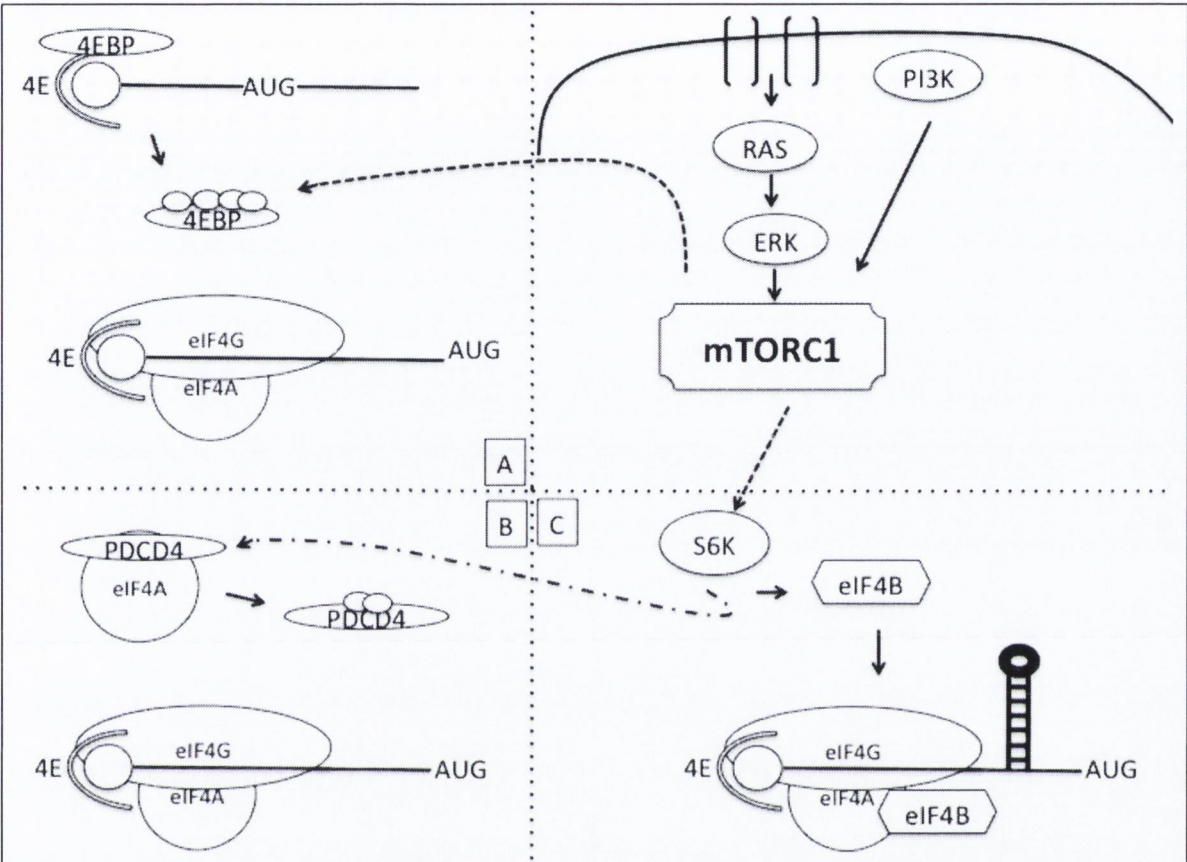


Figure 1.10: The mTORC1 pathway and cap-dependent translation Growth factors such as insulin, insulin-like growth factor 1(IGF1), TNF α , and LPS are known to stimulate cellular translation, cell survival and cellular growth through the mammalian target of rapamycin complex 1 (mTORC1). Growth factors activate mTORC1 via the PI3K-AKT pathway or via RAS-ERK pathway. A) eIF4E-BP represses eIF4E from interacting with eIF4G and thereby blocks eIF4F complex formation. Activated mTORC1 causes phosphorylation of eIF4E-BP, which dissociates eIF4E-BP from eIF4E. eIF4E then interacts with eIF4G and cap-dependent translation can initiate. B) PDCD4 blocks eIF4A from interacting with the eIF4F complex. Activated mTORC1 causes phosphorylation of PDCD4 via S6K1, which dissociates PDCD4 from eIF4A. Then, eIF4A interacts with the eIF4F complex and cap-dependent translation can initiate. C) eIF4B enhances the RNA helicase activity of eIF4A, which promotes 5' to 3' scanning and removes secondary structures present in the 5'UTR. mTORC1 activation promotes phosphorylation of eIF4B via S6Kinase. Phosphorylated eIF4B enhances eIF4A helicase activity.

Although rapamycin and its analogs appear to be good candidates for the use in cancer therapy, and are currently in clinical trials (Easton and Houghton, 2006) it is not clear if their effects are a consequence of influencing translation and/or other cellular pathways under their governance. There is as yet little information on how inhibition of mTORC1 signaling exerts its 'anticancer' effects.

1.14 Cap-independent translation

A number of mechanisms exist for translation initiation. Specifically, differences lie in how the mRNA is recruited and brought to the ribosome for translation. Although the majority of mRNAs are translated by cap-dependent scanning mechanism starting from the first initiation codon encountered by the ribosome (Kozak, 1983) other methods exist. In cap-dependent translation, eIF4F is responsible for the task. It has been estimated that around 10% of mRNAs have the potential for their translation to be initiated by internal ribosome entry (Spriggs et al., 2008). Cellular stress and nutrient deprivation leads to a reduction in cap-dependent translation, or at times a complete shut-down by targeted modification and proteolysis of components of initiation complex. Cellular stress has also been implicated with formation of stress granules (SGs) that act as storage sites for mRNAs and the translational machinery until the cell recovers (Anderson and Kedersha, 2006). Under harsh condition, the cell responds by impairing cap-dependent translation whilst maintaining translation of a subset of mRNAs required for cellular homeostasis.

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A number of alternative mechanisms exist that allow translation to continue in a cap-independent manner. For instance certain mRNA have alternative start codons. In principle the first AUG encountered by the 48S PIC is the start site of translation, however certain mRNA can have upstream start codons (uAUGs) or upstream open reading frames (uORF) before the real coding sequence. Such sequences are found in proto-oncogenes and cellular growth genes. Ribosomal shunting is another rare mechanism that allows 48S PIC scanning to circumvent inhibitory secondary structures in the 5'UTR. However the most well understood alternative process is internal ribosome entry site (IRES). The 5'UTR of the mRNA with internal ribosome entry is capable, in the presence of *trans*-acting factors of adopting a highly complex structure, called an IRES, that allows the ribosome to be recruited upstream of the translation start site (Komar and Hatzoglou, 2005, Stoneley and Willis, 2004). All the proteins involved in the process are not fully known, however there is evidence that viral IRESs require polypyrimidine tract binding protein (PTB) for activity (Mitchell et al., 2005, Sawicka et al., 2008). Furthermore, recent studies suggest that eIF4A is not required for IRES-mediated translation, instead eIF4A-resistance is now being used as an approach to detect an IRES site in the 5'UTR of certain gene transcripts; however certain viral IRESes do require eIF4A (Olson et al., 2013, Bordeleau et al., 2006c). IRES containing transcripts have been shown to outcompete cap-dependent transcripts under patho-physiological conditions (Svitkin et al., 2005, Marr et al., 2007). Furthermore, it has been shown that during polio-virus infection, apoptosis, mitosis, genotoxic stress and heat shock, cellular IRESes rather than cap-dependent translation are preferentially used (Johannes et

al., 1999, Stoneley et al., 2000, Qin and Sarnow, 2004, Subkhankulova et al., 2001, Coldwell et al., 2001).

Cellular survival proteins are synthesized in a cap-independent manner, e.g. via IRES especially under condition where cap-dependent translation is impaired (Spriggs et al., 2008, Holcik, 2004). A number of proteins involved with the translation apparatus are selectively expressed or repressed at the level of translation in a growth dependent manner. The mRNAs of these proteins contain a cytosine residue in their 5' cap followed by a string of 4-14 pyrimidine residues or terminal oligopyrimidine tract (TOP). This 5'TOP acts as a cis-regulatory element that rapidly switches translation on or off depending on the stimuli and cellular nutrient status. It has been recently observed that inflammatory condition induce IRES-dependent translation (Rubsamen et al., 2014).

1.15 IL-10

As described above, Interleukin-10 is under the control of PDCD4, which repress IL-10 by sequestering eIF4A. The translational control of IL-10 is explored extensively in this thesis and IL-10 will now be described in detail. IL-10 is an anti-inflammatory cytokine that plays a central role in infection by limiting the immune response and thereby preventing damage to the host. IL-10 is produced at different stages of the inflammatory response by numerous cells of the immune system. Originally IL-10 was identified as a T helper 2 (T_H2)-type cytokine (Fiorentino et al., 1989), however

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we now know that IL-10 is expressed by numerous inflammatory cells. A number of adaptive immune system cells express IL-10 and these include: T_H1 , T_H2 , T_H17 , T_{Reg} , $CD8^+T$ and B cells. Cells of the innate immune system also express IL-10 and these include dendritic cells (DCs), macrophages, mast cells, natural killer (NK) cells, eosinophils and neutrophils.

IL-10 participates in numerous processes to mediate an anti-inflammatory response. Interaction of IL-10 with the IL-10 receptor (IL-10R) leads to activation of signal transducer and activator of transcription (STAT) 3 (Niemand et al., 2003). Furthermore, IL-10 also has an autocrine inhibitory effect on macrophages and DCs whereby it suppresses T_H2 cell proliferation and inhibits T_H1 -type responses (Saraiva and O'garra, 2010). Additionally IL-10-producing T_{Reg} cells are stimulated by IL-10, which provides a feedback loop to limit innate effector functions of macrophages and DCs (Barrat et al., 2002). The importance of IL-10 is also highlighted by the fact that the absence of IL-10 leads to detrimental immunopathology to the host (Ejrnaes et al., 2006, Brooks et al., 2006). This is evident by IL-10 or IL-10R-deficient studies where, IL-10- or IL-10R-deficient mice develop colitis in the presence of microorganisms whilst the wild-type mice do not (Spencer et al., 1998, Kuhn et al., 1993). This cytokine is under tight regulation and holds great significance for the immune system.

1.15.1 IL-10 induction

Several layers of regulation exist to control IL-10 expression. The first layer exists in the changes in chromatin structure at the *IL-10* locus. IL-10 is also regulated at a transcriptional level by enhancers and silencers. Lastly, post-transcriptional mechanisms to regulate IL-10 exist, that include mRNA stabilization/degradation and translational control.

Macrophages and DCs produce IL-10, *in vitro*, in response to PRR activation. TLR2 agonists are particularly effective in inducing IL-10 expression from most APCs. Macrophages produce IL-10 following stimulation with TLR-3, TLR4 and TLR9 (Boonstra et al., 2006, Dillon et al., 2004). Dectin 1 and CLEC4M also induce IL-10 in macrophages (Rogers et al., 2005b, Geijtenbeek et al., 2003). In addition ligation of Fc receptors (FcRs) in TLR-stimulated macrophages and CD40 ligation in TLR-stimulated or dectin 1-stimulated DCs enhance IL-10 production (Gerber and Mosser, 2001, Edwards et al., 2002, Rogers et al., 2005a).

1.15.2 Regulation of IL-10 expression in Macrophage and DCs

It has been shown that efficient IL-10 production in response to LPS requires the activation of both TRIF- and MyD88- dependent pathways (Boonstra et al., 2006). Furthermore TNFR-associated factor-3 (TRAF-3), which is a regulatory component for type I IFN production, also mediates LPS-induced up-regulation of IL-10 (Hacker et al., 2006).

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Activation of ERK in response to TLR stimulation positively correlates with IL-10 expression. LPS-stimulated macrophages, which show strong activation of ERK, produce high levels of IL-10. In contrast, DCs have a lower activation of ERK and also produce less IL-10 (Kaiser et al., 2009b). Also, ERK-deficient DCs show a decreased production of IL-10 in response to TLR activation (Agrawal et al., 2006). This therefore suggests that ERK is an important positive regulator of IL-10 production. ERK activation in response to TLR2 and TLR4 ligands is mediated by MyD88 and Tumour progression locus -2 (TPL-2) activation. TPL-2 activates MEK1/MEK2, which activate ERK. Apart from MAPK activation, MyD88 can also activate NF κ B. NF κ B1 (p105) has also been implicated in IL-10 regulation. It was shown that NF κ B1-deficient macrophages have low IL-10 expression in response to CpG or LPS (Banerjee et al., 2006b). Banerjee and colleagues found that NF κ B1-mediated IL-10 expression occurs in both an ERK-dependent and ERK-independent manner (Banerjee et al., 2006). Furthermore, The LPS/TLR4 mediated IL-10 regulation via NF κ B and p38 MAPK pathways lead to the activation of mitogen- and stress-activated protein kinases, MSK1 and MSK2, resulting in cAMP-responsive-element-binding protein (CREB) and AP-1 phosphorylation. The CREB family of transcription factors consists of cAMP-responsive activators in mammalian systems including CREB, cAMP response element modulator (CREM), and activating transcription factor 1 (ATF1) (Mayr and Montminy, 2001). Mal was identified as a key upstream regulator of CREB (Mellett et al., 2011). CREB is involved in regulation of IL-10 in a number of cells.

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NFκB and ERK mediated IL-10 regulation is demonstrated in Figure 1.11. NFκB has been found to be directly involved in ERK signaling, since the upstream signaling component TPL-2 is usually present in a complex with NFκB1. This interaction with NFκB1 prevents TPL-2 degradation and thus enhances ERK activation (Beinke and Ley, 2004). Furthermore, TPL-2-deficient macrophages and myeloid DCs as well as NFκB1-deficient macrophages produce low levels of IL-10 (Kaiser et al., 2009a, Banerjee et al., 2006a). In NFκB1-deficient mice, this repression of IL-10 production could be partially restored by ERK activation. This suggests that with regard to IL-10, one of the important effects of NFκB1 is to enhance ERK signaling. However, since IL-10 production was only rescued partially, factors other than ERK are likely to play a role as well.

As mentioned above, IL-10 is also produced in response to Dectin 1 activation. Dectin 1- signaling involves spleen tyrosine kinase (SYK) where dectin 1 phosphorylation recruits SYK, which initiates an ERK-dependent signaling cascade that induces IL-2 and IL-10 (Rogers et al., 2005) (Slack et al., 2007).

p38 is also involved in IL-10 regulation. It was shown that LPS- or CpG- induced IL-10 expression in macrophages, primary DCs or human peripheral blood monocytes is p38-dependent, as p38 inhibition leads to lower IL-10 levels (Ma et al., 2001, Yi et al., 2002, Kim et al., 2008, Park et al., 2005, Jarnicki et al., 2008, Foey et al., 1998). In TLR4-stimulated macrophages, p38 regulates IL-10 through post-transcriptional mechanisms. It was shown that TLR4 signaling protects IL-10 mRNA degradation in

a TRIF- and p38-dependent manner (Teixeira-Coelho et al., 2014). This leads to greater levels of IL-10 production in TLR4 but not TLR2 stimulated macrophages (Teixeira-Coelho et al., 2014).

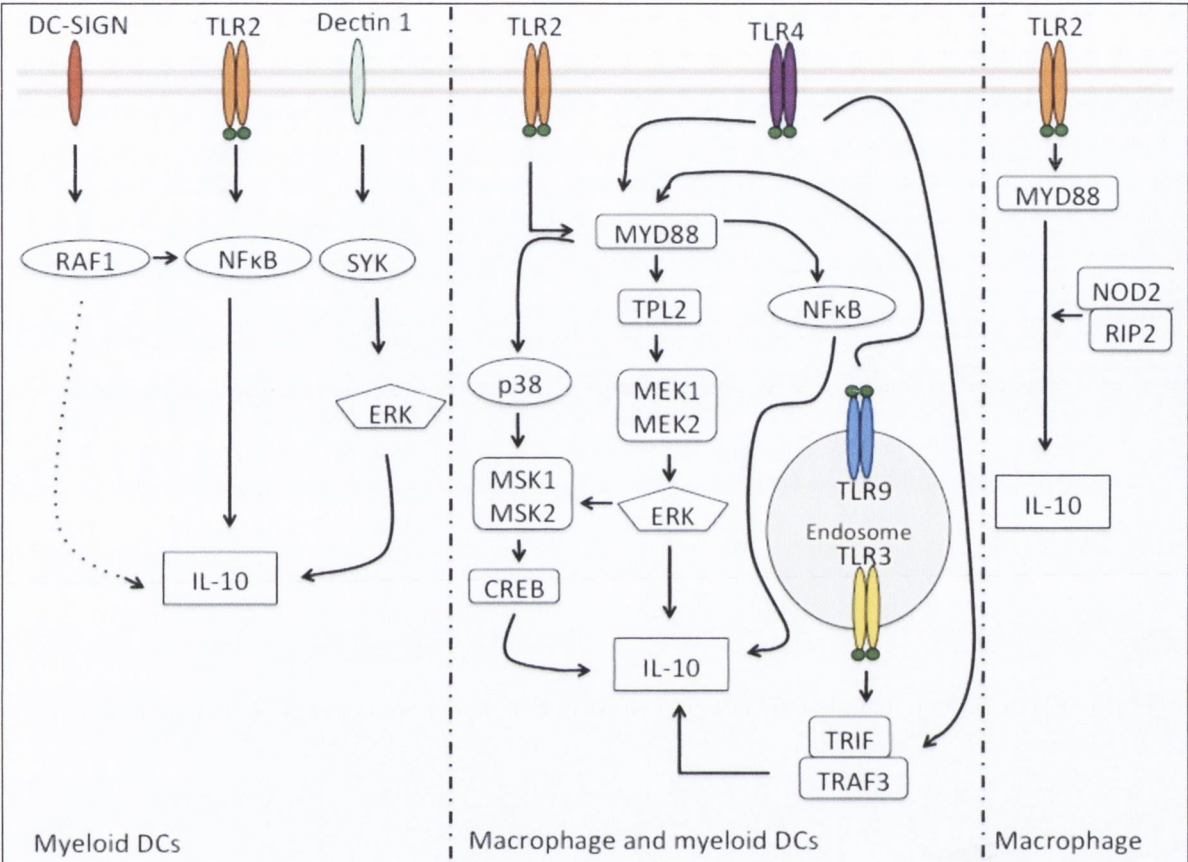


Figure 1.11 Interleukin-10 expression in macrophage and dendritic cells.

TLR or non-TLR signaling can mediate the expression of IL-10 in macrophages and dendritic cells. TLR stimulation results in the activation of the ERK1, ERK2, p38 and NFκB pathways via the adaptor proteins MyD88 and TRIF. These pathways participate in the induction of IL-10 expression, in addition to the expression of pro-inflammatory mediators such as IL-6 and TNFα. Specifically in myeloid DCs, signaling through DC-specific ICAM3-grabbing non-integrin (DC-SIGN) and RAF-1 can augment TLR2-mediated IL-10 expression. In addition, Dectin 1 signaling via SYK and ERK also mediates IL-10 expression in myeloid DCs. In macrophages, specifically, a role for NOD2-TLR2 crosstalk has been identified in IL-10 production. IL-10 can be produced via MyD88-dependent and MyD88-independent mechanism in macrophage and DCs.

KEY: IL-10R: IL-10receptor; MEK: MAPK/ERK1 kinase; MSK: mitogen- and stress-activated protein kinase; RIP2: receptor-interacting protein 2; TRAF3: TNFR-associated factor 3; CREB: cAMP response element-binding protein.

1.15.3 Regulation of IL-10 expression in the cells of the adaptive immune system

T_H1, T_H2, T_H9, and T_H17 cells have been reported to produce IL-10 (Jankovic et al., 2007, Anderson et al., 2007, Gabrysova et al., 2009, McGeachy et al., 2007, Veldhoen et al., 2008). The exact underlying mechanisms are not fully understood, however it is known that T_H1, T_H2 and T_H17 cell activation depends on DC- and macrophage-derived factors. These factors are downregulated by IL-10. Which indicates that a negative feedback loop exists that prevents immunopathology by these cells.

IL-10 producing T_H1 cells are differentiated in the presence of endogenous IL-12 and high levels of T cell receptor antigen (Gabrysova et al., 2009). As is the case for macrophage and DC, T_H1 also require ERK for IL-10 production in addition to STAT-4 (Saraiva et al., 2009a). In T_H2 cells IL-10 production is regulated by IL-4, STAT-6 and GATA binding protein 3 (Zhu et al., 2004, Shoemaker et al., 2006, Chang et al., 2007). In T_H17 cells IL-10 production seems to be STAT-3 and in some cases STAT-1-dependent (Stumhofer et al., 2007, Xu et al., 2009). IL-10 induction from T_H1, T_H2 and T_H17 is ERK- but not p38- dependent, which contrasts the requirements of macrophage and DCs (Saraiva et al., 2009a). In addition it has also been found that IL-21 and IL-27 induce ERK activation, where IL-21 can enhance IL-10 expression in CD4⁺ T cells and IL-27 enhances IL-10 expression in T_H1, T_H2 and T_H17 cells (Xu et al., 2009, Fitzgerald et al., 2007, Stumhofer et al., 2007, Batten et al., 2008, Pot et al., 2009, Fuqua et al., 2008, Owaki et al., 2006). However it is not fully understood if IL-21 and IL-27 mediated IL-10 regulation is ERK-dependent.

Literature on IL-10 production by T_{Reg} cells is very conflicted; where most studies have found evidence that T_{Reg}s do not produce IL-10 *in vitro* or *ex vivo*, some studies show IL-10 production in response to IL-2 and IL-4 by T_{Reg}s (Barthlott et al., 2005, Hori et al., 2003, Vieira et al., 2004, de la Rosa et al., 2004). Furthermore, it has also been found that *ex vivo* stimulated T_{Reg} expressing factor forkhead box P3 (FOXP3) transcription factor do not produce IL-10 (Vieira et al., 2004). However, T_{Reg}s isolated from the gut do produce IL-10 (Maynard et al., 2007).

CD8+ T cells and B cells have also been implicated in IL-10 production. CD8+ T cells produce IL-10 upon TCR activation or CD40 ligand interaction from activated pDCs, whilst B cells produce IL-10 in response to auto antigens, TLR4 and 9 ligands and vitamin D3 (Tanchot et al., 1998, Gilliet and Liu, 2002, O'Garra et al., 1992).

1.15.4 IL-10 Promoter

Human and mouse *IL-10* promoters share high homology and are of similar structure. Specific protein (SP) 1, SP3, IRF1, STAT3 and CCAAT/enhancer binding protein β (C/EBP β) transcription factors have been proposed to bind and activate IL-10 in macrophages and T cell lines (mouse and human) (Brightbill et al., 2000, Tone et al., 2000, Brenner et al., 2003, Liu et al., 2003, Ziegler-Heitbrock et al., 2003). Furthermore homodimers of NF κ B p50 have been shown to activate IL-10 transcription by binding to proximal *IL-10* promoter in primary mouse macrophage

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where p50-deficient macrophages show impaired IL-10 production in response to LPS (Cao et al., 2006). Since p50 lack the transactivating domain, it homodimerize and form a complex with the transcriptional co-activator CREB-binding protein to activate transcription (Cao et al., 2006). This is in addition to its role in modifying ERK signaling as mentioned in section 1.16.2. Also, it has been found that NFκB p50 subunit binds to the *IL-10* promoter of human T-cell lymphoma cell line (Mori and Prager, 1997). In addition, NFκB p65 subunit also binds to κB site on *IL-10* promoter and enhances its expression in response to LPS in mouse macrophage (Saraiva et al., 2005).

Two homeobox family cofactors (HOX), pre-B-cell leukaemia transcription factor 1 (PBX1) and PBX-regulating protein 1 (PREP1) have also been identified as IL-10 regulators in mouse macrophages (Chung et al., 2007). PBX1 and PREP1 were found to bind apoptotic cell-response element (ACRE) in the *IL-10* promoter. CREB and ATF1 also bind IL-10 promoter in response to LPS-induced MSK1 and MSK2 activation (Ananieva et al., 2008). IL-10 also regulates the pathway for TLR-induced NFκB activation through the production of glycogen synthase kinase-3β (GSK-3β), which increases the binding of CREB and decreases the binding of CBP/p300 to the RelA subunit of NFκB. CREB interacts with its coactivator protein, CREB-binding protein (CBP), or p300 to initiate transcription of CREB-responsive genes.

IL-10 induction in response to double-stranded RNA has been reported to be protein kinase R (PKR) dependent and NFκB regulated (Chakrabarti et al., 2008). A

role for C/EBP β (CCAAT/enhancer binding protein- β) in cAMP-mediated IL-10 production has been identified in *Escherichia coli* infected primary mouse macrophages, where C/EBP β -deficient macrophages failed to produce IL-10 (Csoka et al., 2007). TGF β 1 has been implicated in IL-10 production via SMAD4 binding to *IL-10* promoter in mouse T_H1 cells (Kitani et al., 2003), however TGF β 1 is also known to repress T_H1 and T_H2 cells *in vitro* and thereby block IL-10 (Saraiva et al., 2009b). GATA3 has been identified as the master regulator of IL-10 in T_H2 cells, whereby GATA3 can initiate chromatin modification at the *IL-10* locus and has one binding site in *IL-10* promoter, however GATA3 alone cannot transactivate IL-10 (Chang et al., 2007, Shoemaker et al., 2006). Finally, MAF binding and presence has also been implicated in IL-10 production in a number of cell types including T_H1, T_H2, T_H17, mouse macrophage and FOXP3⁺ T_{Reg} cells (Cao et al., 2005, Pot et al., 2009, Saraiva et al., 2009b, Xu et al., 2009).

A number of transcription factors participate in IL-10 regulation, and the list is expanding. Its transcription is not simple and differs in different cell types, with some common factors participating in both adaptive and innate immune systems. However there seems to be some different molecular mechanisms in innate vs adaptive systems. For example, the κ B site present 4.5kb upstream of *IL-10* start site has a role only in LPS- CpG- or zymosam- stimulated macrophages and DCs, however this is not the case in T cells (Saraiva et al., 2005). Furthermore, there are some differences in IL-10 regulation between T_H1 and T_H2 cell types, where mouse T_H2 cells have a role for JUN in IL-10 regulation whilst T_H1 cells do not (Wang et al.,

2005c). Table 1.2 illustrates kinases and transcription factors involved in IL-10 regulation in different cell types.

Macrophage	Dendritic cell	T _H 1	T _H 2	T _H 17
ERK	ERK	ERK	ERK	ERK
p38	p38	STAT4	STAT6	STAT3
TPL2	TPL2	MAP	GATA3	MAF
NFκB	SYK	SMAD4	JUN	
C/EBPβ	RAF1		MAP	
SP1	NFκB			
MAF				
CREB				
PREP1				
PBX1				
ATF1				

Table 1.2 IL-10 regulatory factors (signaling components and transcription factors). A number of signaling molecules and transcription factors regulate IL-10 expression in antigen-presenting cells and CD4⁺ T-cells. The table above illustrates data that was generated using cell lines and primary cells knock-down.

1.15.5 Negative regulation of IL-10 expression

IFNγ inhibits ERK- and p38- dependent IL-10 expression in macrophages and DCs (Hu et al., 2006). IFNγ mediates its effect in a number of ways, 1) it directly blocks TLR-induced MAPK activation, and 2) it induces the release of glycogen synthase kinase 3 (GSK3) by antagonizing PI3K-AKT activation which limits AP1 binding on the *IL-10* promoter (Hu et al., 2006). IL-10 itself negatively regulates its production by inducing expression of DUSP1, which in turn negatively regulates p38 (Hammer et al., 2005). Furthermore MHC class II transactivator (CIITA) is also involved in repressing IL-10 expression in mouse DCs and it blocks the activity of the *IL-10* promoter in a mouse macrophage cell line (Yee et al., 2005). In addition, STAT1 is also implicated in negative regulation of IL-10 expression in human monocytes

(Kallioli and Ivashkiv, 2008, VanDeusen et al., 2006). B cell lymphoma-3 (BCL-3) has repressive activity on IL-10 expression in macrophages, although this may not be a direct effect (Riemann et al., 2005). In addition, BCL-6 has been found to have repress IL-10 production in T_H2 ; however no BCL-6 binding site has been found in *IL-10* promoter, suggesting it may act indirectly (Kusam et al., 2003).

ETS1 also has been described as a negative regulator of IL-10 production in mouse T_H1 cells. ETS1-deficient T_H1 cells produce more IL-10 in support of this (Grenningloh et al., 2005). Furthermore, T_H1 cell-specific transcription factor T-bet (TBX21) has also been suggested to be a negative regulator of IL-10 in T_H1 cells, and its deletion results in higher IL-10 production (Sullivan et al., 2005). Negative regulation of IL-10 is show in Figure 1.12.

1.15.6 Post-transcriptional regulation of IL-10

mRNA stability, regulated by the 3'UTR is of critical importance for the production of several cytokines. The 3'UTR of a number of cytokines are quite long and contain class II adenosine-uridine-rich elements (ARE) that target mRNA for rapid degradation. The IL-10 3'UTR contain various ARE sites and has been recently found to be a target for tristetraprolin (TTP), an RNA binding protein involved in mRNA destabilization (Powell et al., 2000, Stoecklin et al., 2008). In addition miR-106a targets the IL-10 3'UTR and induces its degradation (Sharma et al., 2009).

Cap-dependent ribosomal scanning depends on the 5'UTR length and structure (Willis, 1999, Kozak, 1989, van der Velden and Thomas, 1999). A number of different classes of mRNA have been described on the basis of lengths and structures found in the 5'UTRs (van der Velden and Thomas, 1999). The first class

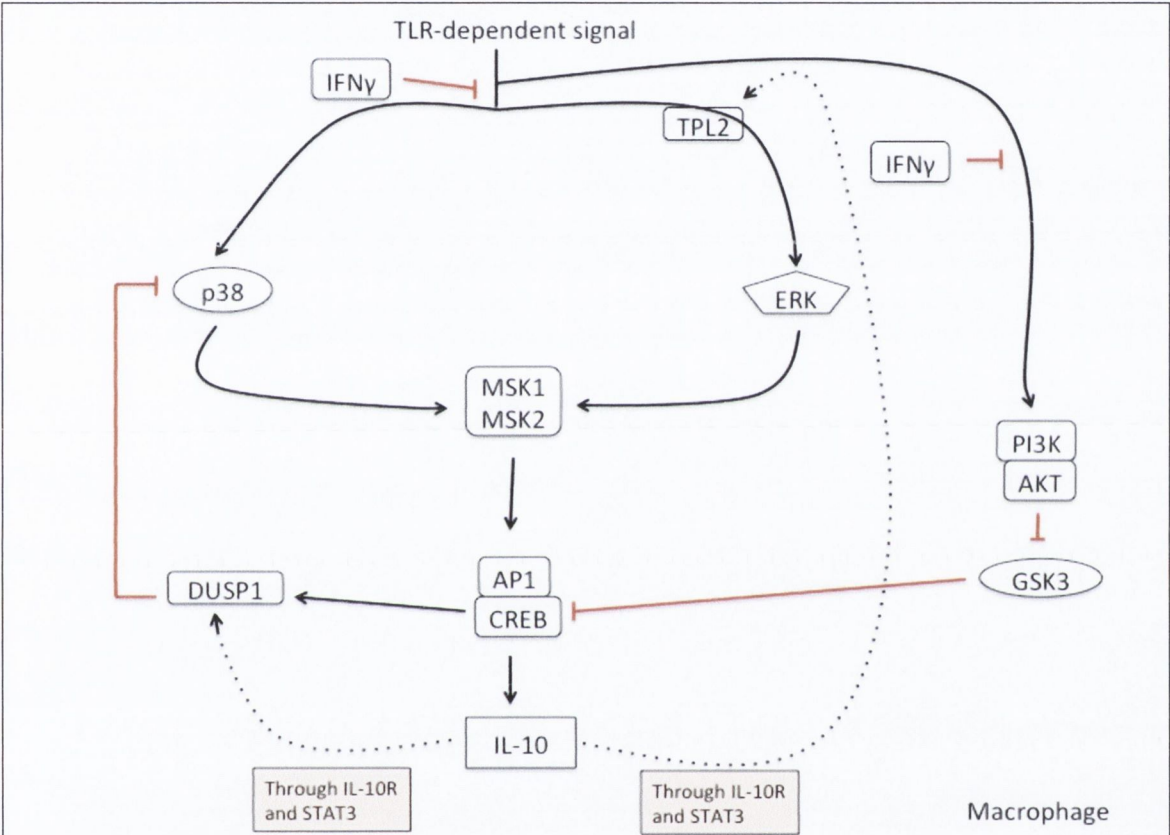


Figure 1.12. Regulation of IL-10 production in macrophages

IFN γ is involved in negative regulation of p38- and ERK- dependent pathways. In addition IL-10 itself negatively regulates its expression via these pathways as described above. IL-10 induces the expression of dual-specificity protein phosphatase 1 (DUSP1) which negatively regulates p38 phosphorylation and blocks IL-10 production. There is also evidence suggesting that IL-10 positively regulates its expression by upregulating TPL-2. IFN γ also blocks the PI3K/AKT. PI3K/AKT pathway usually blocks glycogen synthase kinase 3 (GSK3); GSK3 blocks CREB and AP1 and thereby limit IL-10 expression. IFN γ limits IL-10 expression through this pathway as well.

contain long structured 5'UTR, which are poorly translated. The second class contains oligopyrimidine terminal tracts (TOP) and are regulated in a growth-dependent manner, discussed in section 1.14. The third class of mRNA have short 5'UTR that allow efficient translation. Although two different isoforms of the IL-10 5'UTR have been identified in human peripheral blood mononuclear cells (Forte et al., 2009), the 5'UTR mediated post-transcriptional regulation of IL-10 is not well characterized.

1.15.7 IL-10 and immunosuppression

IL-10 is involved in a positive regulatory loop with Transforming growth factor β (TGF β), where TGF β upregulates IL-10 and IL-10 enhances the expression of TGF β (Mocellin et al., 2005). TGF β has been suggested to be the principal immune-suppressive factor secreted by tumour cells (WojtowiczPraga, 1997). TGF β 1 knockout mice get multi-organ inflammatory syndrome as a result of lethal autoimmunity (Prud'homme, 2007).

IL-10 participates in immune-response suppression by inhibiting antigen presentation, MHC class II expression, and the upregulation of costimulatory molecules CD80 and CD86. Furthermore, IL-10 prevents the production of the Th1-associated cytokines IL-2 and IFN γ from antigen-presenting cells (APCs). Physiologically, IL-10 significantly suppresses the major pro-inflammatory cytokines IL-1, IL-6, IL-12, and TNF α (Mosser and Zhang, 2008).

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As mentioned in section 1.16.2 and 1.16.3, although IL-10 is produced by both innate and adaptive immune systems, it seems to act primarily on DCs and macrophages to inhibit the differentiation and the antigen-presenting properties of macrophages and DCs. It mediates repression of immune detection by blocking the expression of HLA-DR and costimulatory molecules, thereby limiting immuneresponse (Mocellin et al., 2005). Furthermore, IL-10 inhibits the differentiation of DCs from monocyte precursors, and it also inhibits DC maturation.

The other profound effect of IL-10 is to inhibit the production of proinflammatory cytokines IL-1, IL-6, IL-12, and TNF α and mediators from macrophages and DCs. Inflammatory chemokines of both the CC and CXC type are also suppressed by IL-10. Furthermore, IL-1 receptor antagonist secretion is induced by macrophages in response to LPS to limit inflammation (Akdis and Blaser, 2001).

IL-10 signals through two-receptors, IL-10R1 and IL-10R2 (Yoon et al., 2006). IL-10R1 has high affinity for IL-10 whereas IL-10R2 has comparatively lower affinity and only makes a marginal contribution to ligand binding (Yoon et al., 2006). The two receptors together allow the signal transduction. The activation of IL-10R1/IL-10R2 complex activates Janus tyrosin kinases JAK1 and Tyk2. JAK1 and Tyk2 phosphorylate the cytoplasmic tails of the receptor which allows recruitment of STAT3 to IL-10R1 (Bogdan et al., 1992). STAT3 homodimers form at the receptor, which are later released. The STAT homodimers translocate into the nucleus where they bind and transcribe genes with STAT-binding elements in the promoter region

(Pauleau et al., 2004). Additionally IL-10 also activates PI3K pathway and is understood to repress NF κ B activation (Lang et al., 2002). These processes are involved in transcriptional regulation whereby pro-inflammatory genes are dampened down and anti-inflammatory genes are up regulated.

IL-10 via STAT3 increases the TLR-mediated expression of tristetraprolin (TTP). Many mRNAs have AU rich elements in their 3'UTR, which is targeted by TTP for degradation (Schaljo et al., 2009). Therefore, IL-10 is also involved in post-transcriptional regulation of mRNAs via TTP. Known physiological target mRNAs for TTP include TNF α , granulocyte-macrophage colony-stimulating factor, and interleukin-2 β . Additionally, IL-10 also induces DUSP1, which dephosphorylates p38 and thereby inactivates it (Hammer et al., 2006). p38 negatively regulates TTP by phosphorylation, therefore IL-10 inactivates p38 to further boost TTP-mediated mRNA destabilization.

A number of other factors are also induced by IL-10, including Bcl-3 (B-cell leukaemia-3), Sbn2 (strawberry notch homolog 2 [*Drosophila*]), ETV3 (ETS family member- transcriptional repressor) (helicase family co-repressor) and I κ BNS (I κ B- δ), which work in coordination to suppress the production of TLR-induced genes (Smallie et al., 2010, Smith et al., 2011). ETV3 and SBNO2 are components of the pathways that contribute to the downstream anti-inflammatory effects of IL-10, partially by blocking NF κ B (El Kasmi et al., 2007). I κ BNS disrupts the docking between activated NF κ B and IL-6 promoter site, and inhibits IL-6 production after

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LPS stimulation (Yamamoto and Takeda, 2008). Bcl-3 effectively inhibits K48 polyubiquitination of the p50 homodimer and stabilizes its interaction with DNA through the formation of a stable Bcl-3:(p50)₂:DNA complex. By controlling stimulatory NFκB dimer loading and dimer exchange at the site of gene promoters, the stabilized Bcl-3:(p50)₂:DNA complex confers a state of long-lasting promoter hypo-responsiveness (tolerance) to TLR signaling.

As mentioned previously, miR-155 is pro-inflammatory miRNA and it is involved in a number of processes to promote inflammation. It stabilizes TNFα mRNA and blocks SHIP1 production (Alam and O'Neill, 2011). IL-10 blocks the production of miR-155 and thereby limits inflammation (McCoy et al., 2010). However IL-10 does not block miR-146a which has cell-intrinsic anti-inflammatory effects (McCoy et al., 2010)(Alam and O'Neill, 2011).

Furthermore, IL-10 also regulates IL-1 signalling. IL-10 induces IL-1R antagonist IL-1RA in LPS treated macrophages via the activation of STAT3 (Carl et al., 2004). IL-1RA binds to IL-1R with approximately equal affinity as IL-1α and IL-1β and has no agonist activity (Hannum et al., 1990).

The JAK-STAT signalling pathway also transcriptionally regulates suppressors of cytokine signalling (SOCS) molecules. SOCS act as a negative feedback signal by inhibiting JAK and STAT activation and phosphorylation (Kile and Alexander, 2001, Krebs and Hilton, 2001). SOCS-3 inhibits expression of many genes in cells,

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including endotoxin-inducible cytokines such as IL-6, $\text{TNF}\alpha$ and IL-1 (Nishinakamura et al., 2007, Williams et al., 2007). There are 8 members of the CIS-SOCS family (CIS and SOCS1-7) all of which contain central SH2 domain, a SOCS box and an amino-terminal domain of variable length and divergent sequence. SOCS-box-containing molecules are understood to function as E3 ubiquitin ligases and mediate the degradation of proteins via interactions through N-terminal regions. In addition to ubiquitin-mediated degradation of proteins (mostly signalling complex proteins), SOCS1 and SOCS3 can interact via their kinase inhibitory region and suppress cytokine signals (Kubo et al., 2003). SOCS3 protein is strongly induced by both IL-6 and IL-10 in the presence of LPS, but IL-6 signalling is selectively inhibited via SOCS3 binding to the IL-6R subunit GP130 (Yasukawa et al., 2003). It is widely accepted and shown that IL-10 induced SOCS3 is specifically anti-inflammatory but not IL-6.

However, not all the IL-10 activities are immunosuppressive. IL-10 can costimulate B-cell activation, prolong B-cell survival, and contribute to class switching in B cells. It can also costimulate natural killer (NK) cell proliferation and cytokine production (Cai et al., 1999). Furthermore it has been shown that IL-10 can also act as a growth factor to stimulate the proliferation of certain subsets of CD8^+ T cells (Rowbottom et al., 1999). There is still a lot to be explored to understand the molecular mechanism for IL-10 mediated resolution of inflammation. To date, a number of pathways have been identified but there are a lot more that will be discovered over time.

1.15.8 IL-10: The two-edged sword

As mentioned thus far, IL-10 is an important cytokine however it is a two-edged sword where too much IL-10 is lethal and too little is detrimental. A number of studies using IL-10 deficient mice have illustrated the importance of this cytokine in limiting autoimmune pathologies. IL-10 knockout mice or those treated with blocking anti-receptor antibodies succumb to what would normally be sublethal doses of LPS (Berg et al., 1995). In addition, bacterial and parasitic infections that are normally cleared in wild-type mice result in lethal autoimmune mortality in IL-10-deficient mice (Hunter et al., 1997, Gazzinelli et al., 1996). IL-10 knockout mice result in a dramatically exacerbated disease in almost all mouse models of autoimmunity, including experimental autoimmune encephalitis, rheumatoid arthritis, and inflammatory bowel disease. From these studies and others the power of IL-10 in limiting an over-exuberant immune response and preventing autoimmunity can be well iterated.

On the other hand, IL-10 over-expression is also dangerous for host. IL-10 over-expression results in lethality from bacteria that are normally cleared in wild-type mice. In addition viral infections mediate their survival by encoding IL-10 homologs to suppress immunity and persist in an otherwise immunocompetent host, and there is now evidence that tumors and tumor-associated macrophages produce IL-10 to contribute to the immunosuppressive environment of the tumor.

1.16 Aims and objectives

The inflammation process is clearly highly complex and tightly regulated. The overall goal of this thesis was to analyze further the role of miRNA and translation in the regulation of TLR signaling. A particular focus being the anti-inflammatory cytokine IL-10. The specific aims were as follow:

1. The first aim of this study was to identify miRNAs that are down regulated in response to LPS. These would be predicted to target positive regulators of TLR signaling.
2. The second aim of this project was to identify target proteins regulated by LPS-repressed miRNAs, through various miRNA target prediction software. From the analysis a list of potential targets could be generated, grouping any targets that would belong to the same family of proteins, thereby providing evidence for co-ordinate regulation.
3. From this approach I found evidence for eIF4A regulation. The third aim thereby became to identify the functional importance of eIF4A in LPS-induced gene expression.

My project has uncovered an important role for eIF4A1 in the control of IL-10 translation in LPS signaling, providing new insights into the complex regulation of IL-10 as a negative regulator of TLR signaling.

Chapter 2

Materials & Methods

2.1 Materials and methods

2.1.1 PRR stimuli

Ultrapure rough LPS (from *E. coli*, serotype EH100) was purchased from Alexis. Pam₃Cys-Ser-(Lys)₄ (Pam3CSK4) was obtained from Calbiochem. R848, low molecular weight polyinosine-polycytidylic acid [Poly (I:C)], and Polyadenylic-polyuridylic acid [Poly (A:U)] were purchased from Sigma-Aldrich.

2.1.2 Animals

Wild type C57/BL6 mice were obtained from the bioresources unit at Trinity College Dublin and some C57/BL6 were purchased from HARLAN, UK. All mice used were between ages 8-12 weeks. All the animal protocols used in this study were in accordance with the Irish Department of Health.

2.1.3 Plasmids

The firefly luciferase reporter pGL3-control vector was a gift from Stephany Micallef, School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, TCD, Ireland. The image of the vector is shown below.

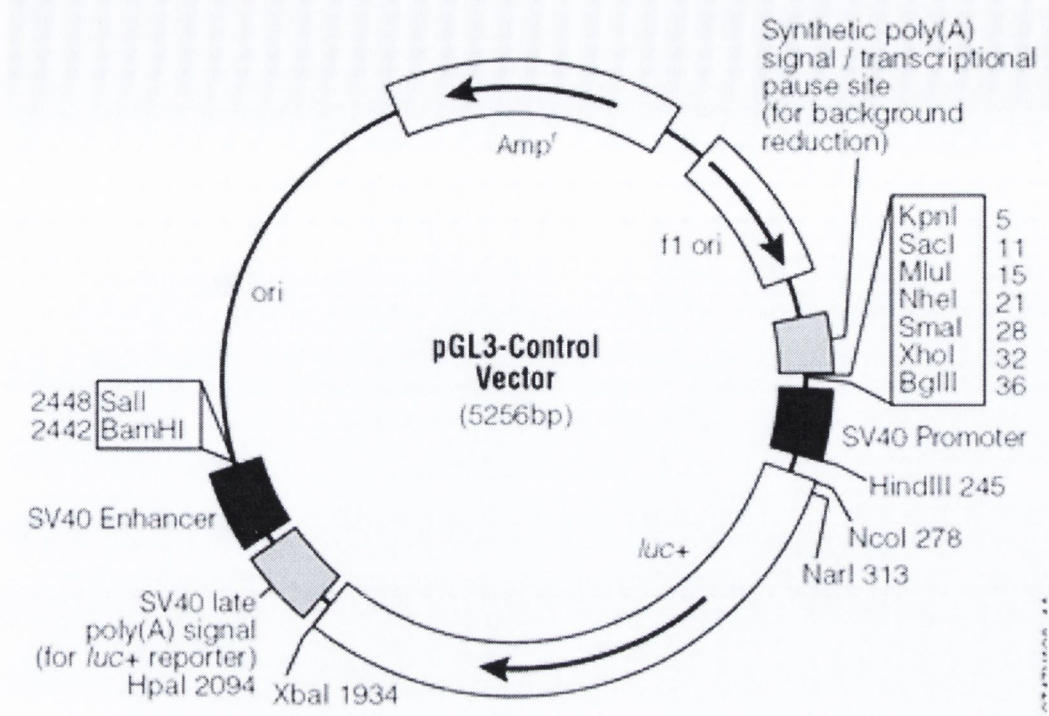


Figure 2.1.3 pGL3-Control Vector

2.1.4 Antibodies

Rabbit polyclonal antibody eIF4A1 (ab31217), eIF4A2 (ab31218) MCL-1 (ab25955) and eEF2 (ab33523) were purchased from ABCAM. The β -actin, Anti-mouse IgG and Anti-rabbit IgG were obtained from Jackson Sigma-Aldrich (Poole, Dorset, UK). IgG from rabbit serum (I8140) was purchased from Sigma.

2.1.5 Cell lines and culture reagents

The human embryonic kidney cell line 293T (HEK293T) and L929 cell lines were gifts from Dr. Kate Fitzgerald, University of Massachusetts, USA. The HEK293/TLR4-MD2-CD14 was purchased from Invivogen. The immortalised BMDM were a kind gift from Prof. Douglas Golenbock, University of Massachusetts, USA. RPMI 1640 +

Glutamax and Dulbecco's modified eagle medium (DMEM) + Glutamax cell culture media were obtained from Life Technologies. Fetal calf serum (FCS) was obtained from Biosera. Penicillin, streptomycin (PS), puromycin and trypsin were obtained from Sigma-Aldrich. Normocin™, Blasticidin and HygroGold™ were from Invivogen.

2.1.6 Inhibitors

Hippuristanol and Silvesterol were a kind gift from Prof. Jerry Pelletier, Department of Biochemistry, McGill University, Montreal, Quebec, Canada. PS1145, IκBkinase inhibitor was purchased from Santa Cruz Biotechnology. PS1145 efficiently inhibits both basal and induced NF-κB activity. Parthenolide was purchased from ENZO Lifesciences (BML-T113-0050), and Rapamycin was purchased from LC Laboratories (R-5000).

2.1.7 DNA purification and PCR reagents

Plasmid purification Maxiprep kits and RNeasy Mini RNA purification kits were purchased from Qiagen. Wizard® Plus SV miniprep DNA purification kits were from Promega. Primers were ordered from Eurofins MWG Operon. High capacity cDNA reverse transcription kit was purchased from Applied Biosystems. GoTaq® qPCR mastermix was from Promega. TaqMan® Fast Universal PCR Master Mix and TaqMan® Gene Expression assays for eIF4A1, eIF4A2, IL-10, IL-6, TNFα, 18s ribosomal RNA control, GAPDH, let-7c-1-3p, let-7b-3p, miR-98-3p, and snoRNA202 were from Applied Biosystems.

2.1.8 Miscellaneous reagents

General laboratory chemicals were purchased from Sigma-Aldrich with the exception of: Broad range pre-stained protein marker from New England BioLabs Ltd. 20X LumiGLO® chemilluminiscent reagent was from Cell Signaling. Protein A Sepharose CL-4B beads immunoprecipitation reagent was from Santa Cruz Biotechnology. Murine TNF- α , murine IL-10, and murine IL-6, ELISA Duosets were purchased from R&D Systems. GeneJuice® was purchased from Novagen. Lipofectamine 2000™ was from Invitrogen. Coomassie (Bradford) Protein assay reagent was purchased from ThermoScientific™.

2.2 Cell culture

2.2.1 Growth and maintenance of cell lines

HEK293TLR4-MD2-CD14, I-BMDM and RAW 264.7 cell lines were cultured in DMEM medium containing 10% FCS, and 1% P/S. HEK293TLR4-MD2-CD14 cells were supplemented with Normocin™ (100 μ g/ml) and the selective antibiotics Blastidicin (10 μ g/ml) and HygroGold™ (10 μ g/ml). L929 cells were cultured in RPMI medium containing 10% FCS, and 1% P/S. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. For continuing cell culture, all cell lines were seeded at 1-5x10⁵ per ml and sub-cultured two or three times a week. HEK293T, I-BMDM and L929 cells were removed from the surface of the flask by incubation with 5 ml of trypsin-EDTA (0.05 mg/ml). RAW 264.7 cells were removed with a cell

scraper. 10 ml of complete media was then added to the cells and they were centrifuged at 453 x g for 3 min before resuspending the pellet in fresh media. Cell viability was determined using the dye Trypan blue, which is excluded from healthy cells but taken up by non-viable cells. Cells were counted using a haemocytometer under a bright light microscope.

2.2.2 Cryo-preservation of cells

For cryo-preservation, cells were grown to sub-confluency and were harvested and counted as previously described in section 2.2.1. Cells were centrifuged at 453 x g for 3 min and the pellet was resuspended in 90% FCS and 10% DMSO such that each 1 ml aliquot contained 2×10^6 cells. These aliquots were placed in 1.5 ml cryovials and a cryo-freezing container at -80°C overnight before being transferred to liquid nitrogen for storage.

2.2.3 Generation of bone marrow-derived macrophages

Running hot water followed by ethanol spray was used to rinse all the utensils for bone marrow extraction. Laminar flow hood was used to harvest the bone marrow. C57BL/6 mice were euthanized in a CO_2 chamber followed by cervical dislocation. The abdomen and hind legs were sprayed with 70% ethanol. An incision was made in the midline of the abdomen and the skin was pulled down to expose the hind legs. Muscle was removed from the femur and tibia using sharp scissors. The femur was cut just below the hip joint and just above the knee joint. The tibia was cut just below the knee joint and just above the ankle. The bones were removed to a 10 cm

dish containing 10 ml of cold DMEM. Using a 10 ml syringe filled with phosphate buffered saline (PBS) and a 24-gauge needle the bone marrow was flushed out into a 50 ml tube by inserting the needle into the bone cavity. After flushing all of the bones, the cells were resuspended by pipetting up and down using a 10 ml pipette to bring the cells into single-cell suspension. The cells were passed through a cell strainer and centrifuged at $453 \times g$ for 3 min. Cells were resuspended in 5 ml of red blood cell lysis buffer for 5 min. 20 ml of PBS was added to the cells, which were then centrifuged at $453 \times g$ for 3 min. Cells were resuspended in DMEM and counted as previously described in section 2.2.1. Cells were seeded at $1 \times 10^6/\text{ml}$ in non tissue culture coated sterile 10 cm dishes in DMEM containing 10% FCS, 1% P/S and 20% L929 cell media. The cells were cultured for 7 days, additional 10% L929 media was added on day 3, before being set up for experiments at $0.5-1 \times 10^6/\text{ml}$.

2.3 Western blotting

2.3.1 Preparation of whole cell lysates

Cells were washed in ice cold PBS before being lysed. 5X Laemmli sample buffer (10% (v/v) glycerol, 2% (w/v) SDS, $200 \mu\text{g}/\text{ml}$ bromophenol blue, 125mM Tris pH 6.8 and 5% DTT (that was added just before use)) was used to prepare cell lysates. Samples were then removed to microcentrifuge tubes and boiled for 5 min at 95°C . The samples were either analyzed or frozen at -20°C freezer for analysis at a later time point.

To quantify protein content BRADFORD assay was used. For analysis of proteins by BRADFORD, the cell lysates were prepared by lysis in low stringency lysis buffer [50 mM Hepes pH 7.5, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.5% Nonidet P40 (NP-40), 1 mM phenylmethanesulphonyl fluoride (PMSF), 1 µg/ml leupeptin, 11.5 µg/ml aprotinin and 1 mM sodium orthovanadate] on ice for 10 min. Lysates were transferred to microcentrifuge tubes and centrifuged at 1610 x g for 20 min at 4°C. Bradford was performed on these samples.

2.3.2 Bradford method for determination of protein concentration in cell lysates

Samples were diluted 1:5 with dH₂O to give a final volume of 5 µl and 250 µl Bradford Reagent (0.01 % (w/v) Coomassie Brilliant Blue G250, 4.7 % (v/v) ethanol, 8.5 % (v/v) orthophosphoric acid) was added to each. The reaction was allowed to develop for 5min after which time the absorbance at 595 was read using a FLUOstar Optima (BMG LABTECH). Protein concentrations were determined using a standard curve constructed using BSA concentrations in the range 1 - 1500µg/ml.

2.3.3 Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Bio-Rad® apparatus was used to prepare gels. A 12% or 15% resolving gel was poured first. To make two gels 10mls was prepared as follows: For 12% 3.3 ml H₂O or for 15% 2.3 ml H₂O, for 12% 4 ml 30% (v/v) acrylamide mix or for 15% 5 ml

30% (v/v) acrylamide mix (29.2% acrylamide and 0.8% N,N'-methylene-bis-acrylamide), 2.5 ml 1.5M Tris-HCL (pH 8.8), 100 μ l 10% (w/v) SDS, 100 μ l 10% (w/v) ammonium persulphate (APS) and 4 μ l N,N,N',N'-tetramethylethylenediamine (TEMED). After the resolving gel was set, the stacking gel was poured. For two gels, 4 mls were prepared as follows: 2.7 mls H₂O, 0.67 mls 30% (v/v) acrylamide mix, 0.5 ml 1M Tris (pH 6.8), 40 μ l 10% (w/v) SDS, 40 μ l 10% (w/v) APS and 4 μ l TEMED. The gels were placed in a Bio-Rad® gel running apparatus containing running buffer (1X made from 10X stock which is prepared as follows: 30.3g 25mM Tris, 144g 192mM Glycine, 10g 0.1% SDS and made up to 1L with distilled H₂O). The protein samples and a molecular weight standard were added to the gels. The gels were then electrophoresed using a constant current of 25 mA per gel, until the dye front from the sample buffer ran off the end of the gel.

2.3.4 Electrophoretic transfer of proteins using a wet transfer system

The resolved proteins were transferred to Immobilon™ polyvinylidene difluoride (PVDF) membrane using a wet transfer system. A piece of PVDF membrane was soaked in methanol to activate it. All other components were soaked beforehand in transfer buffer (25 mM Tris pH 8.0, 0.2 M glycine, 20% methanol). The gel was placed on two sheets of filter paper and sponge overlaid with the membrane. Two more sheets of filter paper were placed on top followed by a second sponge. The entire assembly was placed in a cassette, the chamber was filled with transfer buffer and a constant current of 150 mA was applied for 2 hours.

2.3.5 Electrophoretic transfer of proteins using a semi-dry transfer system

The resolved proteins were transferred to Immobilon™ polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer system. A piece of PVDF membrane was soaked in methanol to activate it followed by soaking in buffer 2 (40ml 3M TRIS pH 10.4, 80ml MeOH, 280 dH₂O). All other components were soaked beforehand in three different buffer. From bottom up, 1 piece of filter paper was soaked in buffer 1 (3.3ml 3M TRIS pH 10.4, 80ml MeOH, 316.7 dH₂O) followed by another piece of filter paper on top soaked in buffer 2. The membrane was then placed followed by the gel. Two pieces of filter paper were then soaked in buffer 3 (10ml 1M TRIS pH 9.7, 80ml MeOH, 1.9ml Caproic Acid (8.6M), 308.1ml dH₂O). The gel was placed on two sheets of filter paper and sponge overlaid with the membrane. The entire assembly was placed in semi-dry transfer with a constant current of 15 V (800mA and 300W) applied for 35 minutes.

2.3.6 Blocking of non-specific binding sites

The membrane was incubated in 5% non-fat dried milk powder (Marvel) to block non-specific binding of protein to the in 0.01% (v/v) Tris buffered saline (TBS)-Tween-20® for one hour at room temperature (RT) on a slow shaker. TBS-Tween-20® was prepared from a 10X stock which was made up as follows: 12.11g Tris, 87.6g NaCl, 10ml Tween-20® and made up to 1L in distilled H₂O.

2.3.7 Antibody probing

The membranes were then incubated with the antibody to the protein of interest diluted 1 in 1000 in 5% Marvel or BSA (w/v) in 0.1% (v/v) TBS-Tween-20®. The rotating incubation was either carried out overnight at 4°C or for 2 hrs at RT depending on the antibody. After the incubation the membranes were washed 4 times in 0.1% (v/v) TBS-Tween-20® for 10 min at RT. The membranes were then incubated with either anti-mouse, or anti-rabbit (1 in 2,000 dilution in 5% Marvel in 0.1% (v/v) TBS-Tween-20®) IgG horse radish peroxidase conjugate antibody, depending on the primary antibody that was used, for an hour at RT. The membranes were then washed 4 times in 0.1% (v/v) TBS-Tween-20® for 10 min at RT. Chemiluminescent substrate was then added to the surface of the membranes for 1 min. The chemiluminescent substrate was prepared according to the manufacturers instructions (Cell Signaling). The membranes were then placed in a cassette together with Scientific Imaging Film. The film was developed and the position of the antibodies visualised.

2.3.8 Stripping and reprobing of PVDF membranes

A membrane may be probed for multiple proteins if it is stripped of the initial antibody and reprobed with a new antibody of interest. The membrane was washed in 0.1% (v/v) TBS-Tween-20® and then incubated for 5-15 mins in Restore™ PLUS Western blot stripping buffer. The membrane was washed in 0.1% (v/v) TBS-Tween-20® and blocked in 5% (w/v) Marvel in 0.01% (v/v) TBS-Tween-20® for

20 min at RT. The membrane was then probed and developed as described in section 2.3.6.

2.4 Plasmid DNA preparation

2.4.1 Plasmid transformation

E. coli DH5 α cells were transformed with DNA. 50 ng of plasmid DNA was placed in a micro centrifuge tube and 50 μ l of cells were added. The cells were incubated on ice for 30 min. Cells were then placed at 42°C for 45 seconds and then on ice for 2 min. All of the cells were then plated on Lysogeny broth (LB) agar plates supplemented with ampicillin or kanamycin. Agar plates were incubated at 37°C for 18 hrs.

2.4.2 Plasmid purification

After transformation, an individual colony was picked for a starter culture and added to 2 ml of LB medium containing the appropriate selective antibiotic for 8 hours at 37°C, shaking at 300 rpm. 100 μ l of this starter culture was added to 100 ml of LB medium containing the appropriate selective antibiotic overnight at 37°C, shaking at 300 rpm. The bacterial cells were harvested by centrifugation at 3220 $\times g$ for 30 min at 4°C. Plasmid DNA was purified using a Maxiprep kit. The cultures were transferred to 50 ml tubes and centrifuged at 6000 $\times g$ for 15 min at 4 °C. The bacterial pellets were resuspended in 10 ml of buffer P1. 10 ml of buffer P2 was added and the tubes were mixed vigorously. They were incubated at room temperature for 5 min. 10 ml of chilled Buffer P3 was added to the lysate, and mixed thoroughly. The lysate was poured into the barrel of a QIAfilter cartridge and

incubated for 10 min. The cap was removed from the cartridge, the plunger was inserted and the cell lysate was placed into a clean 50 ml tube. 2.5 ml of buffer ER was added to the lysate and mixed and inverted 10 times. This was then incubated on ice for 30 min. A QIAGEN tip was equilibrated by applying 10 ml of Buffer QBT. The column was left to empty by gravity flow. The filtered lysate was added to the QIAGEN tip and left to empty by gravity flow. The tip was washed with 2×30 ml of Buffer QC. The DNA was eluted with 15 ml of Buffer QN. The DNA was precipitated by the addition of 10.5 ml of isopropanol. This was centrifuged at $6,000 \times g$ for 60 min. The DNA pellet was washed with 5 ml of endotoxin free 70 % ethanol and centrifuged at $6,000 \times g$ for 30 min. The supernatant was removed and the pellet was left to air dry for 10 min and subsequently resuspended in between 200-500 μ l of endotoxin free Buffer TE.

2.5 Transfection

2.5.1 Transient transfection using DNA plasmids

HEK293TLR4-MD2-CD14 and RAW264.7 cell lines were transfected with large DNA plasmids using GeneJuice. Briefly, cells were plated 1 -2 days before transfection. When the HEK293TLR4-MD2-CD14 cell line had reached 80 % confluency it was transfected with DNA. When the RAW264.7 cell line reached 60 % confluency, transfection proceeded. A mixture of 6 % GeneJuice in serum-free DMEM was prepared and incubated at room temperature for 10 min. Endotoxin-free plasmid DNA, prepared as outlined in 2.4.1-2, was plated into empty wells of a 96-well plate or into an eppendorf tube. For luciferase assays, all samples were transfected with

the same amounts of control phRL-TK (Promega) and Firefly luciferase-reporter vector, in triplicate, to a total of 200 ng DNA per point. Regarding the 6 % GeneJuice-DMEM mixture, 1/10th the volume of the cell/media mixture was added on top of the DNA. For 100 μ L cells in a 96-well plate, 10 μ L of transfection reagent-OptiMEM was added to the appropriate volume of DNA. For 500 μ L cells in a 24-well plate, 50 μ L of transfection reagent-OptiMEM was added to the appropriate volume of DNA. Complexes were incubated for 15 min at room temperature. Following this, DNA-GeneJuice-DMEM mixture was added on top of the cells and the plates were gently rotated by hand for a few seconds before being returned to the incubator. For most DNA transfection experiments, cells were left between 24-48 h to recover before stimulation with TLR ligands for the appropriate times.

2.5.2 Transient transfection using small RNA

Oligonucleotides were resuspended in the appropriate volumes of RNase-free water, upon receipt of lyophilized RNA, to obtain a 50 μ M stock for miRNA oligonucleotides and siRNAs. RNA was aliquoted and stored frozen at - 20 °C. For transient transfections with RNA oligonucleotides, cells were plated 1 – 2 days before transfection. Cells were transfected with small RNA when they had reached 60 -80 % confluency. For every RNA transfection, equal total concentrations of RNA were transfected per reaction, consisting of two RNA species – a non-targeting negative control and the “target” RNA e.g. -eIF4A1 or eIF4A2 siRNA. For transfection with siRNA, LIPOFECTAMINE 2000 was used as the transfection reagent. Firstly, a mixture of 2 % transfection reagent in OptiMEM media (serum-

free) was made up and incubated at room temperature for 5 min. The appropriate volume of RNA for each transfection reaction was pipette into an RNase-free eppendorf or empty well of a 96-well plate, as appropriate. The total volume of RNA used in each transfection reaction was also kept constant. Dilutions to RNA stocks were made up in pre-warmed OptiMEM media. To the RNA, a volume of the 2 % transfection reagent-OptiMEM mixture was added, 1/10th the volume of the cell/media mixture to which it would be added. For 100 µL cells in a 96-well plate, 10 µL of transfection reagent-OptiMEM was added to the appropriate volume of RNA. For 500 µL cells in a 24-well plate, 50 µL of transfection reagent-OptiMEM was added to the appropriate volume of RNA. This RNA-transfection reagent-media mixture was incubated at room temperature for 20 min before being added on top of the cells. Following addition to plates, the cells were gently rotated by hand for a few seconds before being returned to the incubator. For most RNA transfection experiments, cells were left 24 h to recover before stimulation with TLR ligands for the appropriate times. The table below illustrates volumes used for a reaction mixture of total 250µl.

Tube A	125µl Opti-mem	4 µl Lipofectamine 2000
Tube B	125µl Opti-mem	1 µl miRNA/mimic/scramble

2.6 RNA analysis

2.6.1 Trizol method for purification of total RNA

800µl of Trizol reagent was added to 120 µl of Preswell Protein A Sepharose CL- 4B beads with protein + RNA from 1×10^7 cells containing 0.1% SDS and 10 µl proteinase K (20 mg/ml). It was then mixed for 45 seconds by vortexing. The homogenate was let sit at room temperature for 5 min, followed by addition of 160 µl of Chloroform (Sigma). The mixture was then inverted and let sit for 5 min. The mixture was then centrifuged at $12000 \times g$ for 15 min at 4 °C. The top aqueous layer was carefully removed and placed into an RNase-free ependorf tube. The RNA was subsequently precipitated from this mixture by ethanol precipitation. Briefly, 1.5 volume (~400µl) isopropanol was added to the extract and left for 5 min at room temperature. This was centrifuged at $12000 \times g$ for 15 min. Supernatant was removed and excess isopropanol removed carefully without disturbing the pellet. The pellet was subsequently washed twice with 800 µl of 70 % EtOH. It was centrifuged at full speed for 5 min. The resulting RNA pellet was briefly air-dried and resuspended in 15-30 µl RNase-free water.

2.6.2 Modified Qiagen RNeasy kit to extract total (including small) RNA

All steps were carried out in RNase-free conditions and on ice. Media was removed from the cells and they were washed briefly in 1X PBS. Cells were lysed in an appropriate volume of Qiagen Buffer RLT, supplemented with 10 % β-mercaptoethanol (β-ME), ($<1 \times 10^7$ cells – 350µL, $>1 \times 10^7$ cells – 500µL). 1.5 volumes of 100 % Ethanol was added to the lysate and the mixture was vortexed gently. The

mixture was applied, 700 μL at a time, to a Qiagen RNeasy Column, and passed through by centrifugation at 10,000 rpm for 30 sec. Following application of the lysate, the column was washed twice using 500 μL Qiagen Buffer RPE. Following this the column was placed in a clean, RNase-free eppendorf. 20 μL of RNase-free water was applied to the column which was subsequently centrifuged at full speed for 1 min to elute the RNA.

2.6.3 Normalisation and dilution of RNA

The RNA was quantified using a Nanodrop 2000 micro-volume UV-vis spectrophotometer and normalized using nuclease free water. For mRNA 100 ng/ μL final volume was used and for miRNA 10 ng/ μL was used.

2.7 Reverse transcription PCR

2.7.1 microRNA specific reverse-transcription

A unique reverse-transcription step specific for each microRNA (miRNA) was performed on each RNA sample using the Applied Biosystems TaqMan miRNA reverse transcription kit and a stem-loop primer specific to each miRNA. The 15 μL reaction consisted of 1.5 μL 10X reverse transcription buffer, 0.15 μL 100 mM dNTP mix, 0.19 μL RNase inhibitor, 1.0 μL MultiScribe reverse transcriptase, 3.0 μL miRNA-specific stem-loop RT primer, 4.16 μL RF-H₂O and 5 μL 2 ng/ μL RNA sample (10 ng total). Following assembly of reactions in a 96-well plate, plates were sealed and centrifuged at 1000 rpm for 20 sec. Plates were placed in a 96-well Thermal-

Cycler (Mastercycler gradient, Eppendorf), and the following program ran: 16 °C – 30 min, 42 °C – 30 min, 85 °C – 5 min, 4 °C HOLD.

2.7.2 miRNA specific real-time PCR amplification

TaqMan miRNA specific probes were used to quantify miRNA expression in real-time (Applied Biosystems). For PCR, an Applied Biosystems 7500FAST or 7900 HT machine was used in 96-well FAST format. Reactions were performed in quadruplicate in the case of the 150 miRNA screen and subsequently in triplicate. Reactions were set up as follows:. For the 7500FAST: 10 µL reactions were prepared as follows: 5.0 µL 2X TaqMan FASTmix, 3.5 µL RNase-free-H₂O, 0.5 µL 20X miRNA specific primer/probe mix and 1.0 µL miRNA-specific RT product. Alternatively, 5 µL reactions were performed in which the volumes of all components used were halved. For the 7500FAST cycling was performed as follows: 40 cycles of 95 °C – 5 sec, 60 °C – 30 sec. Reactions were prepared in appropriate optical-well plates (FAST format 96-well). Plates were sealed following addition of RT product and centrifuged at 1000 rpm for 30 sec. Plates were loaded into the appropriate machine. Applied Biosystems SDS software was used to program the appropriate cycle. All probes used for miRNA-PCR were FAM-labelled. Amplification plots for each PCR reaction were generated using the SDS software from which Cycle Threshold (C_t) values could be derived. Relative quantification was performed and the $\Delta\Delta C_t$ method was used to calculate fold changes in mRNA expression relative to mRNA from unstimulated samples, as described in 2.7.5. Figure 2.7.2 illustrates stem-loop TaqMan PCR for miRNA expression.

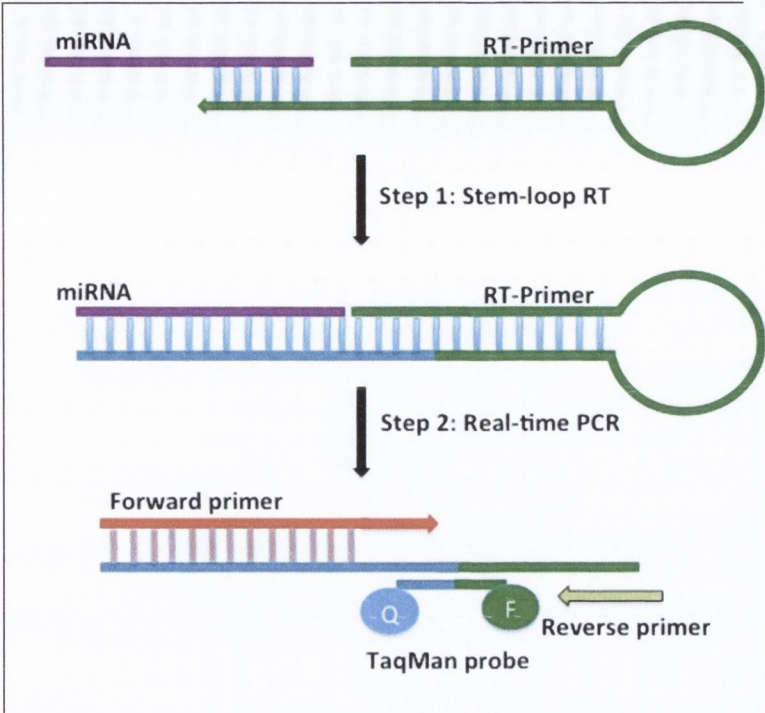


Figure 2.7.2 Stem-loop TaqMan PCR for miRNA expression. A miRNA specific primer, containing a stem-loop structure at the 5' end and a miRNA specific 3' end binds the target miRNA. Extension occurs at the 3' end by reverse transcriptase resulting in a long DNA molecule which contains the universal stem-loop structure and a miRNA

specific antisense region. The resulting cDNA is quantified by real-time PCR. The high temperatures of real-time PCR denature the stem-loop structure. Forward and reverse primers bind unique miRNA complementary site and the stem-loop structure. Quantification of PCR process is carried out by the use of a specific TaqMan probe which binds the RT product. The TaqMan probe contains a Fluorophore and a Quencher, (FAM labeled). With each amplification step, the 3' – 5' exonuclease activity of the Taq polymerase enzyme degrades the TaqMan probe, releasing fluorescence which can be monitored at the wavelength specified for FAM. This allows quantification of starting cDNA/RNA.

2.7.3 Gene specific reverse transcription PCR

For generation of cDNA libraries from RNA samples the High Capacity cDNA Archive Kit II (Applied Biosystems) was used. 20 μL reactions were prepared, containing 2 μg of each RNA sample in a volume of 10 μL . To this 2.0 μL 10X Reverse Transcription buffer, 0.8 μL 100 mM dNTP mix, 2.0 μL 10X Random Primer mix, 1.0 μL MultiScribe Reverse Transcriptase and 4.2 μL RF-H₂O were added. Reverse transcription was performed in a 96-well plate. When the plate was sealed it was centrifuged at 1000 rpm for 30 sec. It was then placed in a Thermalcycler (Mastercycler gradient, Eppendorf) and the following cycle was performed: 25 $^{\circ}\text{C}$ – 10 min, 37 $^{\circ}\text{C}$ – 2 h, 4 $^{\circ}\text{C}$ – HOLD.

2.7.4 Gene specific real-time PCR amplification

Gene-specific FAM labelled probes and primers were designed by Applied Biosystems (TaqMan gene expression assays). The endogenous control probe, murine GAPDH, was VIC labelled. PCR reactions were carried out on an AB7500FAST machine in 96-well FAST format. Briefly the reactions were prepared as follows: 5.0 μL 2X TaqMan FASTmix, 3.5 μL RF-H₂O, 0.5 μL 20X gene specific primer/probe mix and 1.0 μL cDNA. Cycling was performed as follows: 40 cycles of 95 $^{\circ}\text{C}$ – 5sec, 60 $^{\circ}\text{C}$ – 30sec. Amplification plots for each PCR reaction were generated using the SDS software from which Cycle Threshold (C_t) values could be derived. Relative quantification was performed and the $\Delta\Delta C_t$ method was used to calculate fold changes in mRNA expression relative to mRNA from unstimulated samples, as described in 2.7.5.

2.7.5 Real-time PCR data analysis

Amplification plots for each PCR reaction were generated using the SDS software from which Cycle Threshold (C_t) values could be derived. Relative quantification was performed and the $\Delta\Delta C_t$ method was used to calculate fold changes of each miRNA (or mRNA) relative to miRNA (or mRNA) from unstimulated samples. Briefly summarised, the C_t values for each reaction – endogenous control and test probe for calibrator and test samples, were calculated using the SDS software. For each detector/probe, the effect of loading and RNA normalisation was corrected by subtraction of the C_t value for the endogenous control for each reaction/sample. This gives the ΔC_t value. Then, the ΔC_t value of the calibrator sample is subtracted from the ΔC_t of each test sample. The resulting $\Delta\Delta C_t$ value is normalised using the formula $2^{(-\Delta\Delta C_t)}$. The resulting value is the fold-change in expression for a test sample relative to the calibrator sample for that particular detector/probe normalised using an endogenous control reference.

2.8 Enzyme-linked immunosorbent assay

Supernatants were analysed for murine IL-10, TNF- α , and IL-6 using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems). The optical density values were measured at 450 nm and concentrations were calculated using a standard curve. The plates were read at using a FLUOstar Optima (BMG LABTECH) plate reader.

2.8.1 Coating plate with capture antibody

Nunc F96 Maxisorp immuno-plates or Corning 96 well EIA/RIA plates were used for ELISA analysis. The capture antibody, an antibody specific to a particular epitope on the protein-of-interest, was diluted to the appropriate concentration (usually 1/180) in sterile 1X PBS. 50 μ L of capture antibody solution was pipetted onto each well and left overnight at room temperature. The next day each well was washed in triplicate with 0.05 % Tween in 1X PBS. Following this, each well was blocked with 200 μ L 0.5 % BSA in 1X PBS for 2 h at room temperature. Following this each well was washed three times with 0.05 % Tween in 1X PBS.

2.8.2 Application of standards and samples

Supernatants were collected from each experiment and either applied directly to an ELISA plate or stored frozen at -80 $^{\circ}$ C until ready to analyse. Standards for each molecule were prepared on the day. These consisted of defined amounts of recombinant protein-of-interest. A top standard, usually of 2000 pg/ml for murine ELISA was prepared in 0.5 % BSA in 1X PBS to be used at 3 X 50 μ L of each concentration. When the standards and plates were prepared standards were applied in triplicate along the left-hand side of each plate. A blank was also applied containing no recombinant protein-of-interest, with only 50 μ L 0.5% BSA in 1X PBS. Samples were also applied in triplicate and their positions on the plate noted. Samples and standards were incubated either overnight at 4 $^{\circ}$ C or for 2 h at room temperature.

2.8.3 Detection and determination of cytokine content of samples

Following incubation of the appropriate standards and the samples, each well was washed three times using 0.05 % Tween in 1X PBS. Detection antibody, a biotin-labelled antibody specific to another epitope on the protein-of-interest, was diluted to the appropriate concentration (usually 1/180) in 0.5 % BSA in PBS. 50 μ L of the detection antibody solution was applied to each well and left at room temperature for two hours. Following this each well was washed three times using 0.05 % Tween in 1X PBS. Streptavidin-horseradish peroxidase conjugate was then applied. This was diluted 1/200 in 0.5 % BSA in 1X PBS and 50 μ L of the solution applied to each well. This was left covered for 30 min at room temperature. Following this the plate was washed three times using 0.05 % Tween in 1X PBS. TMB substrate reagent (BD OptEIA) was used as the substrate for the horseradish peroxidase. Equal volumes of substrate A and B were mixed together and 50 μ L of the mixture applied to each well. The plate was subsequently covered and left in the dark briefly. When the bottom standards turned a light blue colour 25 μ L 1 M Sulphuric Acid was applied to each well. This terminated the HRP/TMB reaction and also turned the colour in each well yellow. The plate was then read using a FLUOstar Optima (BMG LABTECH) microplate reader at 450 nm. Microsoft Excel was used to generate a standard curve for the protein-of-interest, from which the concentration of protein-of-interest in each sample was determined. The data is presented as mean of three determinations $\pm$ SEM.

2.9 Immunoprecipitation for RNA extraction

An RNA-Immunoprecipitation (RNA-IP) assay was used to pull down specific protein and detect mRNA binding. 10 millions cells were cultured in 10cm non-tissue culture treated plates. The assay was carried out over a period of 3 days. On day 1 the beads were prepared. For each condition, 200 µg of Preswell Protein A Sepharose CL- 4B beads were soaked in 1ml 1X PBS 1X for 30 minutes on ice. Following this, the beads were washed. This involves centrifuging the beads for 3 minutes at 3000 g at 4°C. This step was repeated three times and then the supernatant was removed and the pellet of beads was resuspended in an equal volume (100 µl) of buffer 2 (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM MgCl₂; 0.5% NP-40; and milliQ water) and kept on ice. 5µl of 1mg/ml immunoprecipitating antibody anti-eIF4A1 (specific to the protein) or control Rabbit IgG was added to preswollen Preswell Protein A Sepharose CL- 4B beads. Preswell Protein A Sepharose CL- 4B + antibody were incubated overnight at 4°C, rocking end-over-end. On Day 2, the sample and control cells were prepared. The cells were washed three times with ice cold PBS and placed on ice. The cells were scraped in ice cold PBS and kept on ice and were pelleted and resuspended in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4; 0.5% NP-40; 150 mM NaCl; 1 mM MgCl₂; 0.25 mM phenylmethanesulfonylfluoride; 0.5 mM DTT and milliQ water). A cocktail of protease inhibitors (Roche) and 1µl (40U/µl) RNase inhibitor (Invitrogen) were added additionally to prevent any protein and mRNA degradation. The cells were then vortexed and passed through 1cc U-100 Syringe 28 G1/2 (0.36mm x 13mm), 5 times. The resulting lysed cells were left on ice for 15 minutes. At this stage 50 µl of

each condition was stored to analyse later by western blot for analysis of protein content in cell lysate. A separate sample of 50µl was also stored for RNA extraction to calculate total RNA concentration of gene of interest present in sample. The lysate was then centrifuged at 8000 g for 10 minutes at 4°C. The supernatant containing the cell lysate was collected. The preswollen Preswell Protein A Sepharose CL- 4B beads + antibody were washed three times in 1 ml of Buffer 2, Centrifuged for 3 minutes at 3000 g at 4°C and kept on ice. The supernatant was pre-cleared with no-antibody containing preswollen Preswell Protein A Sepharose CL- 4B beads for 1 hour at 4 °C. The beads were then centrifuged at 3000 g for 5 minutes. The supernatant containing cell lysate was added onto the Preswell Protein A Sepharose CL- 4B beads + anti-eIF4A1 or Rabbit IgG. The cell lysate was mixed with the beads by gentle tapping and were incubated for 2 hours at 4 °C with tumble end-over-end. Following this the beads were spun down at 3000 g for 5 minutes. The beads contain the immunoprecipitate. The supernatant was stored to analyse unbound protein. The beads and bound antibody containing protein complex were washed with ice cold lysis buffer and centrifuged at 3000 g for 3 minutes at 4 °C. The wash step was repeated twice. A small sample of beads (30 µl) was taken to analyse protein binding. The rest was used to extract RNA. The protein was digested using digestion buffer (0.1% SDS and 10 µl proteinase K (20 mg/ml)). Following the addition of digestion buffer the resulting mixture was incubated for 30 minutes in a 55°C water bath. RNA was extracted as previously described in section 2.6.1. Figure 2.9 illustrates a diagrammatic representation of RNA-IP.

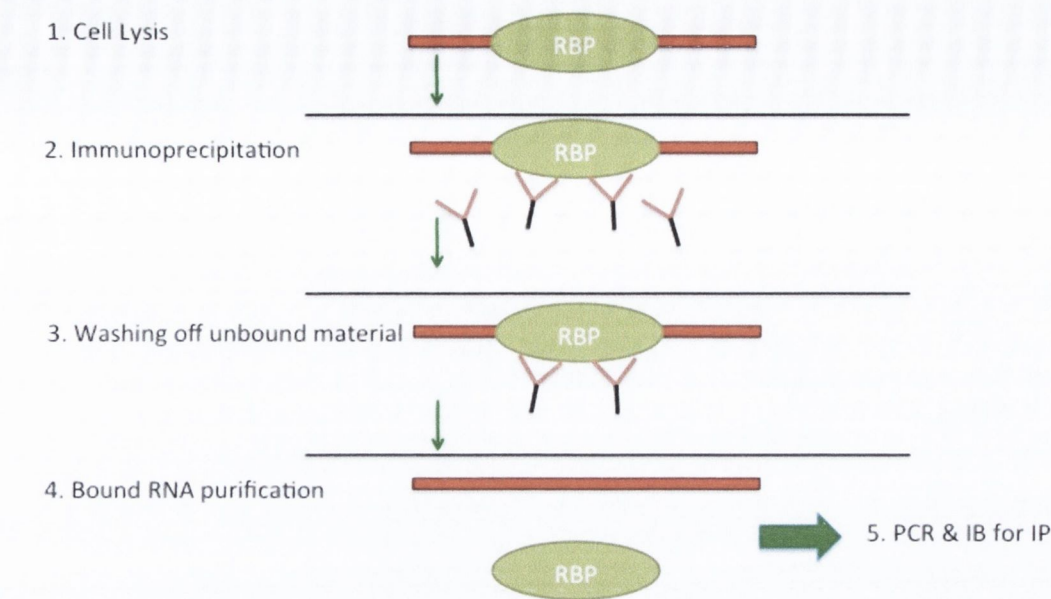


Figure 2.9 Flow diagram of RNA-IP

The figure illustrates step by step process involved in RNA-Immunoprecipitation. The cells are collected with various treatments and lysed in a special lysis buffer. The samples are then immunoprecipitated with protein of interest. The unbound or weakly bound material is washed off. IP is then used to extract RNA. RNA is amplified using PCR and qPCR.

2.10 Luciferase reporter assays

2.10.1 Transient transfections and stimulations

For luciferase assays, cells were set-up in 96-well plates at 2×10^5 cell/ml, 100 μ l/well. When cells had reached 80 % confluency they were transfected with DNA plasmids as described in 2.5.1. Cells were left for 24 hours. The cells were subsequently washed with warm PBS and fresh media was added. The cells were

then left 18 – 24 hours to recover and subsequently stimulated with LPS or vehicle control.

2.10.2 Assay procedure

Cells were washed with PBS then were then lysed for 15 min at room temperature with 50 µl of 1X Passive Lysis Buffer with shaking at 300 rpm. 20 µl of the lysed cell mix was taken and tested for firefly luciferase activity and 20 µl of the lysed cell mix was tested for Renilla luciferase activity. Firefly luciferase activity was assayed by the addition of 40 µl of luciferase assay mix (20 mM tricine, 1.07 mM $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$, 2.67 mM MgSO_4 , 0.1 M EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferin, 530 mM ATP) to the sample and Renilla luciferase was read by the addition of 40 µl of a 1:1000 dilution of Coelentrastazine in PBS. Luminescence was read using a FLUOstar Optima (BMG LABTECH). Firefly luminescence readings were corrected for Renilla activity and expressed as fold stimulation over unstimulated negative control.

2.10.3 IL-10 5'UTR

The IL-10 5'UTR (ACATTTAGAGACTTGCTCTTGCACTACCAAAGCCACAAGGC AGCCTTGCAAGAAAAGAGAGCTCCATC) was cloned in pGL3-control vector between HindIII and NcoI site, upstream of luciferase construct by a Gene synthesis service (MWG). Luciferase assay with the 5'UTR of IL-10 is depicted in Figure 2.10.3.

2.11 Statistical analysis

Statistical analysis was carried out using an unpaired Student’s t test using Prism Software (GraphPad). P values < 0.05 were considered to be statistically significant.

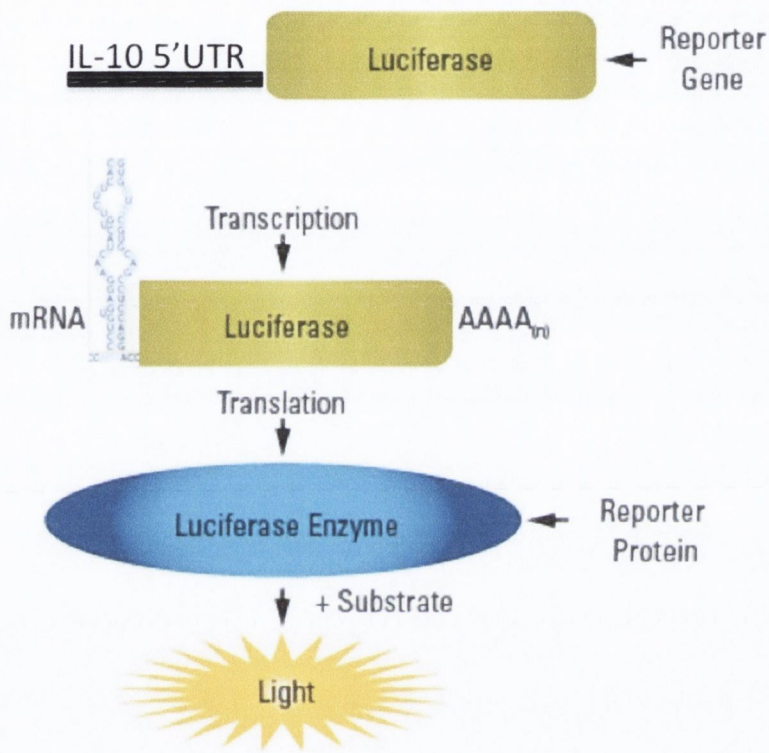


Figure 2.10.3 Flow diagram of Luciferase with 5'UTR of IL-10

IL-10 5'UTR was cloned upstream of luciferase reporter gene. The 5'UTR is predicted to form a secondary hair-pin structure as shown. The hair-pin loop would be transcribed into the mRNA from a constitutive promoter. The translation of the luciferase mRNA with IL-10 5'UTR could be measured by addition of the substrate and measurement of luminescence (depicted by the light). This would then be compared with wild-type control vector.

Chapter 3

Results

Chapter 3

Results

3.1 Introduction

Most studies on the role of miRNA in innate immunity have focused on LPS-inducible miRNA. The role of miRNA that are repressed in response to LPS has been less well-characterised. Even though a few studies have identified miRNA that are repressed in response to LPS, this has not been the main focus of these studies. miR-107, miR-125b and let-7i have been described as LPS-repressed miRNA. These studies have uncovered critical roles that these miRNA play in macrophage biology. miR-107 blocks CDK6 translation and thereby prevents macrophage adhesion. This repression is lifted in LPS-stimulated macrophage. Let-7i blocks TLR4 protein synthesis, and miR-125b blocks TNF α . These breaks are lifted in response to LPS.

We therefore decided to screen for LPS-repressed miRNA, and identify and study the targets of these miRNA. A number of techniques are available to measure differential expression levels of miRNA.

Deep-sequencing was chosen to measure the expression changes. This technology is advantageous because it provides exact copy numbers of miRNA that reflects abundance, in addition it can identify any new miRNAs that may be macrophage-specific.

In this study a number of LPS-repressed miRNA predicted to target eIF4A were identified which led us to examine the eIF4A expression level plus its role in translation of cytokines in response to LPS. Of the three stages of translation, initiation is the rate-limiting step and undergoes the most regulation, of which eIF4A, a protein involved in the eIF4F complex (as described in section 1.12), is a key component.

Various components of eukaryotic initiation factors have been implicated in different types of inflammatory diseases and cancer. Although the translation machinery is very well understood, new insights are revealing further complexity in the link between diseases and translation machinery. Interactions between different components of the eIF4F complex are known, however how the various components are regulated is not fully understood, especially in LPS-treated macrophage.

Here we fully characterize the role of eIF4A in the induction of IL-10 by LPS.

3.2 Results

3.2.1 Deep-sequencing analysis to identify LPS repressed miRNA in BMDM

Deep-sequencing analysis was carried out on BMDM stimulated with LPS. Figure 3.2.1 A and B illustrate that the BMDM responded to LPS as indicated by the increase in IL-6 (Figure 3.1 A) and TNF α (Figure 3.1 B) at 24 hours. Samples from these LPS-responsive cells were then extracted for RNA analysis and sent for deep-sequencing to identify the expression of various different miRNA. A total of 180 miRNA were identified as LPS-repressed (Figure 3.1 C-H). Figure 3.1 C, and D illustrate miRNA that were most strongly repressed, up to 4 fold (log₂ scale), in response to LPS. Figure 3.1 E, F, G and H illustrate miRNA that were repressed less than 1 fold (log₂ scale) in response to LPS. It is worth noting that miR-107 (Figure 3.1 C), and miR-125b (Figure 3.1 D), known to be LPS-repressed, were also LPS-repressed here. These two LPS-repressed miRNA acted as a positive control to validate LPS-based repression. Induction of miR-155, miR-21 and miR-146a, which are known to be LPS-inducible, was also observed (Figure 3.1 I).

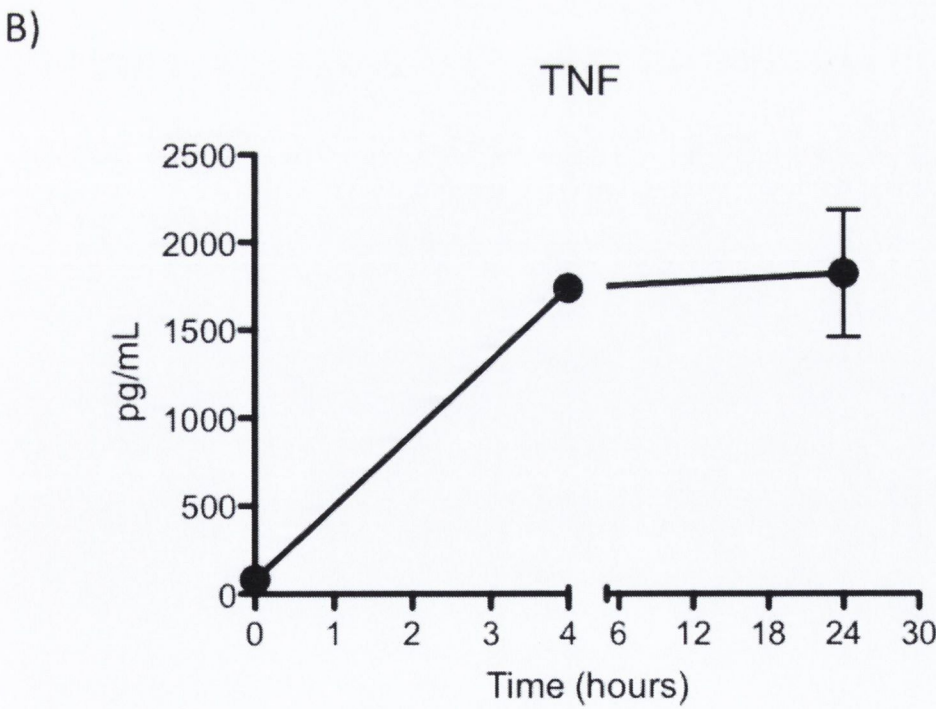
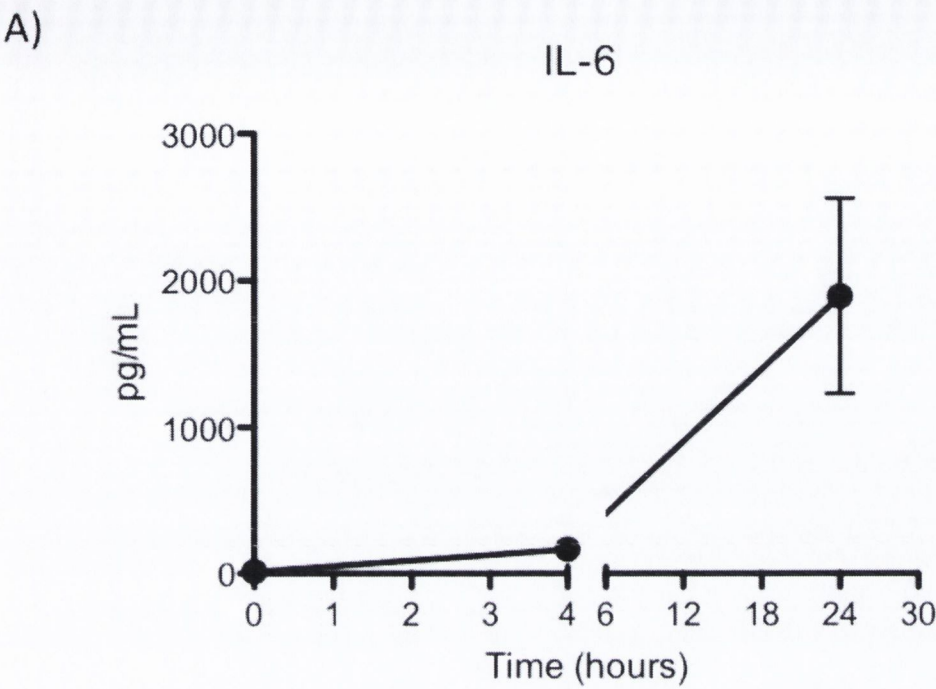
3.2.2 LPS-repressed miRNA target prediction

After generating the list of LPS-repressed miRNA, a literature review was performed to search for any known targets of these miRNA. This did not reveal any immunologically relevant targets. Target prediction analysis on all 200 of the miRNA

in the list was performed next. The goal was to generate a shortlist of miRNA that are predicted to target the key components of the inflammatory response in BMDM. A number of different miRNA target prediction programs were used, to minimize false positives. The programs that were used were: Miranda, miRBase, TarBase, TargetScan and PicTar. Manual prediction was also employed, where seed sequence was searched in the mRNA sequence for verification.

As shown in the Table 3.2 eight key miRNA were identified that were predicted to target various eukaryotic translation initiation family members. Let-7b-3p, miR-98-3p and let-7c-1-3p were predicted to target eIF4A2. miR-5121 and miR-98-3p were predicted to target eIF2C4. miR-5107 and miR-3473 were predicted to target eIF2C1. miR-5117 was predicted to target eIF3j and eIF4E whilst miR-3096b-3p was predicted to target eIF5.

LPS-mediated repression of miRNA targeting eIF machinery would promote translation.



c)

Gene	Control1 TPM	Control2 TPM	Control3 TPM	LPS_Osh1 TPM	LPS_Osh2 TPM	LPS_Osh3 TPM	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_Osh1 DESeq	LPS_Osh2 DESeq	LPS_Osh3 DESeq	Controls LPS_Osh TPM log2 ratio	Controls LPS_Osh DESeq log2 ratio
mmu-miR-1948-5p	3.4	1.3	0.4	0	0	0.3	5.7	1.9	0.7	0	0	0.5	-4.09	-4
mmu-miR-2137	4.6	2.6	3.9	0	1.5	0.6	7.6	3.8	6.3	0	2.4	1	-2.4	-2.38
mmu-miR-700-5p	3.4	5.2	3.1	0	0.8	1.5	5.7	7.7	4.9	0	1.2	2.6	-2.35	-2.27
mmu-miR-264-2-3p	4.6	3.9	1.3	0	1.5	0.9	7.6	5.8	2.1	0	2.4	1.6	-2.03	-1.97
mmu-miR-704	2.9	1.3	0	0	0.8	0.9	4.8	1.9	0	0	1.2	1.6	-1.3	-1.28
mmu-miR-273-5p	160.5	94.5	139.5	55.1	40.7	79.1	268.4	180.4	222	89.6	6.3	135.4	-1.17	-1.13
mmu-miR-343-3p	4.6	1.3	2.6	2.9	0	0.9	7.6	1.9	4.2	4.7	0	1.6	-1.16	-1.13
mmu-miR-215-5p	4	9.1	8.7	1.4	4.6	4	6.7	13.5	13.9	2.4	7.1	6.8	-1.12	-1.07
mmu-miR-107-5p	137.6	209.7	133.4	59.4	76.8	95	230.2	311.6	212.3	96.7	118.8	162.6	-1.06	-1
mmu-miR-122-5p	4.6	3.9	0	4.3	0	0	7.6	5.8	0	7.1	0	0	-0.98	-0.92
mmu-miR-5114	10.8	15.5	7.9	1.4	9.2	7.6	18.1	23.1	12.5	2.4	14.3	13.1	-0.91	-0.86
mmu-miR-30c-2-3p	5.1	2.6	12.7	1.4	3.1	6.4	8.6	3.8	20.2	2.4	4.8	11	-0.9	-0.85
mmu-miR-24-1-5p	8	7.8	13.1	5.8	4.6	5.8	13.4	11.5	20.9	9.4	7.1	9.9	-0.84	-0.79
mmu-miR-28b	8.6	11.6	9.2	5.8	6.9	4	14.3	17.3	14.6	9.4	10.7	6.8	-0.82	-0.78
mmu-let-7b-3p	5.7	5.2	4.4	1.4	0	7.3	9.6	7.7	7	2.4	0	12.6	-0.81	-0.7
mmu-miR-3082-3p	7.4	12.9	7.4	5.8	3.8	6.4	12.4	19.2	11.8	9.4	5.9	11	-0.79	-0.72
mmu-miR-98-3p	17.7	22	42.4	10.1	16.9	21.4	29.6	32.7	67.5	16.5	26.1	36.6	-0.76	-0.71
mmu-let-7f-1-3p	5.1	10.4	10.1	4.3	3.8	7.6	8.6	15.4	16	7.1	5.9	13.1	-0.71	-0.62
mmu-miR-128-1-5p	7.4	3.9	6.1	0	6.9	4.3	12.4	5.8	9.7	0	10.7	7.3	-0.64	-0.63
mmu-miR-27b-3p	10.8	12.9	1567.9	11	18.4	993.7	18.1	19.2	2495.1	21.2	28.5	1701.7	-0.63	-0.53
mmu-miR-188-3p	4.6	3.9	1.7	1.4	4.6	0.6	7.6	5.8	2.8	2.4	7.1	1	-0.63	-0.62
mmu-miR-1933-3p	9.1	7.8	7.9	2.9	7.7	5.5	15.3	11.5	12.5	4.7	11.9	9.4	-0.62	-0.6
mmu-miR-185-3p	6.9	7.8	12.7	4.3	6.9	7	11.5	11.5	20.2	7.1	10.7	12	-0.59	-0.54
mmu-miR-34c-5p	4	3.9	44.6	2.9	2.3	28.9	6.7	5.8	71	4.7	3.6	51.2	-0.58	-0.49
mmu-miR-582-3p	26.3	7.8	26.2	18.8	6.9	15	43.9	11.5	41.8	30.6	10.7	25.6	-0.57	-0.54
mmu-miR-497-5p	6.3	6.5	7.4	5.8	3.8	4	10.5	9.6	11.8	9.4	5.9	6.8	-0.57	-0.53
mmu-miR-508-3p	9.1	24.6	17.1	10.1	13.1	11.9	15.3	36.6	27.1	16.5	20.2	20.4	-0.53	-0.47
mmu-miR-5119	17.7	7.8	5.2	15.9	4.6	0.9	29.6	11.5	8.4	25.9	7.1	1.6	-0.52	-0.52
mmu-miR-879-5p	13.1	15.5	17.5	11.6	15.4	5.5	22	23.1	27.8	18.9	23.8	9.4	-0.5	-0.49

D)

Gene	Control1 TPM	Control2 TPM	Control3 TPM	LPS 04h1 TPM	LPS 04h2 TPM	LPS 04h3 TPM	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	Control vs LPS_04h1 log2-ratio	Control vs LPS_04h2 log2-ratio	Control vs LPS_04h3 log2-ratio
mmu-let-7f-2-3p	12.6	18.1	16.6	7.2	13.1	13.1	21	26.9	26.4	31.8	20.2	22.5	-0.5	-0.45	-0.45
mmu-miR-3470b	14.8	18.1	11.8	20.3	6.1	5.5	24.8	26.9	18.8	33	9.5	9.4	-0.69	-0.64	-0.64
mmu-miR-26b-3p	22.3	49.2	52	20.3	26.1	42.8	37.3	73.1	82.8	33	40.4	73.2	-0.47	-0.4	-0.4
mmu-miR-341-3p	25.1	25.9	37.2	10.1	33.8	20.2	42	38.5	59.2	16.5	52.3	34.5	-0.45	-0.44	-0.44
mmu-miR-324-5p	11.4	19.4	14	4.3	20.7	7.6	39.1	28.9	22.3	7.1	32.1	13.1	-0.66	-0.61	-0.61
mmu-miR-744-3p	4.6	7.8	5.7	4.3	4.6	4.3	7.6	11.5	9	7.1	7.1	7.3	-0.46	-0.39	-0.39
mmu-miR-32-5p	8	15.5	11.4	7.2	13.1	5.5	33.4	23.1	18.1	11.8	20.2	9.4	-0.44	-0.4	-0.4
mmu-miR-19b-3p	213.6	491.8	255.7	114.6	354.9	225.7	357.3	731	470.5	202.7	610.7	386.5	-0.43	-0.38	-0.38
mmu-miR-152-5p	50.8	37.5	26.7	34.8	33	17.4	85	55.8	42.5	56.6	51.1	29.8	-0.43	-0.41	-0.41
mmu-miR-30a-3p	6.9	11.6	11.4	7.2	7.7	7.3	11.5	17.3	18.1	11.8	11.9	12.6	-0.43	-0.37	-0.37
mmu-miR-1249-3p	37.1	20.7	13.1	27.5	7.7	17.7	62.1	30.8	20.9	44.8	11.9	30.3	-0.42	-0.39	-0.39
mmu-miR-25-5p	12.6	10.4	7.4	5.8	6.9	10.1	21	15.4	11.8	9.4	10.7	17.3	-0.42	-0.37	-0.37
mmu-miR-125b-1-3p	203.3	471.1	286.9	128.9	378	218	340.1	700.2	456.6	209.8	584.5	373.4	-0.41	-0.36	-0.36
mmu-miR-30c-1-3p	34.8	36.2	38.1	26.1	20.7	35.7	58.3	53.9	60.6	42.4	32.1	61.2	-0.4	-0.35	-0.35
mmu-miR-200b-3p	22.1	24.6	38.1	10.1	34.6	19.5	37.3	36.6	60.6	16.5	53.5	33.5	-0.4	-0.38	-0.38
mmu-miR-7c-1-3p	697.8	572	859	614.3	486.3	519.4	1167.3	850.3	1366.9	999.6	752.1	889.5	-0.39	-0.36	-0.36
mmu-miR-5121	16.6	19.4	12.7	24.6	6.9	5.8	27.7	28.9	20.2	40.1	10.7	9.9	-0.38	-0.34	-0.34
mmu-miR-16-2-3p	8	7.8	13.6	2.9	8.5	11.3	13.4	11.5	21.6	4.7	13.1	19.1	-0.37	-0.32	-0.32
mmu-miR-542-5p	4	3.9	2.2	2.9	3.1	1.8	6.7	5.8	3.5	4.7	4.8	3.1	-0.37	-0.34	-0.34
mmu-miR-28c	101.6	129.4	90.5	100	91.4	58.3	170	192.4	144.1	162.7	141.4	99.9	-0.36	-0.33	-0.33
mmu-miR-324-3p	25.7	38.8	32.8	29	30.7	16.2	43	57.7	52.2	47.1	47.5	27.7	-0.36	-0.32	-0.32
mmu-miR-425-3p	30.3	50.5	29.3	30.4	37.6	18.6	50.6	75	46.6	49.5	58.2	31.9	-0.35	-0.3	-0.3
mmu-miR-24-2-5p	648.7	755.8	955.6	576.6	656.3	628.9	1085.1	1123.4	1520.7	938.3	1075.2	1042.8	-0.33	-0.29	-0.29
mmu-miR-3470a	12.6	18.1	10.1	20.3	6.9	5.5	21	26.9	16	33	10.7	9.4	-0.32	-0.27	-0.27
mmu-miR-126-5p	23.4	34.9	20.6	20.3	25.4	18	39.2	51.9	32.7	33	39.2	30.9	-0.31	-0.26	-0.26
mmu-miR-16-1-3p	19.4	14.2	19.2	20.3	15.4	7	32.5	21.2	30.6	33	23.8	12	-0.31	-0.29	-0.29
mmu-miR-429-3p	10.1	16.8	14.9	8.7	9.2	15.9	17.2	25	23.7	14.1	14.3	27.2	-0.31	-0.24	-0.24
mmu-miR-5126	6.1	0	0	4.3	0.8	0	10.5	0	0	7.1	1.2	0	-0.3	-0.35	-0.35
mmu-miR-148b-5p	58.2	85.4	54.2	46.4	67.6	47.3	97.4	127	86.3	75.4	104.6	81.1	-0.29	-0.25	-0.25
mmu-miR-330-5p	58.2	63.4	68.2	34.8	65.3	55.6	97.4	94.3	108.6	56.6	101	95.2	-0.29	-0.25	-0.25

E)

gene	Control TPM	Control2 TPM	Control3 TPM	LPS O4H1 TPM	LPS O4H2 TPM	LPS O4H3 TPM	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS O4H1 DESeq	LPS O4H2 DESeq	LPS O4H3 DESeq	Control vs LPS O4H log2-ratio	Control vs DESeq-log2-ratio
mmu-miR-142-3p	628.7	1119.5	590.2	514.3	1257.6	490.4	1051.7	1664	1575.7	836.9	1944.9	839.9	-0.28	-0.24
mmu-let-7a-1-3p	22.8	28.5	40.2	26.1	20	29	38.2	42.3	64	42.4	30.9	49.7	-0.28	-0.21
mmu-let-7c-2-3p	22.8	28.5	40.2	26.1	20	29	38.2	42.3	64	42.4	30.9	49.7	-0.28	-0.23
mmu-miR-140-5p	52.5	64.7	58.6	49.3	51.5	45.2	87.9	96.2	93.3	80.2	79.6	77.4	-0.27	-0.23
mmu-miR-1947-5p	12.6	12.9	14	8.7	14.6	9.5	21	19.2	22.3	14.1	21.6	16.2	-0.27	-0.24
mmu-miR-671-3p	101.1	60.8	73	81.1	43.8	71.5	169.1	90.4	116.2	132	67.7	122.4	-0.26	-0.22
mmu-miR-200b-3p	22.3	31.1	23.6	27.5	10	26.9	37.3	46.2	37.6	44.8	15.4	46	-0.26	-0.19
mmu-miR-467a-5p	5.7	6.5	13.6	2.9	9.2	9.5	9.6	9.6	21.6	4.7	14.3	16.2	-0.26	-0.21
mmu-miR-467b-5p	5.7	6.5	13.6	2.9	9.2	9.5	9.6	9.6	21.6	4.7	14.3	16.2	-0.26	-0.21
mmu-miR-3964	119.3	243.3	193.7	155	202.8	109	199.6	361.7	308.3	252.2	313.7	186.7	-0.25	-0.21
mmu-miR-140-3p	1729	1532.3	1759.5	1612.5	1412	1223.6	2892.4	2277.6	2759.9	2623.9	2183.7	2095.5	-0.24	-0.21
mmu-miR-223-3p	1079.2	2462.8	2866	1170.6	2489.9	1749.1	1805.4	3640.8	4540.7	1904.8	3850.6	2995.5	-0.24	-0.2
mmu-miR-338-5p	12	20.7	16.2	10.1	16.9	14.4	20.1	30.8	25.8	16.5	26.1	24.6	-0.24	-0.19
mmu-miR-107e-5p	5239	6829.3	6612.7	4565	6323.4	4951.2	8764.1	10151.3	10163.8	7428.3	9779.1	8679.2	-0.23	-0.19
mmu-miR-101c	461.9	551.3	659.1	456.4	649.2	333.7	772.8	819.5	1048.8	742.6	1003.9	554.3	-0.23	-0.2
mmu-miR-106b-3p	186.7	235.5	109.8	160.8	158.3	134.4	312.4	350.1	174.7	261.7	244.7	230.1	-0.23	-0.18
mmu-miR-5117-5p	30.8	55.6	4.8	71	4.6	2.1	51.6	82.7	7.7	115.5	7.1	3.7	-0.23	-0.17
mmu-miR-29a-5p	5.7	9.1	12.2	10.1	7.7	5.2	9.6	13.5	19.5	16.5	11.9	8.9	-0.23	-0.19
mmu-miR-340-5p	1472.1	1940	1141.9	1556	1720.9	643.1	2462.5	2883.6	1817.2	2531.9	2661.3	1101.3	-0.22	-0.19
mmu-miR-3096b-5p	358.6	117.8	161.8	359.3	95.3	93.1	599.9	175.1	257.5	584.6	147.3	159.5	-0.22	-0.21
mmu-miR-181c-5p	86.2	21.2	78.8	85.5	61.5	52.8	144.2	105.8	119	139.1	95	90.5	-0.22	-0.19
mmu-miR-3068-3p	44	67.3	49	46.4	59.9	31.8	73.6	100	77.9	75.4	92.7	54.4	-0.22	-0.18
mmu-miR-872-3p	34.8	41.4	31.9	24.6	45.3	22.9	58.3	61.6	50.8	40.1	70.1	39.2	-0.22	-0.19
mmu-miR-298-5p	21.7	10.4	92.7	20.3	13.1	73.9	36.3	15.4	147.5	33	20.2	126.6	-0.22	-0.15
mmu-miR-3091c-5p	108.9	85.4	152.6	123.1	69.1	80.3	516.8	127	242.9	525.7	106.9	137.5	-0.21	-0.2
mmu-miR-5109	222.1	173.4	192.4	318.7	68.4	121.5	371.6	257.8	306.2	518.6	105.7	208.1	-0.21	-0.17
mmu-miR-1964-3p	31.4	31.1	57.7	30.4	26.9	47.6	52.5	46.2	91.9	49.5	41.6	81.6	-0.2	-0.14
mmu-miR-1983	3.4	7.8	6.1	5.8	3.8	5.5	5.7	11.5	9.7	9.4	5.9	9.4	-0.2	-0.12
mmu-miR-30a-5p	19027.6	19822.7	21496.1	16972.2	17579.8	18479	31830.6	29465.1	34207.3	27617.6	27186.9	31646.7	-0.19	-0.14
mmu-miR-10a-5p	18931.7	13832	11635	13285.1	15782.9	5811.9	31670.2	20560.4	18515.1	21617.9	24408	16803.6	-0.19	-0.17

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gene	Control1 TPM	Control2 TPM	Control3 TPM	LPS_04h1 TPM	LPS_04h2 TPM	LPS_04h3 TPM	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	Control vs LPS_04h log2-ratio	Control vs LPS_04h DESeq log2-ratio
mmu-miR-10b-5p	17140.9	12667.3	10631.4	12210.2	14496.8	9015.8	29008.9	18829.1	16921.3	19868.7	22409.1	15440.3	-0.19	-0.17
mmu-miR-27b-5p	292.9	300.2	299.2	269.5	257.4	254.1	490	446.3	476	438.5	398	435.1	-0.19	-0.15
mmu-miR-128-3p	74.2	142.4	222.2	101.4	112.9	170.7	124.2	211.6	353.6	165	174.6	292.3	-0.19	-0.13
mmu-miR-3473	10.8	15.5	8.7	15.9	9.2	5.5	18.1	23.1	13.9	25.9	14.3	9.4	-0.19	-0.15
mmu-miR-30a-3p	332.3	267.9	160.5	281.1	235.9	154.8	555.9	398.2	255.4	457.3	364.7	265.1	-0.18	-0.15
mmu-miR-152-3p	59.4	80.2	44.6	56.5	69.9	36.6	99.3	119.3	71	91.9	108.1	62.8	-0.18	-0.14
mmu-miR-542-3p	32.5	24.6	20.6	20.3	26.1	22	54.4	36.6	32.7	33	40.4	37.7	-0.18	-0.16
mmu-miR-304-5p	15734.1	14849.2	17285.6	14310.9	12932.7	15432.1	26321	27072.4	27507.2	23287	20000.2	26428.7	-0.17	-0.12
mmu-miR-301a-3p	936.4	1331.7	805.6	850.4	1181.6	709.4	1566.6	1979.5	1282	1383.8	1827.3	1214.8	-0.17	-0.13
mmu-miR-30d-3p	110.2	94.5	120.7	86.9	124.5	77.6	184.4	140.4	192.1	141.4	192.5	132.8	-0.17	-0.15
mmu-miR-339-3p	107.3	137.2	142.1	91.3	145.2	106.9	179.6	203.9	226.2	148.5	224.5	183	-0.17	-0.13
mmu-miR-148a-5p	21.7	14.2	10.9	24.6	10.8	6.1	36.3	21.2	17.4	40.1	16.6	10.5	-0.17	-0.16
mmu-miR-423-3p	1606.2	1163.5	1931.4	1309.7	1150.1	1760.4	2687	1729.4	3073.4	2131.1	1778.6	3014.8	-0.16	-0.11
mmu-miR-30c-5p	917	1466.3	1148.8	960.5	1070.9	1317.9	1534.1	2179.5	2146.4	1563	1656.2	2757.1	-0.16	-0.1
mmu-miR-5107	850.2	1259.2	747.4	818.5	1115.5	667.2	1489.2	1871.7	1189.4	1332	1725.1	1142.7	-0.16	-0.12
mmu-miR-30a-3p	304.3	247.2	143	260.8	215.1	144.4	509.1	367.4	227.6	424.3	332.7	247.4	-0.16	-0.14
mmu-miR-5124	9.1	7.8	1.7	10.1	5.4	1.2	15.3	11.5	2.8	16.5	8.3	2.1	-0.16	-0.14
mmu-miR-151-3p	3208.5	2376.1	1536	2994.6	1987.5	1436.4	5367.4	3531.9	2444.3	4872.9	3073.6	2460	-0.15	-0.12
mmu-miR-101a-3p	2081.3	2165.1	2035.9	2083.3	2473.8	1102.1	3481.8	3218.3	3239.8	3390	3825.6	1887.4	-0.15	-0.13
mmu-miR-29c-3p	1442.4	2280.3	2629	1525.5	2373.9	1828.5	2412.9	3389.5	4183.5	2482.4	3671.2	3131.5	-0.15	-0.1
mmu-miR-378b	308.9	361.7	295.1	298.4	306.5	264.7	516.8	540.6	469.8	485.6	474	453.4	-0.15	-0.11
mmu-miR-186-5p	2998.3	3746.6	3409.6	2332.5	3862.8	2991.3	5015.8	5569.1	5435.8	3795.5	5973.7	5122.9	-0.14	-0.1
mmu-miR-29a-3p	1604	2492.6	2942.5	1716.8	2632.8	2046.2	2683.2	3705	4682.5	2793.6	4071.6	3504.3	-0.14	-0.09
mmu-miR-126-3p	89.6	81.5	158.3	84	116	99.2	150	121.2	251.9	136.7	179.4	170	-0.14	-0.11
mmu-miR-361-3p	62.8	75.1	45.5	46.4	51.5	68.7	105.1	111.6	71.4	75.4	79.6	117.7	-0.14	-0.08
mmu-miR-3096b-3p	8	23.3	4.4	24.6	5.4	2.4	13.4	34.5	7	40.1	8.3	4.2	-0.14	-0.06
mmu-miR-142-5p	15229.3	21036.3	21897.6	16227.6	20409.3	16075.6	25476.6	31254.2	36846.3	26605.9	31562.6	28558.1	-0.13	-0.08
mmu-miR-148b-3p	1610.8	1344.6	1205.8	1457.5	1372.1	979.9	2728.1	1998.7	1918.8	2371.6	2121.9	1678.2	-0.13	-0.11
mmu-miR-322-5p	624.1	680.7	721.2	525.9	719.1	611.6	1044.1	1011.9	1147.7	855.8	1112	1047.5	-0.13	-0.09
mmu-miR-450b-5p	40	41.4	36.7	39.1	43.8	25.3	66.9	61.6	58.5	63.7	67.7	43.4	-0.13	-0.1

G)

gene	Control1 TPM	Control2 TPM	Control3 TPM	LPS O4h1 TPM	LPS O4h2 TPM	LPS O4h3 TPM	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS O4h1 DESeq	LPS O4h2 DESeq	LPS O4h3 DESeq	Control vs LPS O4h DESeq log2-ratio	Control vs LPS O4h DESeq log2-ratio
mmu-miR-127-3p	27.4	10.4	7.4	30.4	4.6	6.4	45.9	15.4	11.8	49.5	7.1	11	-0.11	-0.11
mmu-miR-148a-3p	2043.1	1190.6	1798	1997.8	1362.1	1267.3	3417.8	1769.8	2861.2	3750.9	2106.5	2170.3	-0.12	-0.1
mmu-miR-26b-5p	1923.1	2914.5	2608.8	1754.4	2551.4	2583.2	3217.2	4332.1	4151.5	2854.9	3945.6	4389.7	-0.12	-0.06
mmu-miR-338-3p	69.1	98.4	91.8	63.7	109.1	65	115.6	146.2	146.2	103.7	168.7	111.4	-0.12	-0.09
mmu-miR-194-5p	28	36.7	42	31.9	24.6	41.5	46.8	53.9	66.8	51.9	38	71.1	-0.12	-0.06
mmu-miR-872-5p	123.9	170.8	162.3	115.9	161.3	146	207.3	253.9	258.2	188.6	249.5	250	-0.11	-0.06
mmu-miR-3096-3p	8	23.3	4.4	24.6	6.1	2.4	13.4	34.6	7	40.1	9.5	4.2	-0.11	-0.03
mmu-miR-103-3p	2571.2	3210.8	2231.4	2539.7	2663.5	2264.6	4101.3	4772.7	3550.9	4132.6	4119.1	3878.2	-0.1	-0.06
mmu-miR-101b-3p	1331	1502.5	1452.5	1453.1	1740.1	810.7	2256.6	2233.4	2311.3	2364.5	2691	1388.5	-0.1	-0.07
mmu-miR-322-3p	76.5	88	84	63.7	70.7	96.8	128	130.8	133.6	103.7	109.3	185.8	-0.1	-0.05
mmu-miR-145-3p	9.7	6.5	21.4	10.1	6.1	18.9	16.2	9.6	34.1	16.5	9.5	32.4	-0.1	-0.04
mmu-miR-107-3p	1916.9	2378.7	1644.5	1984.8	1972.1	1616.6	3206.7	3535.7	2616.9	3729.7	3049.8	2768.5	-0.09	-0.05
mmu-miR-24-3p	1493.2	2650.4	2577.3	1716.8	1948.3	2667	2497.9	3939.7	4101.4	2793.6	3013	4567.5	-0.09	-0.02
mmu-miR-93-5p	951.1	1145.9	940.8	945.9	1097.1	1004	1591.4	2000.6	1497	1522.9	1696.6	1719.5	-0.09	-0.04
mmu-miR-450b-5p	12.6	14.2	16.6	11.6	14.6	14.7	21	21.2	26.4	18.9	22.6	25.1	-0.09	-0.04
mmu-miR-16-5p	11536.6	22782.5	13094.5	12023.3	19159.3	13570.6	19299.2	33864.6	20837.6	19564.5	29629.6	23240.7	-0.08	-0.03
mmu-miR-378-3p	4831.3	4761.2	4068.3	4181.1	4406.7	4372.8	8582.1	7077.2	6474	6803.6	6814.8	7488.7	-0.08	-0.04
mmu-miR-511-3p	161	177.1	80	153.6	160.6	80.6	269.4	263.5	127.4	249.9	248.3	138.1	-0.08	-0.05
mmu-miR-362-3p	26.3	31.1	35	26.1	44.6	16.8	43.9	46.2	55.7	42.4	68.9	28.8	-0.08	-0.06
mmu-miR-26a-5p	30083.4	36865.5	31153.8	27626.4	35536.1	30403.1	50325.5	54798.2	49575.9	44564.4	54956	52967.7	-0.07	-0.03
mmu-miR-130b-3p	655.5	839.9	461	696.9	699.9	469.3	1096.6	1248.5	731.6	1133.9	1082.3	801.8	-0.07	-0.01
mmu-miR-15a-5p	545.9	883.9	758.8	557.8	907.3	626.9	913.2	1133.9	1107.5	907.6	1403.1	1073.6	-0.07	-0.02
mmu-miR-28-5p	334	486.6	311	363.6	442.5	269	558.8	723.3	494.8	591.7	684.3	450.7	-0.07	-0.03
mmu-miR-421-3p	268.4	288.6	263.7	233.3	288.9	258.6	449	479	439.7	379.6	446.7	442.9	-0.07	-0.01
mmu-miR-652-3p	247.2	148.1	267.2	265.1	271.2	284.3	413.6	517.5	425.2	431.4	419.4	486.9	-0.07	-0.02
mmu-miR-1843-5p	185	191.5	176.7	204.3	213.6	110.5	309.5	284.7	281.2	332.4	330.3	189.3	-0.07	-0.04
mmu-miR-34a-5p	123.1	137.2	154.4	91.3	125.2	178	206.3	203.9	245.7	148.5	191.7	304.9	-0.07	-0.02
mmu-miR-5097	82.8	99.7	93.2	82.6	141.4	18.5	138.5	148.1	148.2	134.4	218.6	65.9	-0.07	-0.05
mmu-miR-200c-3p	3.4	5.2	1.3	2.9	3.1	3.4	5.7	7.7	2.1	4.7	4.8	5.8	-0.07	-0.03
mmu-miR-503-3p	3.4	6.5	3.5	2.9	3.8	6.1	5.7	9.6	5.6	4.7	5.9	10.5	-0.07	0.01

H)	gene	Control1 TPM	Control2 TPM	Control3 TPM	UPS_04a3 TPM	UPS_04a2 TPM	UPS_04a1 TPM	Control2 DESeq	Control3 DESeq	UPS_04a3 DESeq	UPS_04a2 DESeq	UPS_04a1 DESeq	Control vs UPS_04a TPM log2-ratio	Control vs UPS_04a DESeq log2-ratio
	mmu-miR-75-5p	85136.3	75188.9	87492.7	85090.8	59639.9	95738.5	10421.7	112060.6	139229.8	92212.1	163959.5	-0.06	-0.01
	mmu-miR-425-5p	354	542.3	505.1	463.6	457.1	432	592.2	866	803.9	706.9	722.7	-0.06	-0.01
	mmu-miR-29b-3p	73.1	106.1	124.6	97.1	138.3	55.6	122.3	157.7	198.4	213.9	95.2	-0.06	-0.03
	mmu-miR-93a-3p	52.5	54.4	41.5	59.0	58.0	74.1	87.9	80.8	66.1	90.3	41.3	-0.06	-0.04
	mmu-miR-33-5p	9.7	10.4	11.8	7.2	14.6	8.9	16.2	15.4	18.8	22.6	15.2	-0.06	-0.03
	mmu-miR-27b-3p	136874.6	129550.9	123751.3	125791.6	135888	106087	228972.8	192568.9	196929.2	216334.6	181682	-0.05	-0.02
	mmu-miR-181a-5p	19092.2	16710.2	15017.1	19019.3	15178.3	16703.8	31871.7	24838.7	23897.1	23473	25181.4	-0.05	-0.02
	mmu-miR-484	832	1114.3	1740.7	946	1100.1	1510.3	1391.7	1656.3	2770	1701.3	2586.5	-0.05	0
	mmu-miR-1843b-5p	188.4	192.8	180.2	207.2	218.2	115.1	315.2	286.6	286.7	337.4	197.2	-0.05	-0.03
	mmu-miR-339-5p	161	144.9	124.6	163.7	147.5	103.8	209.4	215.5	198.4	228.1	177.8	-0.05	-0.02
	mmu-miR-181-5p	54.8	50.5	49.9	58	49.2	62.8	91.7	75	79.3	76	73.2	-0.05	-0.01
	mmu-miR-7a-1-3p	5.1	20.7	11.2	10.1	10.8	15.9	8.6	30.8	19.5	16.6	27.2	-0.05	0.04
	mmu-miR-27a-3p	124138.9	117917.7	109858.1	118466.6	128539.4	95435.9	207607.7	175276.8	176820.8	198784.3	163441.2	-0.04	-0.01
	mmu-miR-7a-5p	91633.2	80166.2	92091.5	89055.3	63726.9	103976.6	153190.1	119459.1	166948	98552.8	178067.8	-0.04	0.01
	mmu-miR-182-5p	650.4	480.1	414.2	706.5	431	365.8	1068	713.7	659.1	666.5	626.5	-0.04	-0.01
	mmu-miR-1981-5p	69.7	58.2	30.2	53.6	43.8	56.5	116.5	86.6	48	67.7	96.7	-0.04	0
	mmu-miR-28-3p	56.5	51.8	65.6	56.5	43	69.9	94.6	76.9	104.4	66.5	119.8	-0.04	0.01
	mmu-miR-18a-5p	22.3	23.3	22.7	15.9	36.1	14.4	37.3	34.6	36.2	55.8	24.6	-0.04	-0.02
	mmu-miR-362-5p	12	10.4	10.5	10.1	13.1	8.9	20.1	15.4	16.7	20.2	15.2	-0.04	-0.01
	mmu-miR-7a-5p	3766.4	3460.6	3672.9	3481.4	3217.4	3979.8	6500.6	5143.9	5844.8	4992.1	6815.7	-0.03	0.02
	mmu-miR-151-5p	1457.8	1618.4	849.3	1425.6	1612.6	823.9	2438.7	2435.4	1351.6	2693.8	1410.9	-0.03	0
	mmu-miR-125b-2-3p	890.5	719.6	623.2	660.6	855.1	577.7	1339.2	1069.6	991.8	1322.3	989.4	-0.03	-0.01
	mmu-miR-27-3p	124472.9	128797.7	148217.1	129552.6	139048.3	127817.5	208226.5	191449.3	235862.3	215016.1	218897.1	-0.02	0.02
	mmu-miR-98-5p	775.4	772.6	585.3	746.1	704.5	751.8	1297.2	1148.4	1090.6	1089.5	1287.5	-0.02	0.02
	mmu-miR-30c-5p	770.3	1252.9	1160.3	950.4	1217.7	1001.3	1288.6	1921.8	1846.4	1858.6	1714.8	-0.02	0.03
	mmu-miR-140-3p	110.8	195.4	147	140.5	164.4	160.9	218.7	290.5	233.8	254.2	275.6	-0.02	0.03
	mmu-miR-17-3p	30.8	51.8	38.5	36.2	52.2	30.8	51.6	76.9	61.2	80.8	52.8	-0.02	0.02
	mmu-miR-410-3p	3.4	3.9	1.7	7.2	0.8	0.9	5.7	5.8	2.8	1.2	1.6	-0.02	0.03
	mmu-miR-27-3p	2493	2403.6	1610	2625.9	2213.3	1567.4	4170.5	3574.2	2591.9	3453.8	2684.3	-0.01	0.02
	mmu-miR-532-5p	1294.5	1021.1	1104.3	1257.5	1140.8	991.8	2165.5	1517.8	1757.3	1764.3	1698.6	-0.01	0.02

I)

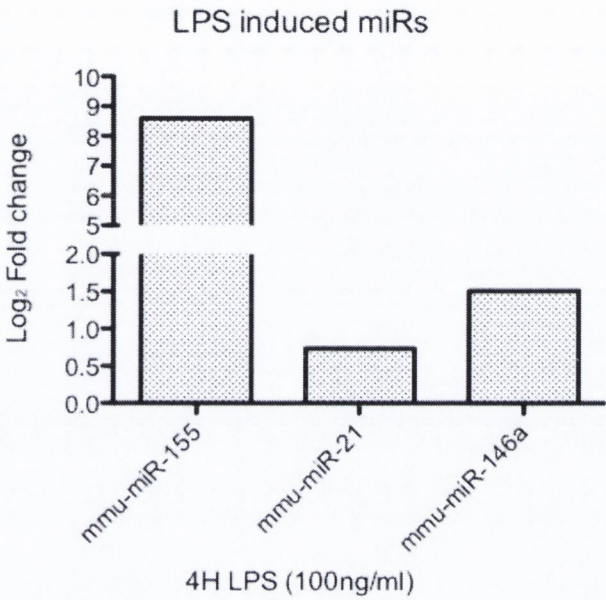


Figure 3.1 LPS-Repressed miRNA in BMDM.

BMDM were seeded at 1×10^6 /mL in a 6 well plate and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. The cells were then left unstimulated or, were stimulated with 100ng/mL LPS for 24 hours. Supernatants were collected and analysed by ELISA for IL-6 (A) and additionally TNF α (B). The data shows the mean $\pm$ S.D. cytokine level from three independent experiments. RNA was extracted using Qiagen RNeasy kit and miRNeasy kit. The samples were then sent for deep sequencing. The samples were analyzed with edgeR, DESeq, and TPM. Charts (C – H) illustrates miRNA data analyzed from edgeR that were LPS-repressed. (I) shows three miRNA that are LPS-induced. The scale is log₂ where a -2 fold change means 75% repression from basal. Data is presented as the mean of 2 independent experiments with $p \leq 0.05$ (two-tailed un-paired t-test).

microRNA	Predicted Target/s	Seed sequence
Mmu-let-7b-3p	eIF4A2	UGUAUA
Mmu-mir-5117-5p	eIF3j, eIF4E3	UAAAGUU
Mmu-mir-5107	eIF2C1	CAGGGAC
Mmu-mir-3096b-3p	eIF5	UGAGGCC
Mmu-mir-5121	eIF2C4	ACAUCUC
Mmu-mir-98-3p	eIF4A2, eIF4g2, eIF2C4	GUAUUGU
Mmu-mir-3473	eIF2C1	GCUCAG
let-7c-1-3p	eIF4A2	AGCUUUC

Table 3.2 eIF family members targeted by LPS-repressed miRNA.

miRNA, in small groups, were run through various miRNA target prediction software (Miranda, miRBase, TarBase, TargetScan and PicTar) to generate a list of targets. The targets shown in the table above were picked because they were predicted targets in most databases and were functionally related.

3.2.3 Validation of LPS repressed miRNA let-7c-1-3p, let-7b-3p, and miR-98-3p

Next, the results obtained from deep-sequencing were tested using RT-PCR and qPCR with TaqMan probes specific for let-7c-1-3p, let-7b-3p and miR-98-3p which were predicted to target eIF4A2. Since three separate miRNA were predicted to target eIF4A2, it increased the chances of finding at least one miRNA-mRNA couple.

Figure 3.3 A shows the time course of let-7c-1-3p repression in response to LPS. Initially, at the 1 hour time point, let-7c-1-3p showed a 2-fold induction. However, from there it was progressively repressed over 24 hours down to 70% less than the basal level. Figure 3.3 B shows the time course of let-7b-3p repression in response to LPS. Let-7b-3p was repressed to 60% below the basal level from 1 hour post LPS treatment and by 4 hours it reached 50% below the basal level and it stayed at that level for 24 hours. Figure 3.3 C shows the time course of miR-98-3p expression in response to LPS. miR-98-3p was not strongly expressed in BMDM, as revealed by high Ct values from qPCR and showed no repression in response to LPS.

3.2.4 eIF4A2 protein induction in response to LPS

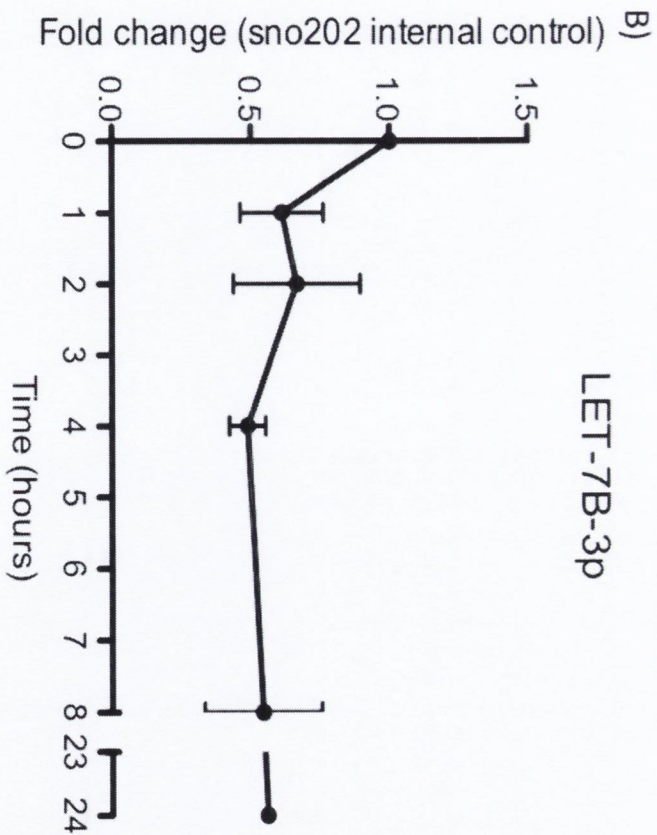
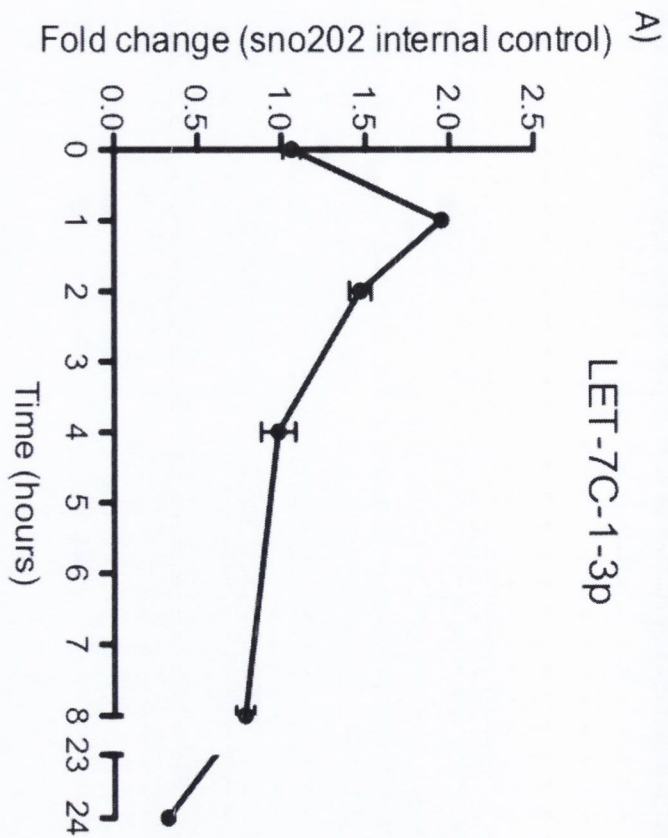
An LPS time course and dose response in BMDM was performed and eIF4A2 protein induction was tested.

As shown in Figure 3.4 LPS induced eIF4A2 in both a dose- and time- dependent fashion. eIF4A2 induction started from as early as 2 hours in samples treated with

100 ng/ml LPS and progressively increased until 24 hours after LPS treatment. For all three doses of LPS tested, eIF4A2 was strongly induced at 8 and 24 hours post LPS treatment, with maximal observed induction occurring at 24 hours after stimulation with 100 ng/ml LPS.

3.2.5 eIF4A2 mRNA induction in response to LPS

The next logical step was to test for the induction of eIF4A2 mRNA in response to LPS. eIF4A2 mRNA was neither induced nor stabilized in response to LPS. Instead, as depicted in Figure 3.5 A, the mRNA was repressed. The mRNA levels of eIF2C1, eIF4E3 and eIF5 were also tested and similar repression was observed in the mRNA levels of all three eIF family members (Figure 3.5 B-D). This result was unexpected since the decrease in miRNA predicted to target these eIFs should boost target eIF. Therefore I did not pursue further the targeting of these eIFs by the repressed miRNA. Instead I focused on the highly novel result of eIF4A2 induction at the protein level by LPS.



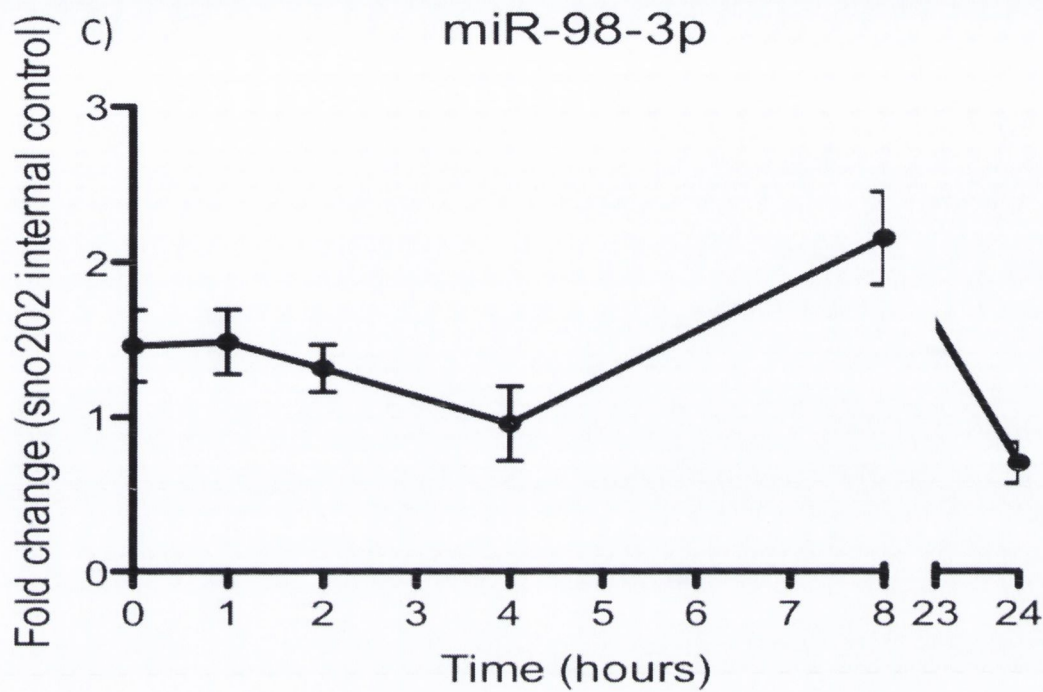


Figure 3.3 Expression levels of let-7c-1-3p, let-7b-3p and mir-98-3p in response to LPS over 24 hours.

BMDM were seeded at 1×10^6 /mL in a 6 well plate and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. The cells were then left unstimulated or, were stimulated with 100ng/mL LPS for 1, 2, 4, 8, or 24 hours. RNA was extracted using Qiagen RNeasy kit. Reverse transcription was carried out followed by qPCR with TaqMan primers and probe specific for let-7c-1-3p, let-7b-3p, mir-98-3p and snoRNA202. let-7c-1-3p (A), let-7b-3p (B) and mir-98-3p (C) expression were normalised to snoRNA202 expression and are presented as fold change relative to expression in un-treated cells. The data shown represent mean relative fold change value $\pm$ S.D. from three separate experiments, each carried out in duplicate.

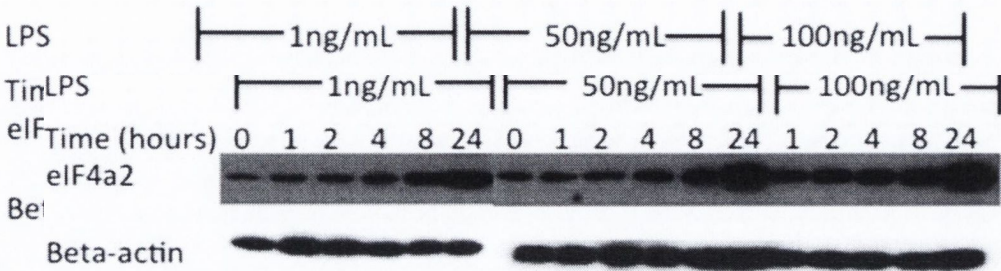
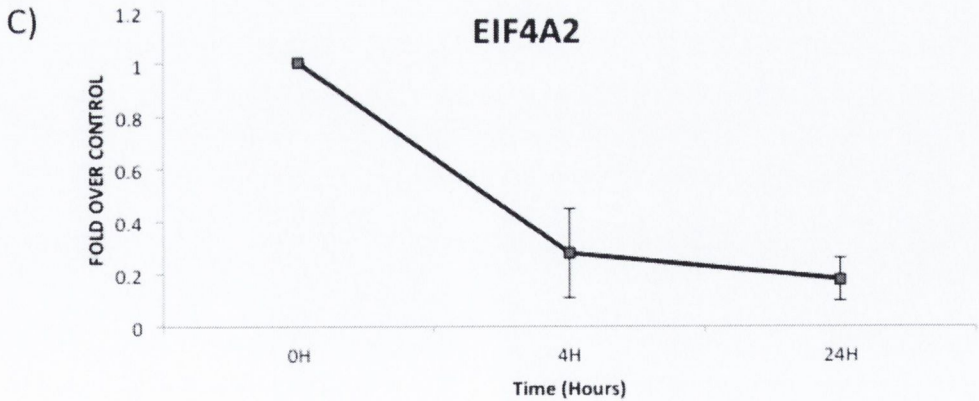
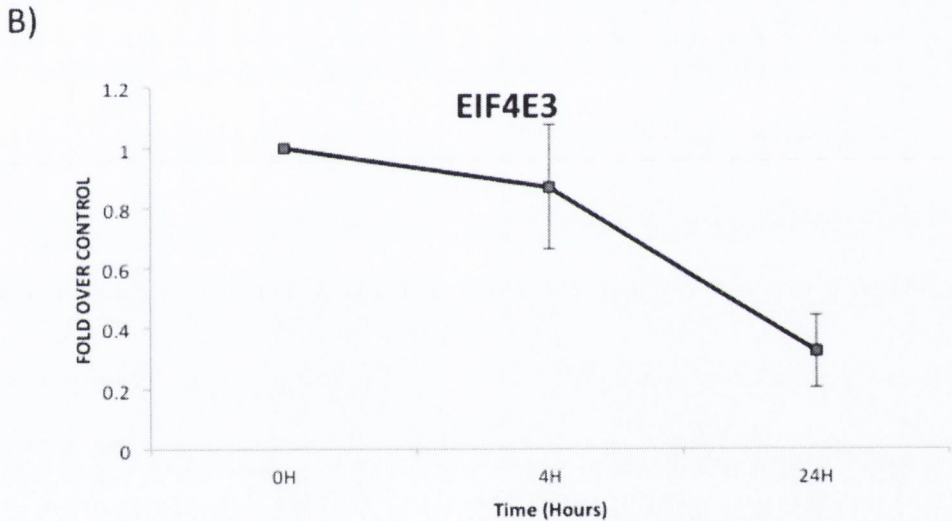
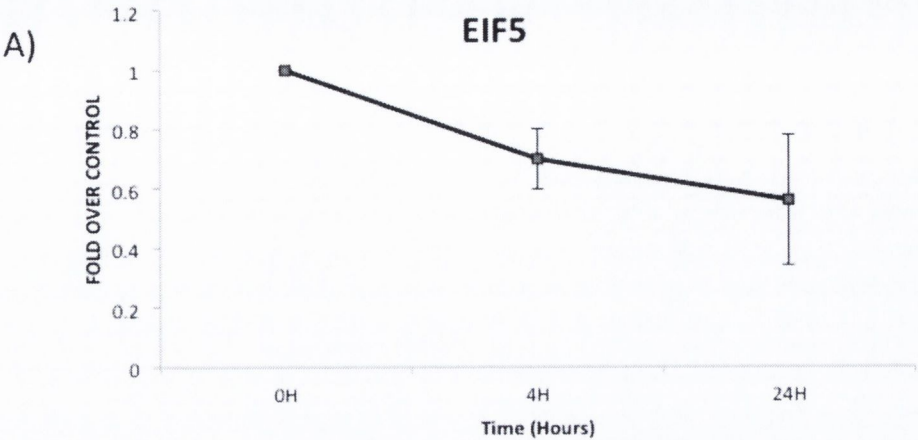


Figure 3.4 eIF4A2 expression in LPS treated BMDM.

BMDM were seeded at 1×10^6 /mL overnight in a 6 well plate and incubated at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. The cells were then left unstimulated or, were stimulated with 1ng/mL, 50ng/mL or 100ng/mL LPS for 1, 2, 4, 8 or 24 hours. Cells were lysed in sample loading buffer and lysates were Western blotted with anti-eIF4A2, and anti- β -actin antibodies. The blot is representative of 3 independent experiments.



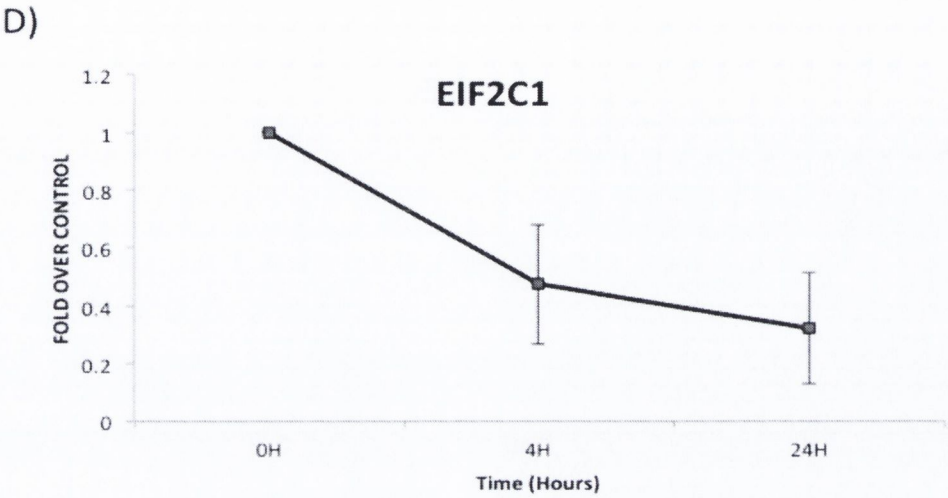


Figure 3.5 Expression levels of eIF4A2, eIF4E3, eIF5 and eIF2C1 in response to LPS.

BMDM were seeded at 1×10^6 /mL in a 6 well plate and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. The cells were then left unstimulated or, were stimulated with 100ng/mL LPS for 4 or 24 hours. RNA was extracted using Qiagen RNeasy kit. Reverse transcription was carried out followed by qPCR using custom made primers specific for eIF4A2, eIF4E3, eIF5 and eIF2C1 and GAPDH. eIF4A2 (A), eIF4E3 (B), eIF5 (C) and eIF2C1 (D) expression were normalised to GAPDH expression and are presented as fold change relative to expression in un-treated cells. The data shown represent mean relative fold change value $\pm$ S.D. from three independent experiments.

3.2.6 eIF4A1 protein induction in response to LPS

Since eIF4A1 is highly homologous to eIF4A2 I examined this protein next. eIF4A1 was strongly induced in response to LPS, as illustrated in Figure 3.6 A. The induction started from as early as 1 hour after stimulation. Similar to eIF4A2, eIF4A1 was also strongly induced at 8 and 24 hours in response to 100 ng/ml LPS (Figure 3.6 A). In addition, eIF4A1 was induced in response to R848 and Poly (I:C) as depicted in Figure 3.6 B and C. Basal levels of eIF4A1 varied somewhat between experiments as can be seen in Figure 3.2.6 A – C at time 0 hours. LPS again induced eIF4A2 (Figure 3.6 D).

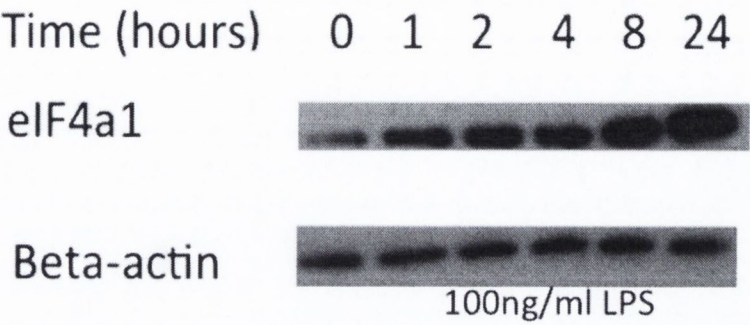
3.2.7 Analysis of the eIF4A1 and eIF4A2 promoter

I became interested in identifying what regulated eIF4A1 in response to LPS. Using JASPAR and TFSEARCH, it was identified that eIF4A1 and eIF4A2 contain 1 and 2 NFκB sites respectively (Table 3.7 A). A few other sites were also identified in the promoter region of eIF4A1 and 2 and it included AP-2α, c-Myc, IRF1, SP1, IRF3, and TAF1 binding sites.

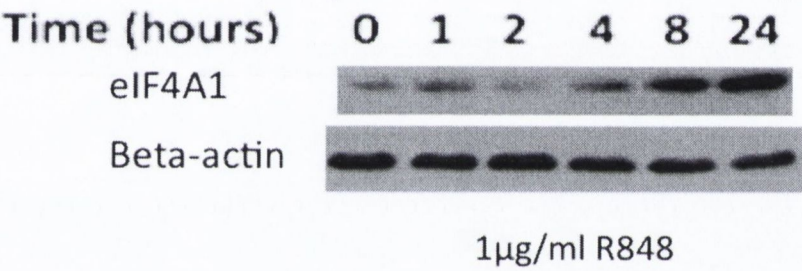
3.2.8 Effects of PS-1145 on LPS-induced eIF4A1 protein

The NFκB inhibitor, PS-1145 (inhibits IκB kinase) was therefore next tested. As observed in Figure 3.8 A, PS-1145 inhibited the induction of eIF4A1 protein in a dose-dependent manner. 24 μM PS-1145 was the most potent dose. A TNFα ELISA was performed as a control to confirm LPS responsiveness and PS-1145-mediated repression, shown in Figure 3.8 B.

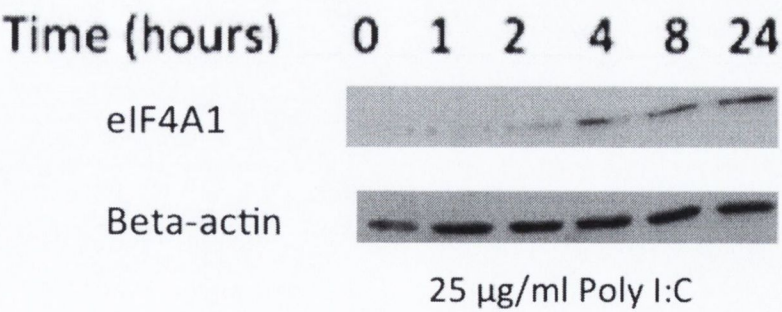
A



B



C



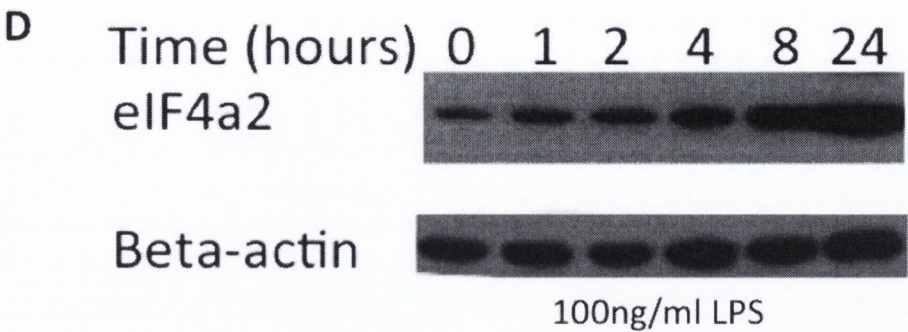
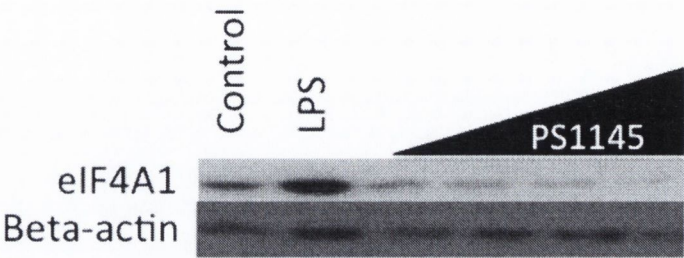


Figure 3.6. eIF4A1 and eIF4A2 expression in response to LPS, Poly (I:C) and R848. BMDM were seeded at 1×10^6 /mL in a 6 well plate and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. The cells were then left unstimulated or, were stimulated with 100ng/ml LPS (**A and D**) , or 25µg/ml Poly (I:C) (**B**) or 1µg/ml R848 (**C**) for 1, 2, 4, 8 or 24 hours. Cells were lysed in sample loading buffer and lysates were Western blotted with anti-eIF4A1 (**A, B, C**), anti-eIF4A2 (**D**) and anti-β-actin antibodies (**A-D**). The blots are representative of 3 independent experiments.

Gene	NFκB site (JASPAR)	NFκB site (TFSEARCH)	Position	Actual site
eIF4A1	1	1	-418 – -408	GGATTCCCC
eIF4A2	2	2	-362 – -353; -281 – -272	GGGGCTTCCC; CGGGGTTTCC

Figure 3.7 A eIF4A1 & 2 have NFκB binding sites at the promoter
JASPAR and TFSEARCH were used to identify NFκB binding site in eIF family members. eIF4A1 had 1, eIF4A2 had 2 predicted sites for NFκB. Amongst the others tested no NFκB putative sites were identified in EIF1, EIF1A, EIF2S1, EIF2S2, EIF2S3, EIF2B1, EIF2B2, EIF2B3, EIF2B4, EIF3A, EIF3B, EIF3E, EIF3F, EIF3G, EIF3H, EIF3I, EIF3K, EIFS8, EIF4A3, EIF4B, EIF43, EIF4G1, EIF4G3, EIF5, EIF5B. NFκB sites were predicted to be present in eIF4H, eIF2S3 and eIF2B3.

A



B

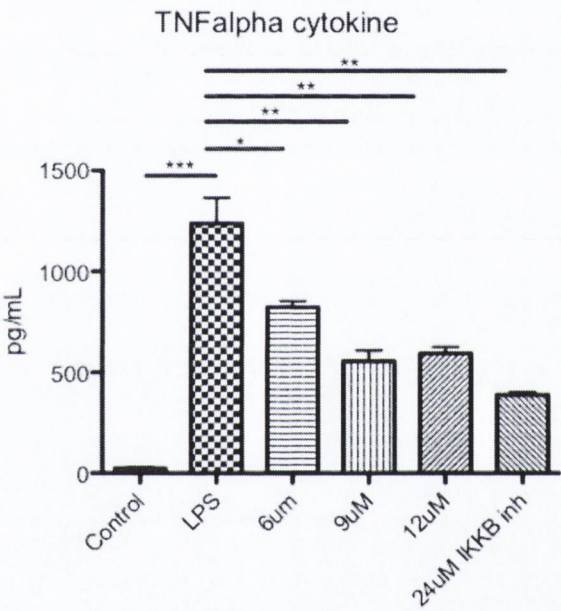


Figure 3.8 Expression changes of eIF4A1 in response to NF κ B-inhibitor PS-1145. BMDM were seeded at 1×10^6 /mL in a 6 well plate and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. The cells were then pre-treated with 6 μ M, 9 μ M, 12 μ M or 24 μ M PS-1145 for 0.5 hours. Following the pre-treatment, cells were left unstimulated or, were stimulated with 100ng/mL LPS for 24 hours. Cells were lysed in sample loading buffer and lysates were Western blotted with anti-eIF4A1 and anti- β -actin antibodies (A). The blot is representative of 3 independent experiments. Supernatants were analyzed by ELISA for TNF α (B). The data shown represent mean $\pm$ S.D. cytokine level from three independent experiments; * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$ (two-tailed un-paired t-test).

3.2.9 Effects of Parthenolide on LPS-induced eIF4A1 protein

Another NF κ B inhibitor, Parthenolide, was investigated next. As illustrated in Figure 3.9 A, Parthenolide inhibited LPS-mediated eIF4A1 induction. With Parthenolide pretreatment before LPS stimulation, eIF4A1 expression was limited to basal levels. A TNF α ELISA was performed as a control to confirm LPS responsiveness and Parthenolide-mediated repression, shown in Figure 3.9 B.

3.2.10 eIF4A1 and eIF4A2 mRNA induction in response to LPS

Previously, we examined the expression of eIF4A2 mRNA at 4 and 24 hours post LPS treatment and observed a repression. Having found the putative NF κ B sites in the eIF4A1 and eIF4A2 promoter regions, and observed the effects of NF κ B inhibitors on eIF4A1 protein it was decided to examine the mRNA induction of eIF4A1 and eIF4A2 at 0.5, 1 and 2 hours post LPS stimulation in BMDM. eIF4A1 was induced up to 1.5 fold in response to LPS up to 2 hours post stimulation, as depicted in Figure 3.10 A. It was induced just over 1.5 fold by 2 hours. eIF4A2 mRNA induction was minimal and it was observed at 0.5 hours post LPS treatment, after which it showed a repression and reached basal level by 120 minutes, as shown in Figure 3.10 B. eIF4A2 showed a similar trend to that observed previously in Figure 3.2.5 A, where a repression below the basal expression level was observed over 24 hours. IL-6 mRNA was also measured as a control to test LPS responsiveness (Figure 3.10 C).

3.2.11 Effect of PS-1145 on LPS-induced eIF4A1 mRNA

We next tested if eIF4A1 mRNA induction was NFκB-dependent. As shown in Figure 3.11 A, eIF4A1 mRNA induction by LPS was blocked with PS-1145 pre-treatment. However only 12μM dose blocked LPS-mediated eIF4A1 induction significantly. Longer pre-treatment with PS-1145 blocked eIF4A1 mRNA induction below the basal level, as shown in Figure 3.11 B. A TNFα ELISA was used as a control to confirm LPS responsiveness and PS-1145-mediated repression, shown in Figure 3.11 C and D. Overall LPS did not strongly induce eIF4A1 mRNA and PS-1145-mediated repression was weak. The role of NFκB in eIF4A1 induction might therefore be indirect.

3.2.12 Effects of Rapamycin on LPS-induced eIF4A1

The results thus far demonstrated that LPS-mediated eIF4A1 induction might not be due to increased transcription. I therefore next tested whether mTOR-dependent translation might be involved.

Figure 3.12 A shows that rapamycin blocked LPS-mediated eIF4A1 induction in a dose-dependent manner. IL-10, IL-6 and TNFα were used as controls. IL-10 is known to be repressed with rapamycin (Weichhart et al., 2008) and as shown in Figure 3.12 B, a similar effect was observed here, whilst IL-6 and TNFα were unaffected as shown in Figure 3.12 C and D.

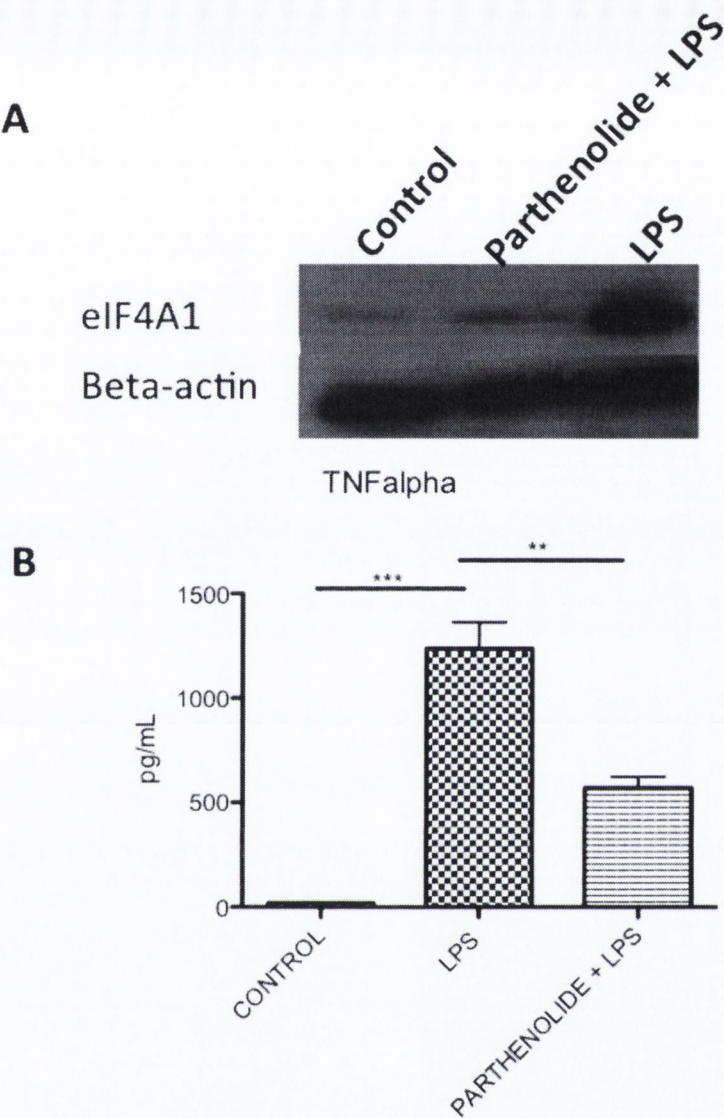


Figure 3.9 Expression changes of eIF4A1 in response to Parthenolide.

BMDM were seeded at 1×10^6 /mL in a 6 well plate and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. The cells were then pre-treated with Parthenolide for 0.5 hours. Following the pre-treatment, cells were left untreated or, were treated with 100ng/mL LPS for 24 hours. Cells were lysed in sample loading buffer and lysates were Western blotted with anti-eIF4A1 and anti- β -actin antibodies (**A**). The blot is representative of 3 independent experiments. Supernatants were analyzed by ELISA for TNF α (**B**). The data shown represent mean $\pm$ S.D. cytokine level from three independent experiments; ** $p \leq 0.001$, *** $p \leq 0.0001$ (two-tailed un-paired t-test).

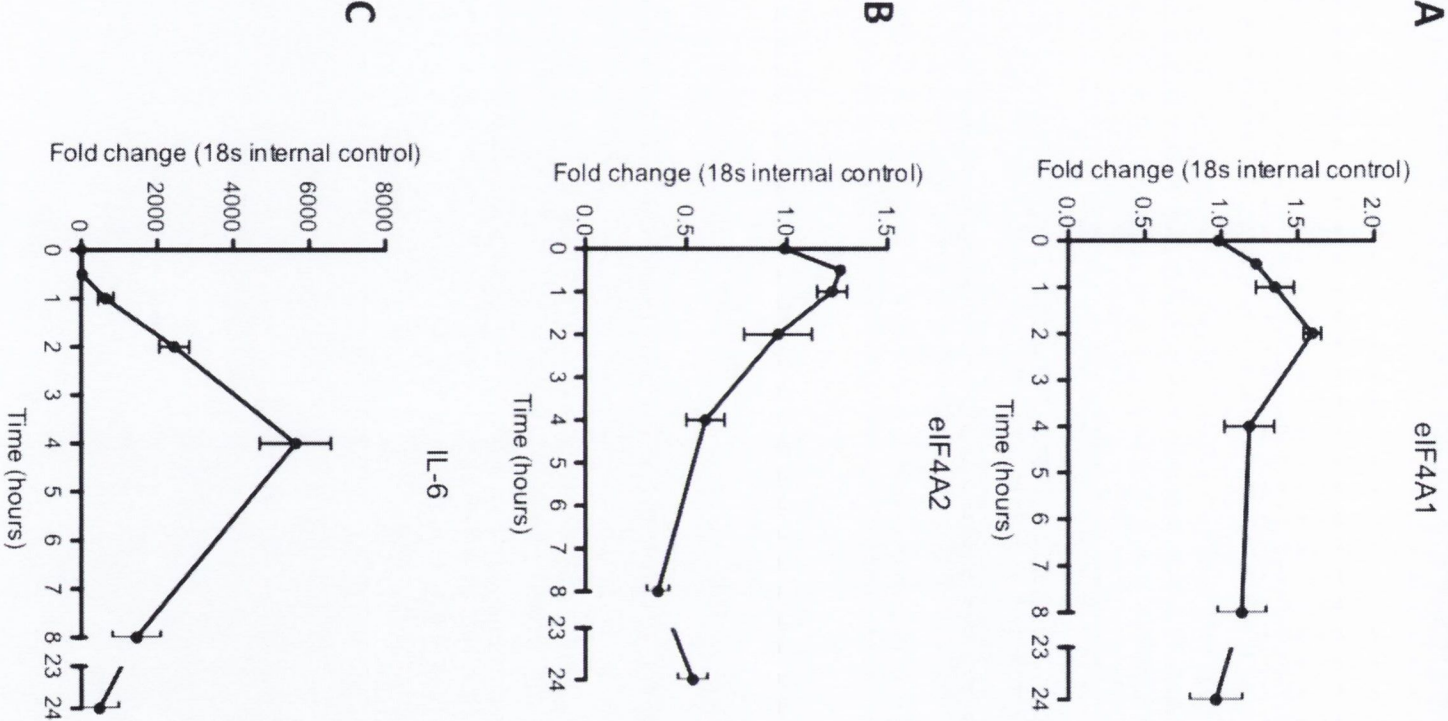
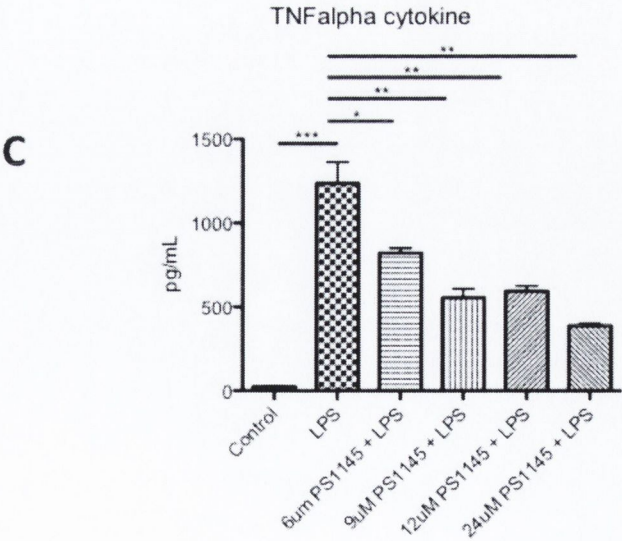
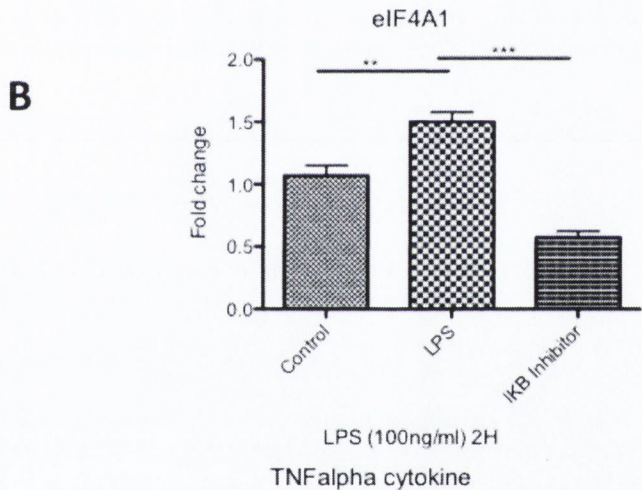
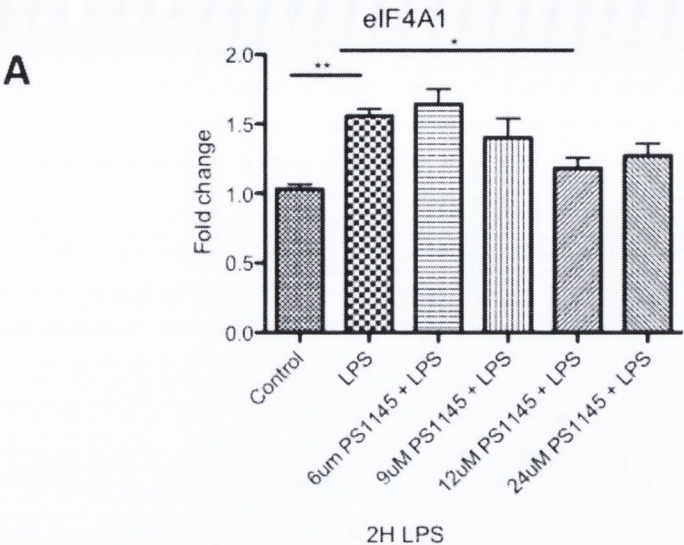


Figure 3.10 Expression levels of EIF4A1 and EIF4A2 mRNA in response to LPS.

BMDM were seeded at 1×10^6 /mL in a 6 well plate and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. The cells were then left unstimulated or, were stimulated with 100ng/mL LPS 0.5, 1, 2, 4, 8 or 24 hours. RNA was extracted using Qiagen RNeasy kit. Reverse transcription was carried out followed by qPCR using primers specific for eIF4A1, eIF4A2, IL-6 and 18s ribosomal RNA. eIF4A1 **(A)**, eIF4A2 **(B)**, and IL-6 **(C)** expression were normalised to 18s ribosomal RNA expression and are presented as fold change relative to expression in un-treated cells. The data shown represent mean relative fold change value $\pm$ S.D. from three independent experiments.



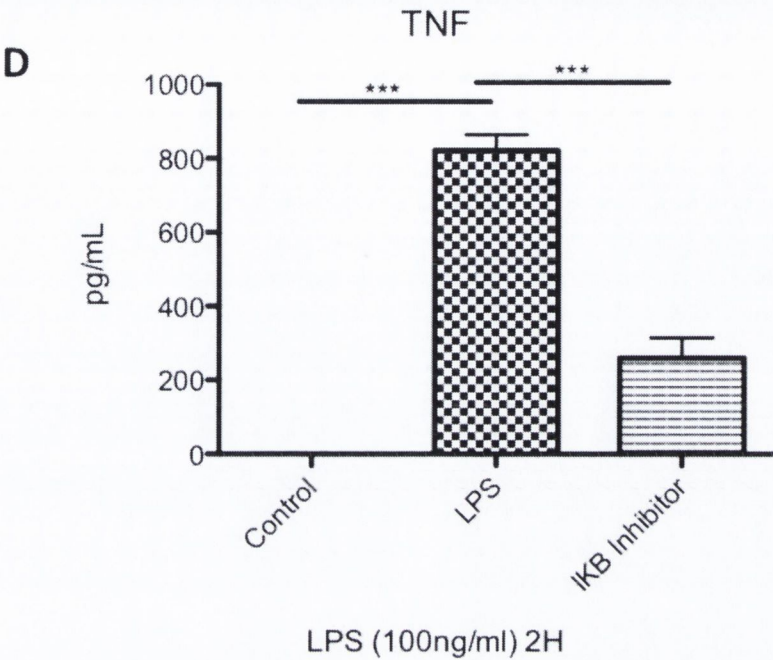
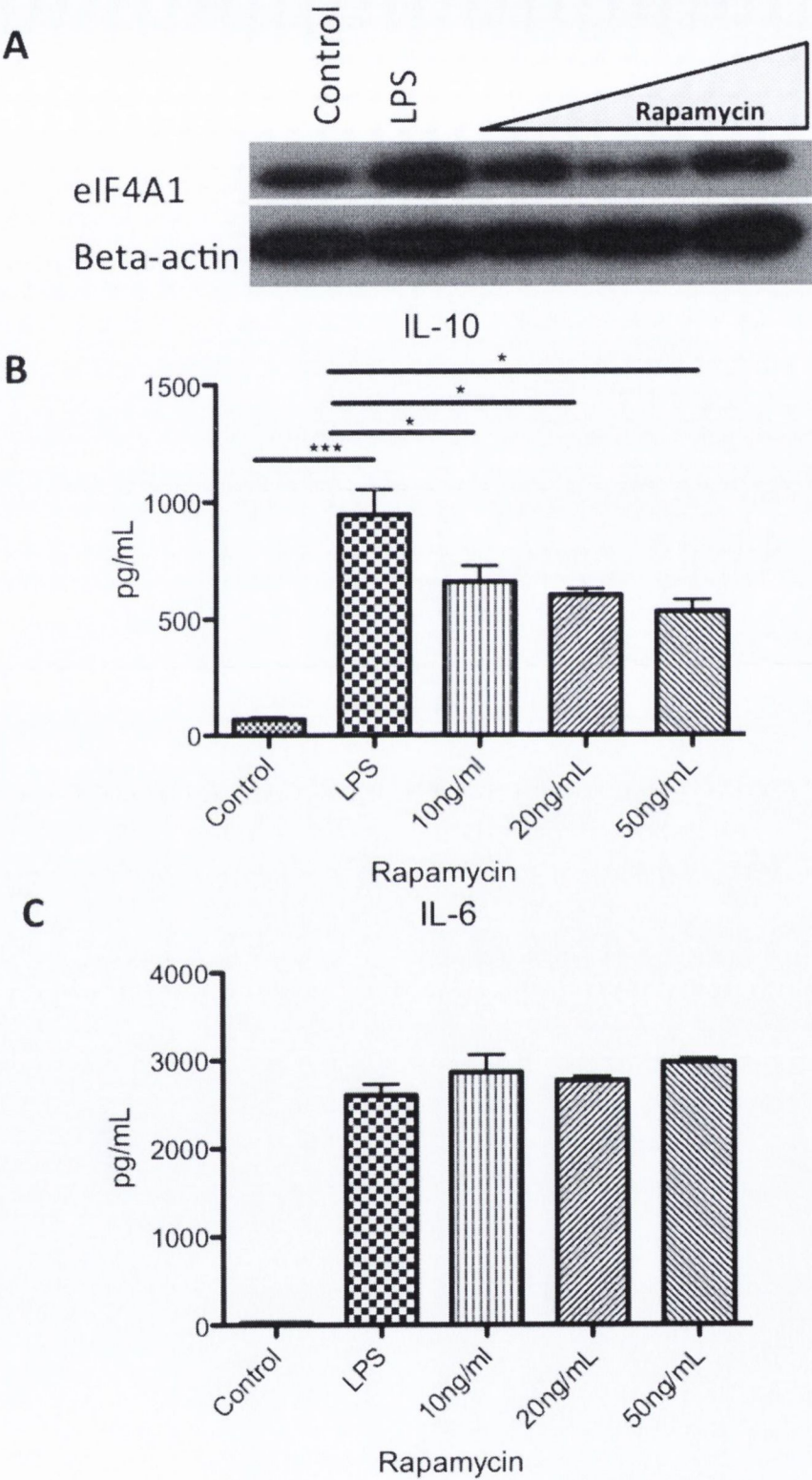


Figure 3.11 Effect of IκB kinase inhibitor-PS1145 on eIF4A1

Primary BMDMs were seeded at 1×10^6 /ml in a 6 well plate and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. **(A)** The cells were then pre-treated with 9μM, 12μM, or 24μM PS-1145 for 0.5 hours. **(B)** The cells were pre-treated with 12μM PS-1145 for 2 hours. Following the pre-treatment cells were left untreated or, were treated with 100ng/ml LPS for 2 hour. RNA was extracted using Qiagen RNeasy kit. Reverse transcription was carried out followed by qPCR primers specific for eIF4A1 and 18s ribosomal RNA. eIF4A1 **(A and B)** expression was normalised to 18s ribosomal RNA expression and are presented as fold change relative to expression in un-treated cells. The data shown represent mean relative fold change value $\pm$ S.D. from three independent experiments. Supernatants were analyzed by ELISA for TNFα **(C and D)**. The data shown represent mean $\pm$ S.D. cytokine level from three independent experiments; * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$ (two-tailed un-paired t-test).



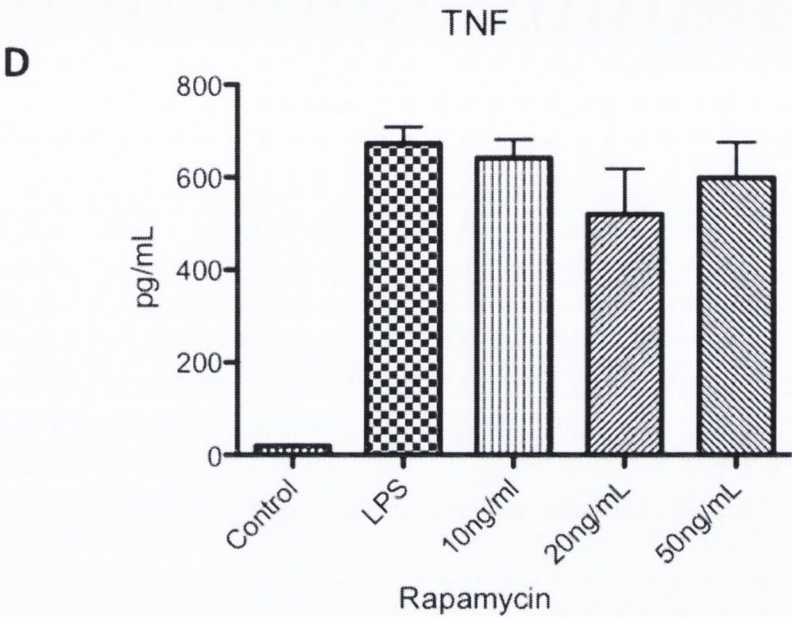


Figure 3.12 mTOR mediated regulation of eIF4A1 expression.

Primary BMDM were seeded at 1×10^6 /mL in a 6 well plate and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. All the cells were then left untreated or, were treated with increasing doses of rapamycin (10, 20, and 50 ng/ml) for 1 hour. Untreated and treated cells were then left untreated or, treated with 100ng/ mL LPS for 24 hours. Cells were lysed in sample loading buffer and lysates were Western blotted with anti-eIF4A1 and anti- β -actin antibodies **(A)**. The blot is representative of 3 independent experiments. Supernatants were analyzed by ELISA for IL-10 **(B)**, IL-6 **(C)**, and TNF α **(D)**. The data shown represent mean $\pm$ S.D. cytokine level from three independent experiments; * $p \leq 0.05$, *** $p \leq 0.0001$ (two-tailed un-paired t-test).

3.2.13 Transcription and translation of IL-10, IL-6 and TNF α in response to LPS

Because Rapamycin could inhibit induction of eIF4A1 and IL-10 in response to LPS, but not TNF α and IL-6, next I examined whether eIF4A1 might regulate IL-10 induction. I first carried out a time course for IL-10, TNF α and IL-6 mRNA induction and protein to detect if there were any significant differences in timings of transcription and translation. It is already known that IL-10 is a cytokine produced at a later time as it participates in resolution phase of inflammation. As can be seen in Figure 3.13 A and B an LPS time course demonstrated that IL-10 and TNF α mRNA were transcribed at the same time, 60 minutes post LPS stimulation, where as IL-6 mRNA peaked at 4 hours and then declined (Figure 3.13 C). IL-10 protein production and secretion was delayed by at least 2 hours, and cytokine production peaks only at 8 hours post LPS treatment (Figure 3.13 D). TNF α protein production was immediate and began from 2 hours and was sustained for 24 hours (Figure 3.13 E). IL-6 protein production followed a similar pattern as TNF α with no delay in translation from the mRNA (Figure 3.13 F). This delay in IL-10 production could be due to dependency on eIF4A.

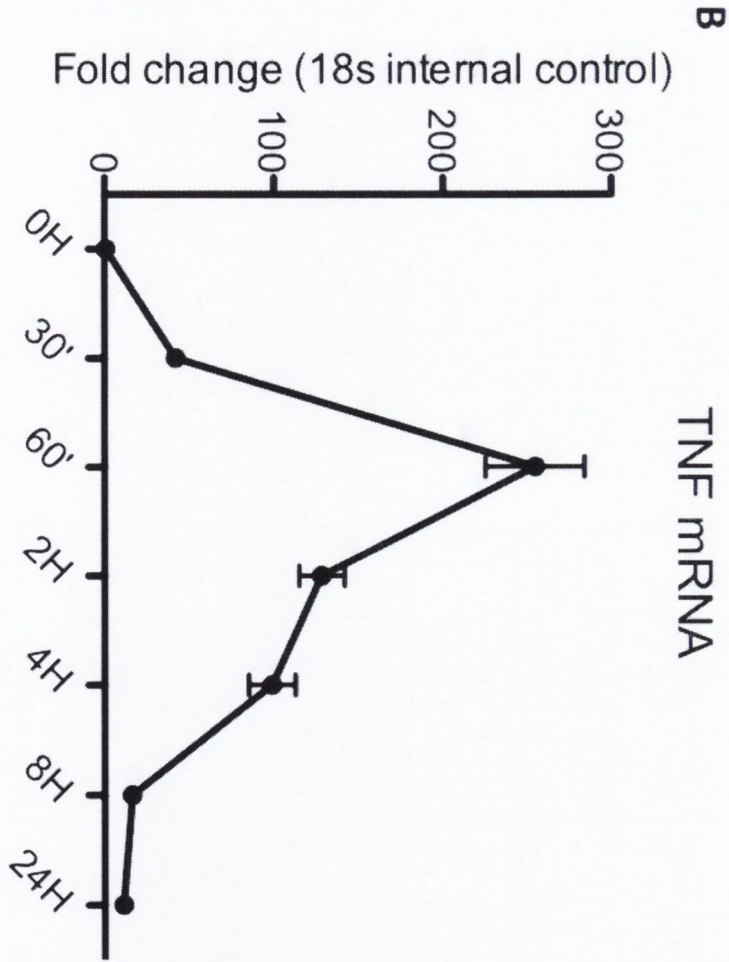
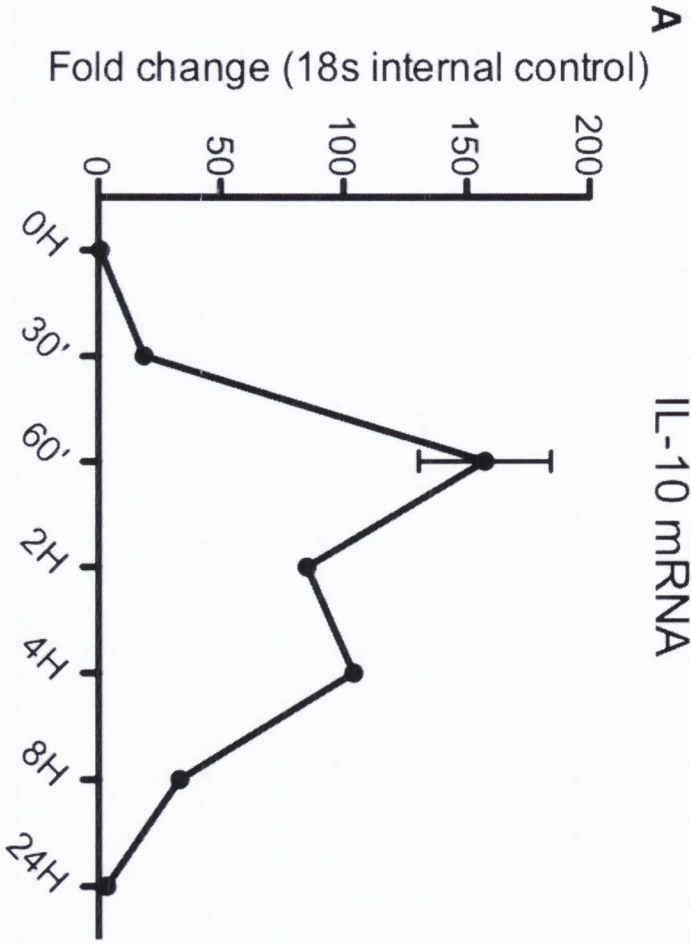
3.2.14 The 5'UTR of IL-10, TNF α and IL-6 genes

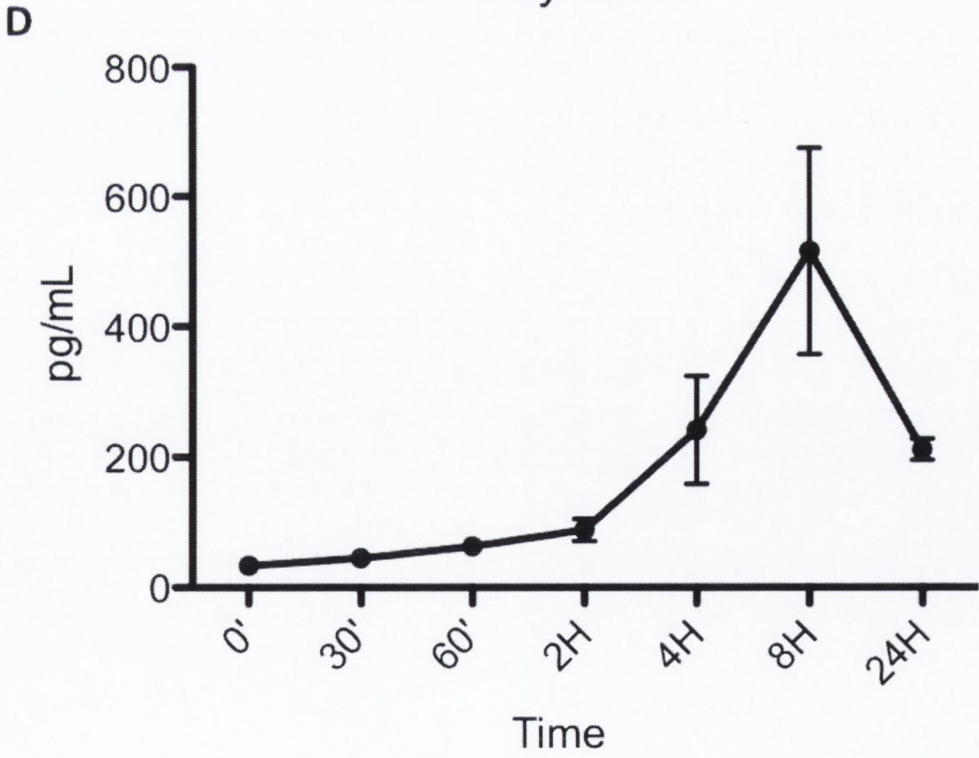
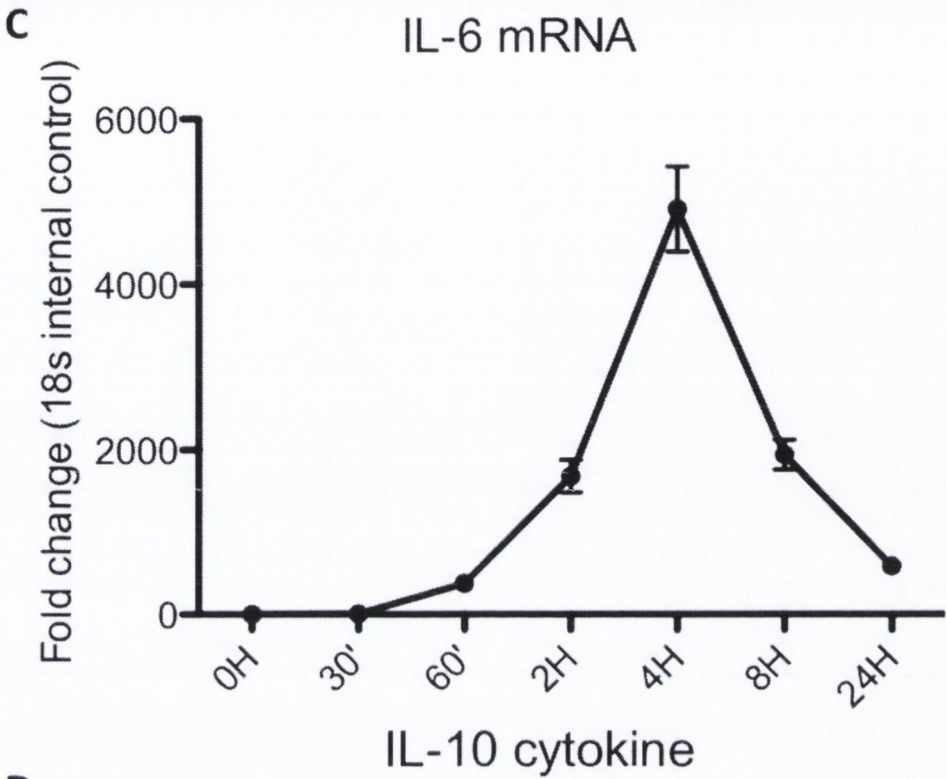
eIF4A-dependency of a transcript is based on the presence of complicated secondary structures in the 5'UTR of the mRNA. Next, we investigated if the 5'UTR of the three cytokines were predicted to form secondary structures. For these purposes the ENSEMBL database was used to obtain the sequences for the IL-10, TNF α and IL-6

5'UTR, shown in Figure 3.14 A. M-fold was used to predict if these sequences could form secondary structures in an energy-free manner. Figure 3.14 B, C and D depicts three structures that the IL-10 5'UTR can form. All three structures have negative Gibbs free energy, suggesting that the formation of such secondary structure would provide stability and therefore would be a likely event. IL-10 has a relatively complicated secondary structure depicted by negative Gibbs free energy that suggests how strong the folding would be in an energy free state. Lower the energy would be the stronger the structure is predicted to form. Additionally it is not predicted to contain *cis* element and is predicted to be eIF4A-dependent.

Figure 3.14 E and F depict two structures that the TNF α 5'UTR can form. Both structures have negative Gibbs free energy, suggesting that the formation of such secondary structures would provide stability and hence would also be a likely event. It is widely accepted that 5'UTR structures with Gibbs free energy of -50 or less, such as TNF α cannot be translated in eIF4A-dependent manner. Such complex structures are generally thought to contain *cis* elements and are translated in eIF4A-independent manner.

In contrast, as shown in Figure 3.14 G, H and I the IL-6 5'UTR cannot form secondary structures without an energy input. Translation from very small 5'UTRs, such as IL-6, are also not eIF4A-dependent and with sequence similarity IL-6 is predicted to contain a Translation Initiation from Short UTR (TISU) site.





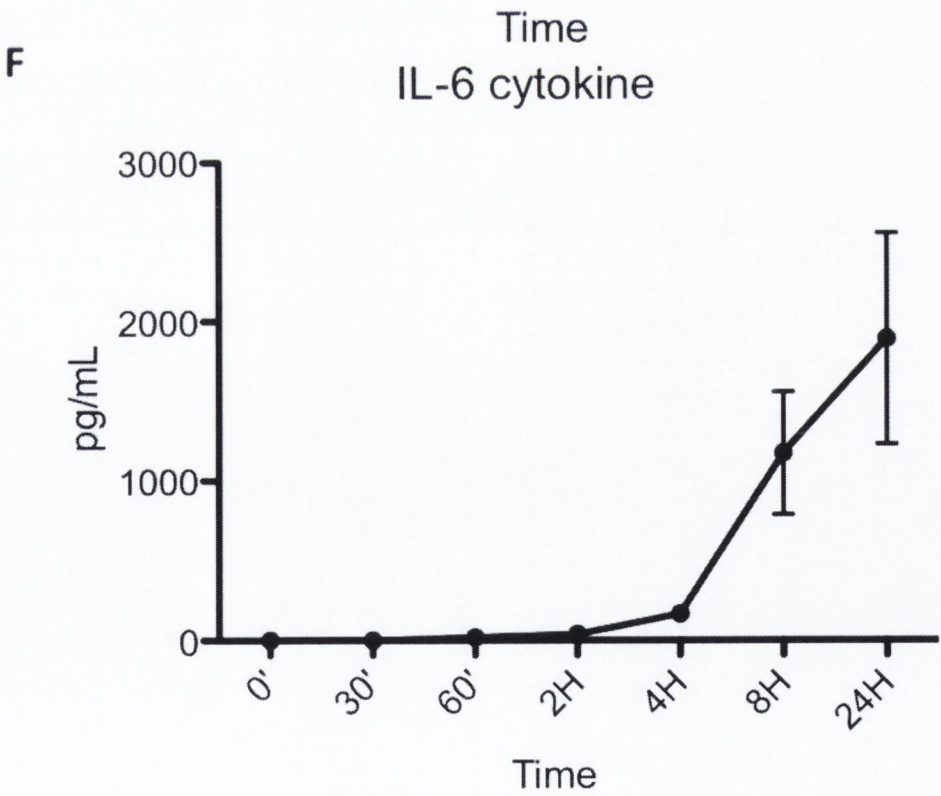
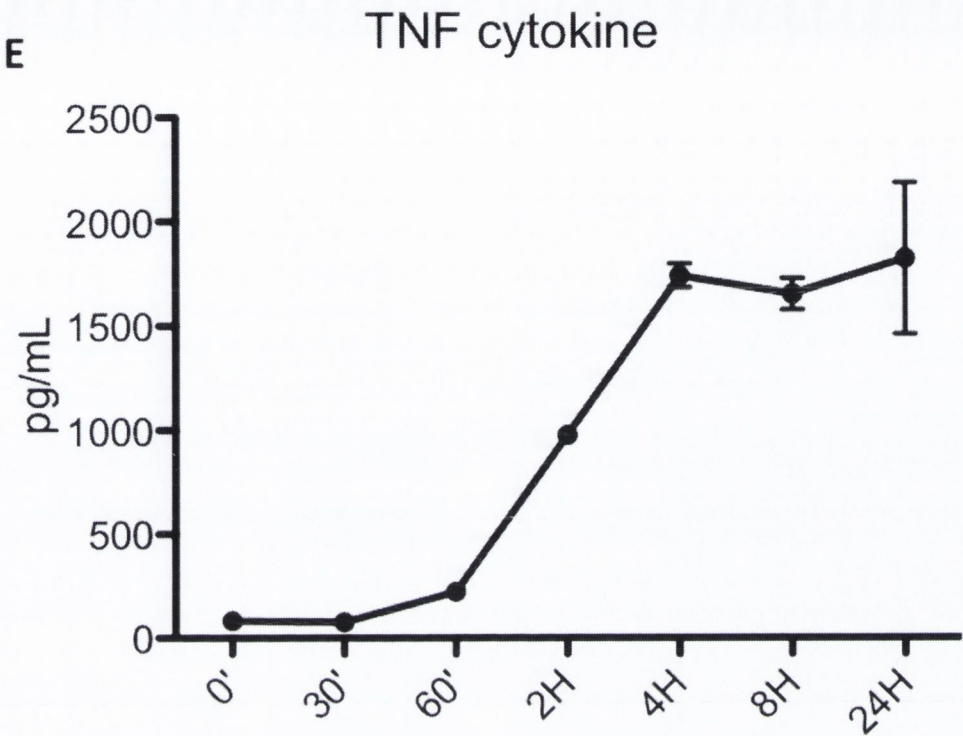


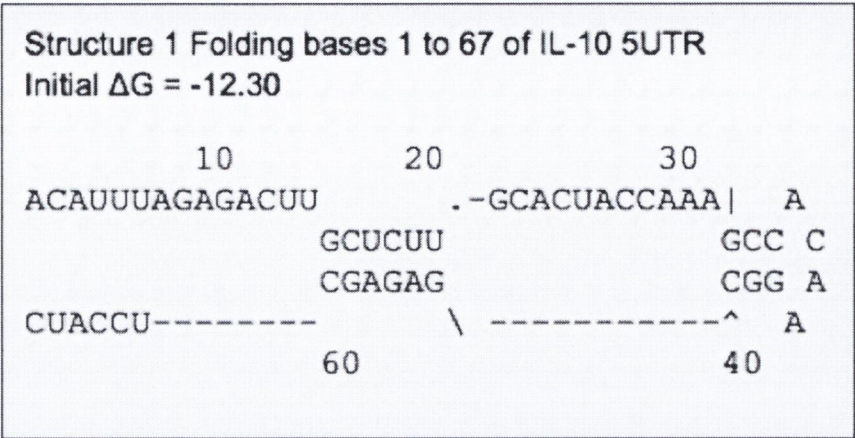
Figure 3.13 LPS time course- IL-6, IL-10, TNF (mRNA & cytokine).

BMDM were seeded at 1×10^6 /mL in a 6 well plate and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. The cells were then left unstimulated or, were stimulated with 100ng/ml LPS for 1, 2, 4, 8, or 24 hours. RNA was extracted using Qiagen RNeasy kit. Reverse transcription was carried out followed by qPCR using primers specific for IL-10, IL-6, TNF α and 18s ribosomal RNA. IL-10 **(A)**, TNF α **(B)**, and IL-6 **(C)** expression were normalised to 18s ribosomal RNA expression and are presented as fold change relative to expression in un-treated cells. The data shown represent mean relative fold change value $\pm$ S.D. from three independent experiments each carried out in duplicate. Supernatants were analyzed by ELISA for IL-10 **(D)**, TNF α **(E)** and IL-6 **(F)**. The data shown represent mean $\pm$ S.D. cytokine level from three independent experiments, each carried out in duplicate.

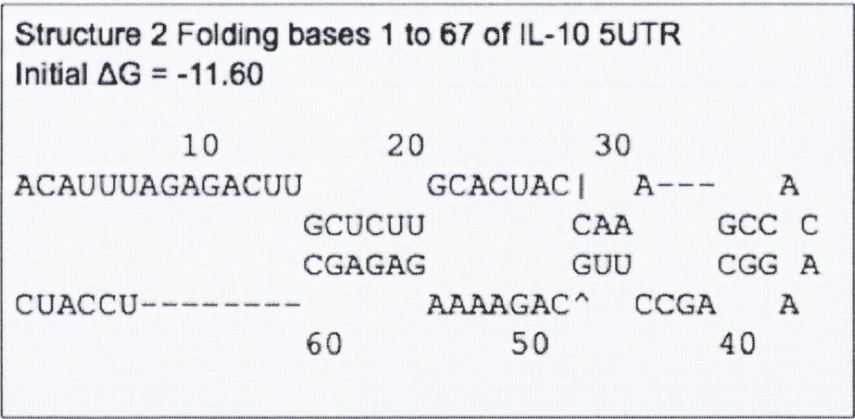
A

Cytokine	5'UTR	Energy requirement for secondary structure formation ΔG
IL-10	ACATTTAGAGACTTG CTCTTGCACTACCAAA G C C A C A AGGCAGCCTTGCAGA AAAGAGAGCTCCATC	= -12.30 kcal/mol
Human IL-10	ACACATCAGGGGCTTG CTCTTGCAAAACCAAAC CACAAGACAGACTTGC AAAAGAAGGC	= -9.6 kcal/mol
IL-6	CCAAGAACGATAGTC AATTCCAGAAACCGC T	= +0.70 kcal/mol
TNF α	AGCAGAAGCTCCCTC AGCGAGGACAGCAAG GGACTAGCCAGGAGG GAGAACAGAACTCC AGAACATCTTGGA TAGCTCCCAGAAAA GCAAGCAGCCAACCA GGCAGGTTCTGTCCCT TTCACTCACTGGCCCA AGGCGCCACATCTCCC TCCAGAAAAGACACC	= -51.00 kcal/mol

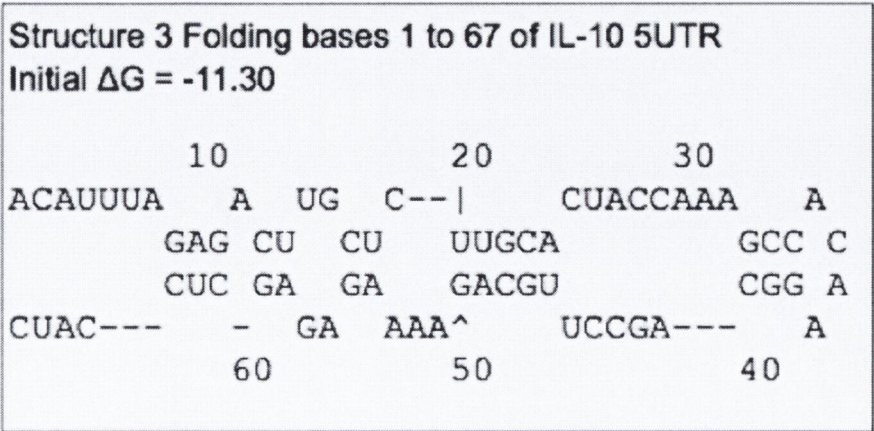
B



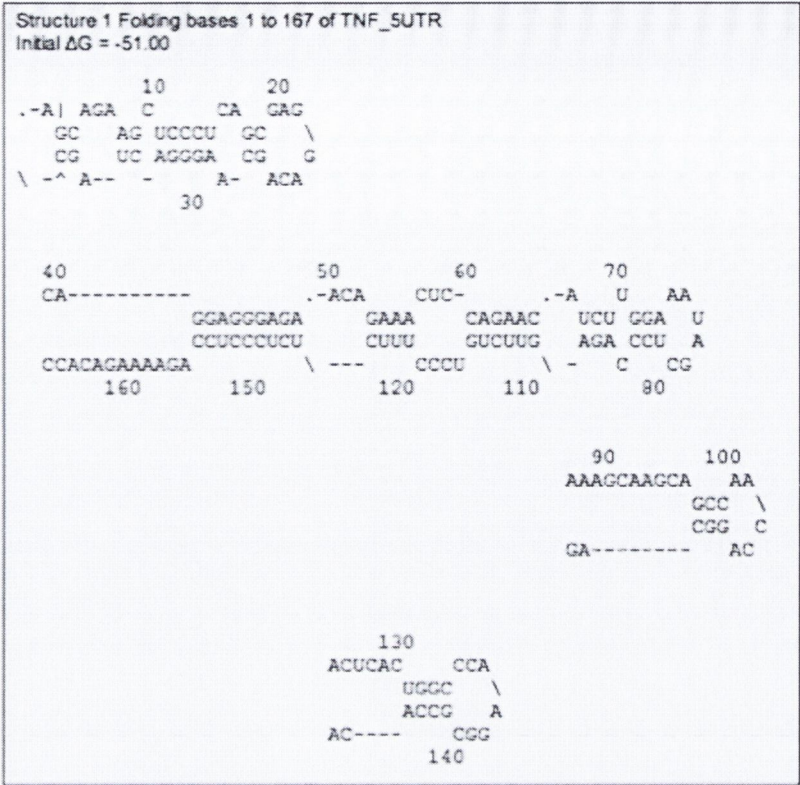
C



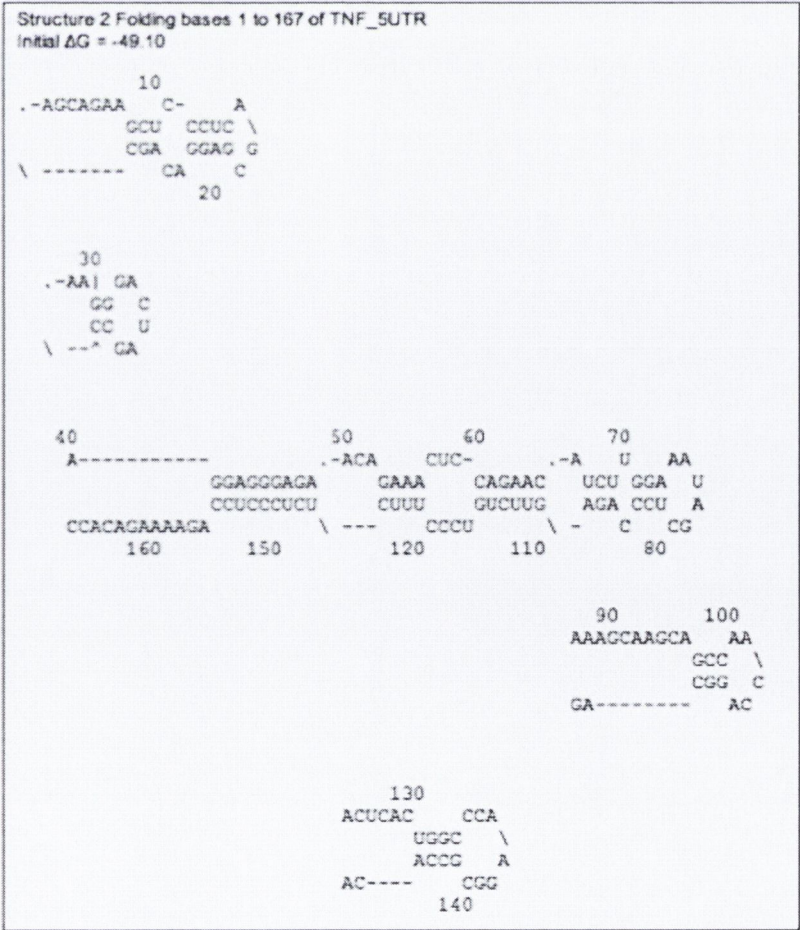
D



E



F



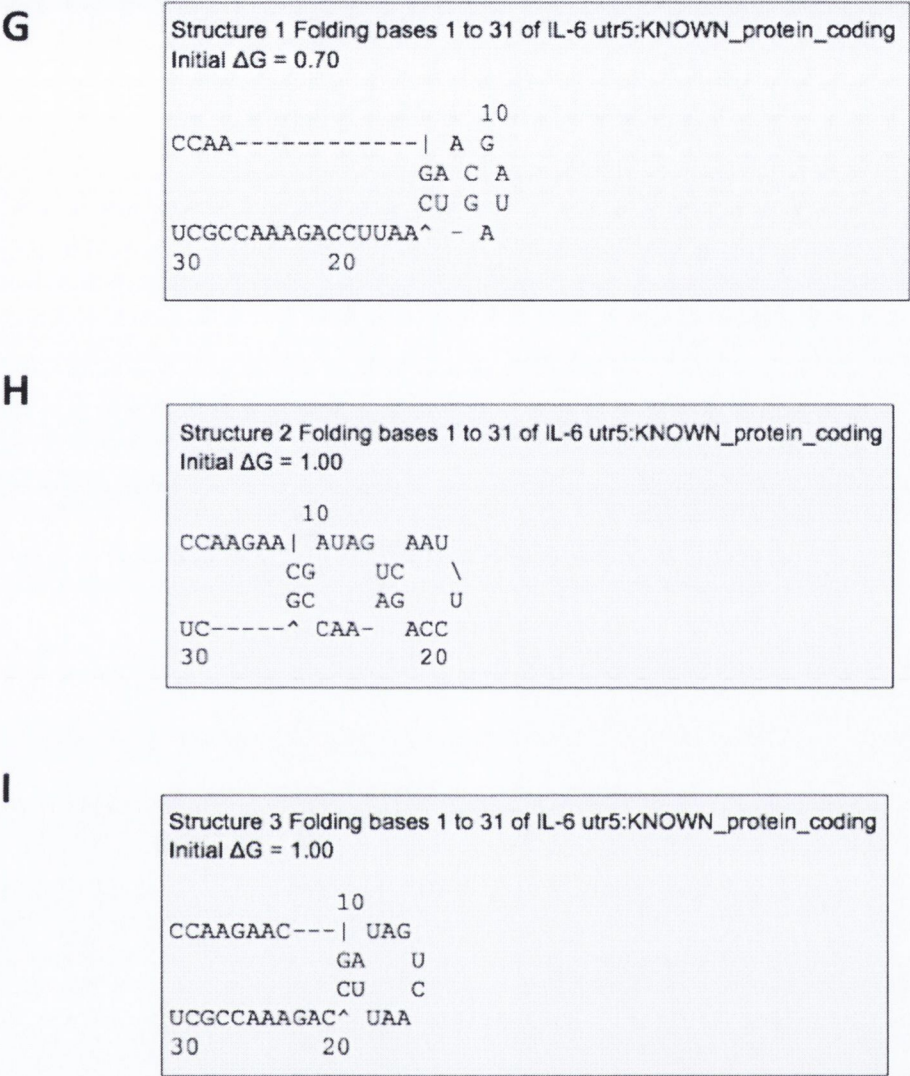


Figure 3.14 Putative secondary structures of 5’UTR of IL-10, TNF α and IL-6 genes.

ENSEMBL was used to obtain 5’UTR sequences of IL-10, human IL-10, TNF α and IL-6 (A). These sequences were then submit into M-fold (<http://mfold.rna.albany.edu/?q=mfold/rna-folding-form>) to predict the folding and any stem-loop structures that could form. (B), (C) and (D) show the three possible structures 5’UTR of IL-10 gene can take, these structures can form in an energy free state. (E) and (F) show the two possible structures 5’UTR of TNF α gene can take, these structures can form in an energy free state. (G), (H) and (I) show the three possible structures 5’UTR of IL-6 gene can take in a non-free energy state.

3.2.15 The effects of Hippuristanol and Silvesterol on IL-10, IL-6 and TNF α translation

IL-10 and TNF α transcription occurs at the same time, but TNF α is immediately translated. Additionally TNF α 5'UTR can form secondary structures and has very low Gibbs free energy and it is predicted to contain a *cis* element in its 5'UTR. The inhibiting effect of rapamycin on IL-10 production, but not on the production of TNF α and IL-6 previously indicated that mTOR was required for IL-10 translation. I therefore next examined if translation of IL-10 but not TNF α or IL-6 was eIF4A-dependent. Two naturally occurring inhibitors of eIF4A, Hippuristanol and Silvesterol, were tested for their effects on IL-10, TNF α and IL-6. The structures of the molecules are shown in Figure 3.15 A.

As can be seen in Figure 3.15 B and C, LPS-mediated induction of IL-10 was blocked in a dose-dependent manner in BMDM by Hippuristanol and Silvesterol pre-treatment. Silvesterol was overall more potent than Hippuristanol, a 25nM dose being sufficient to inhibit IL-10 cytokine production by more than 80%.

As shown in Figure 3.15 D and E TNF α induction was largely unaffected by Hippuristanol and Silvesterol in LPS-stimulated BMDM, however at a 250nM dose of Silvesterol TNF α induction was slightly inhibitory, potentially due to toxicity. This suggested that TNF α translation is not eIF4A-dependent.

As shown in Figure 3.15 F and G LPS-induced IL-6 induction was also not repressed, rather a boost in IL-6 production was observed at 50nM, 75nM, 100nM, and 125nM doses of Silvestrol as well as at 25nM, 50nM, 75nM, 100nM, and 125nM doses of Hippuristanol (Figure 3.15 F and G).

3.2.16 The effects of Hippuristanol on IL-10, IL-6 and TNF α mRNA and protein

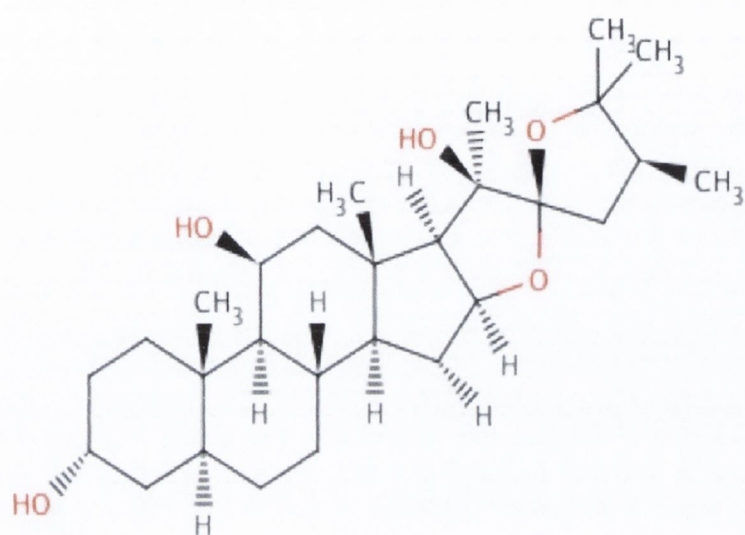
I next tested the production of IL-10, IL-6 and TNF α mRNA in BMDM treated with Hippuristanol and LPS. As can be observed in Figure 3.16 A and B, LPS-induced IL-10 mRNA was not blocked but rather enhanced following Hippuristanol pre-treatment, however protein production was blocked. It was also tested if IL-6 mRNA was affected. A highly significant boost in IL-6 mRNA was observed (Figure 3.16 C), with only a slight boost in IL-6 protein in response to Hippuristanol + LPS (Figure 3.16 D). A boost in TNF α mRNA was also observed in Hippuristanol + LPS treated samples (Figure 3.16 E) whilst the protein translation was largely unaffected.

3.2.17 The role of eIF4A1 and eIF4A2 in IL-10 regulation

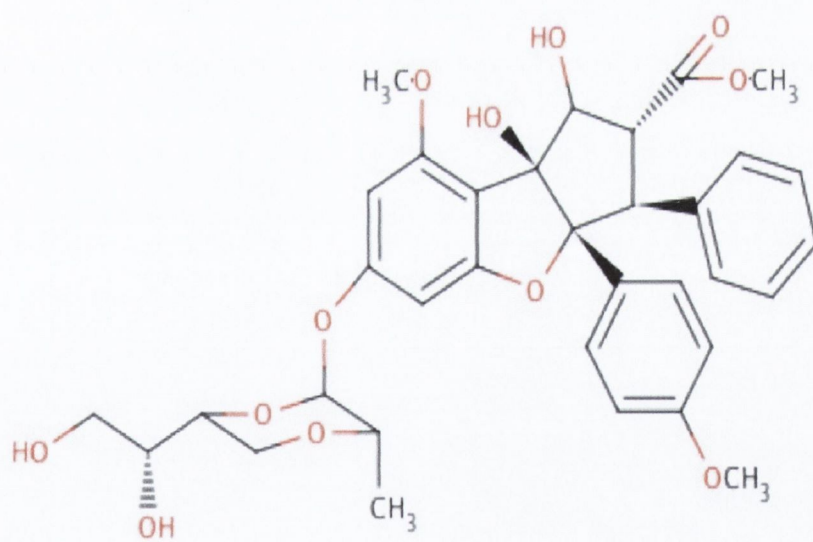
As previously mentioned, eIF4A exists in three isoforms and, of the three eIF4A1 and eIF4A2 are functionally interchangeable as helicases *in-vitro*. Hippuristanol and Silvestrol are known to block all three, I therefore next investigated whether eIF4A1 or eIF4A2 is required for IL-10 translation. Using siRNA, eIF4A1 and eIF4A2 were

knocked down and IL-10 cytokine levels were measured in iBMDM. As can be observed in Figure 3.17 A and B, the knockdown of eIF4A1 and eIF4A2 was successful. IL-10 cytokine production was only blocked in cells in which eIF4A1, and not eIF4A2, had been knocked down, as shown by the ELISA in Figure 3.17 C. This result demonstrated that eIF4A1 was likely to be involved in resolving the IL-10 5'UTR in BMDM. Additionally IL-6 and TNF α were also measured. As shown in Figure 3.17 D TNF α cytokine was also repressed over 30% in eIF4A1 knockdown cells, and as shown in Figure 3.17 E IL-6 cytokine was also repressed in eIF4A1 knockdown cells.

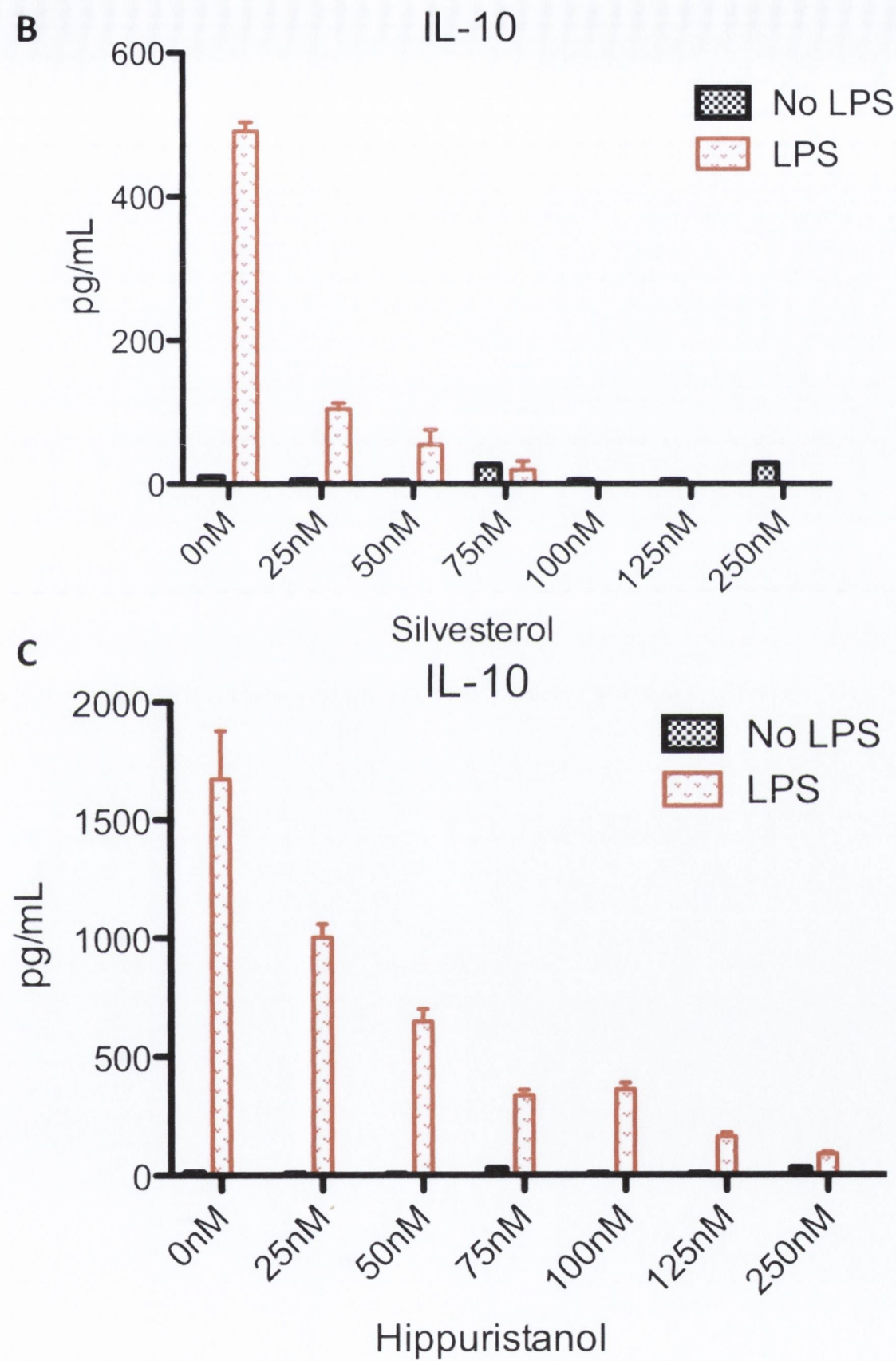
A

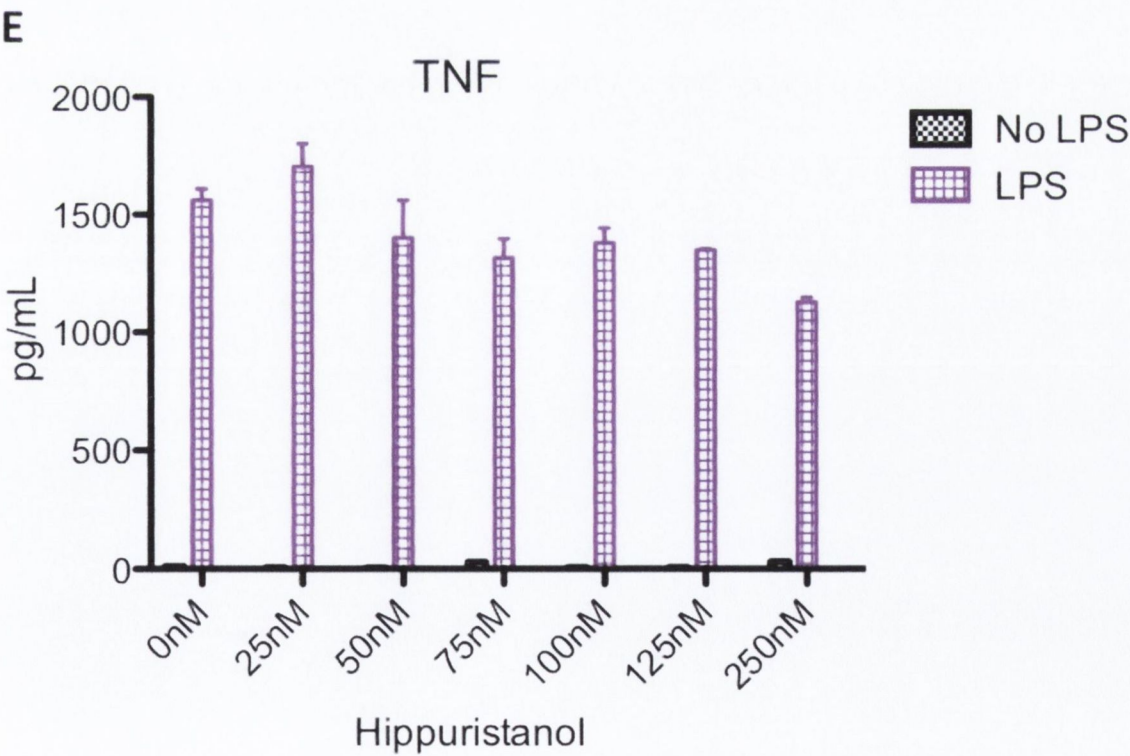
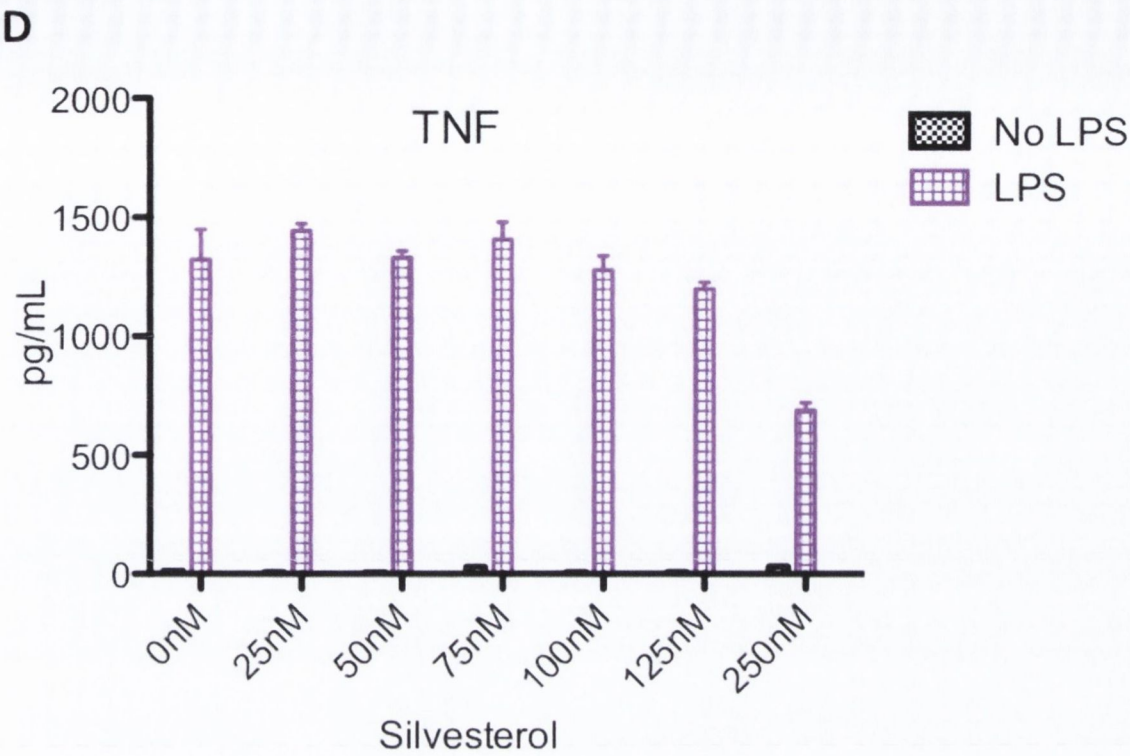


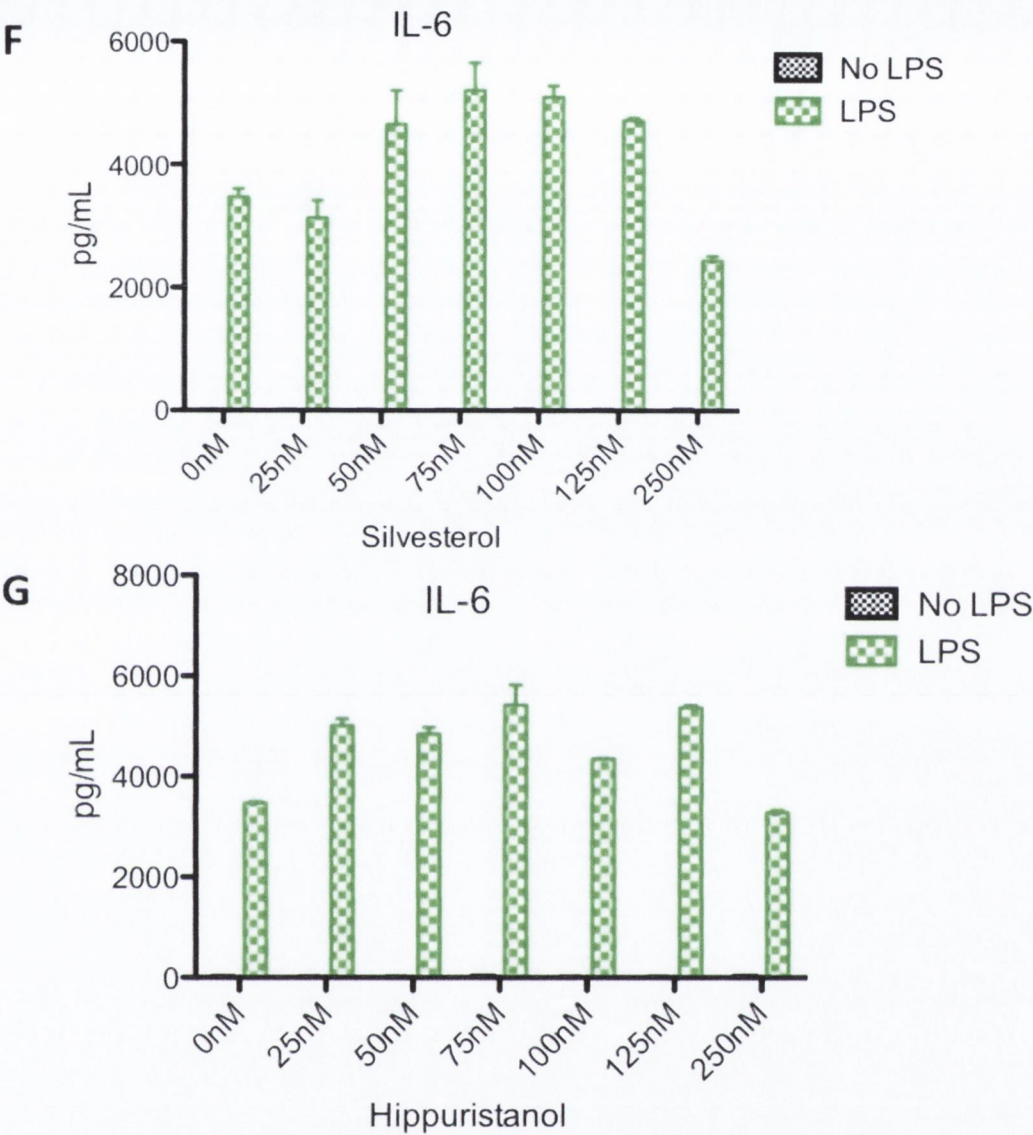
Hippuristanol



Silvesterol

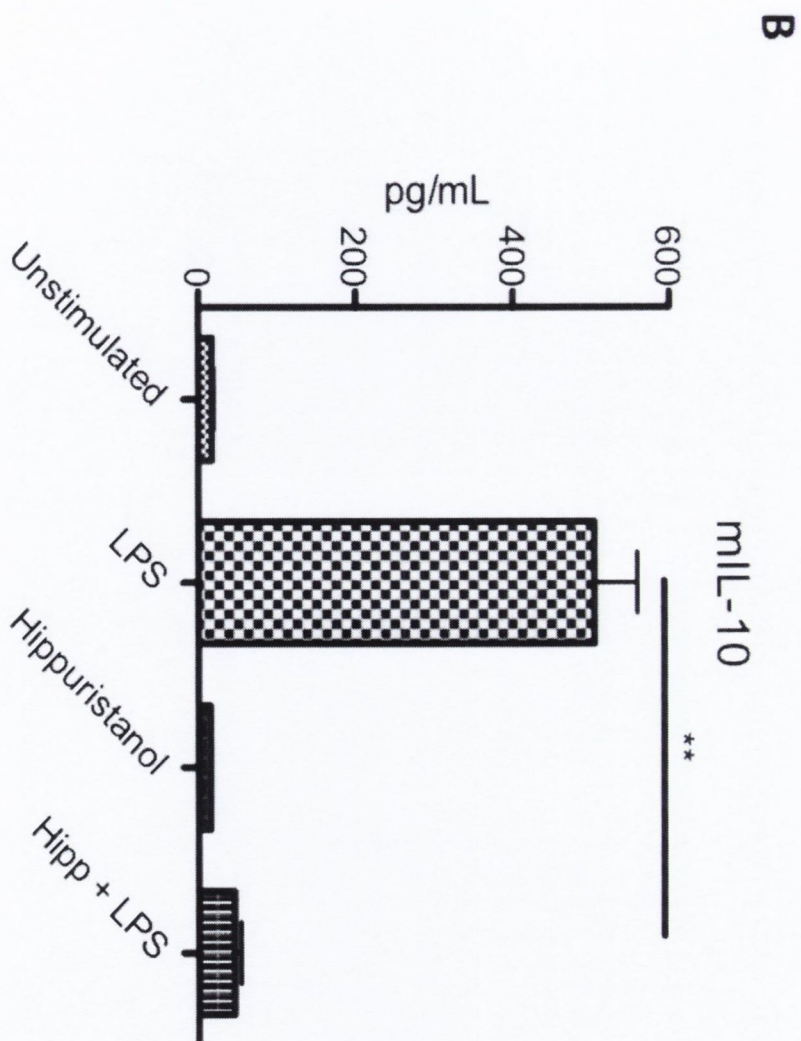
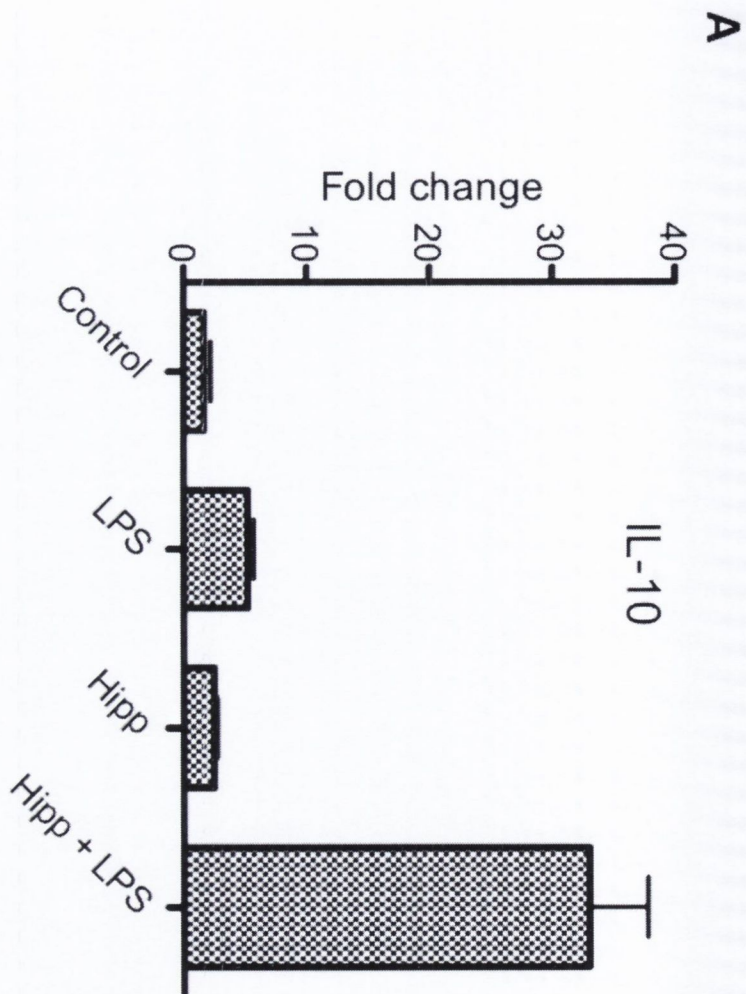


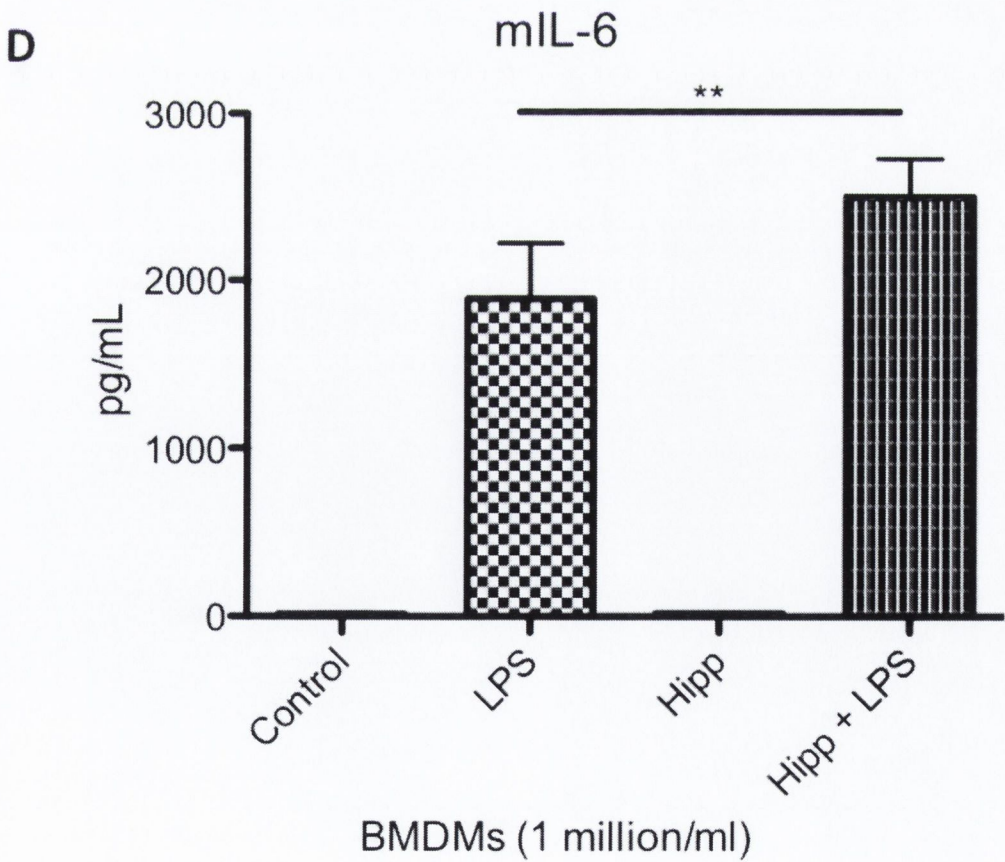
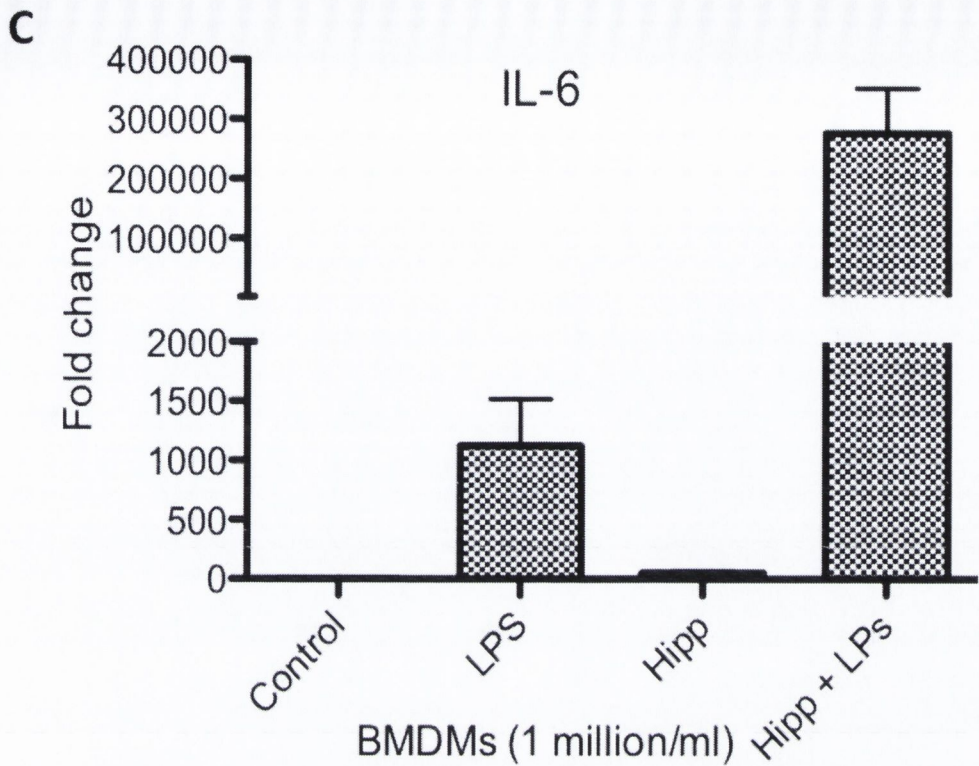




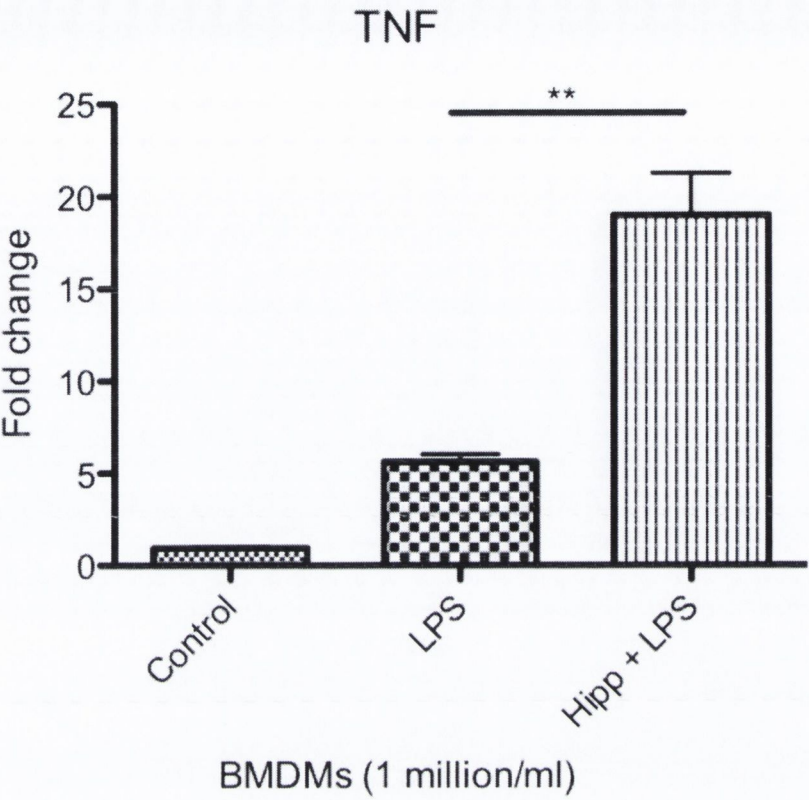
Figures 3.15 Effect of Hippuristanol and Silvesterol on LPS-induced IL-10, IL-6 and TNF α .

(A) The structures of Hippuristanol and Silvesterol. BMDM were seeded at 1×10^6 /mL in a 96 well plate and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. All the cells were then left untreated or, were pre-treated with increasing doses (0nM, 25nM, 50nM, 75nM, 100nM, 125nM or 250nM) of either Hippuristanol or Silvesterol for 30 minutes followed by 100ng/mL LPS for 24 hours. After 24 hours supernatants were analyzed by ELISA for IL-10 (B,C), TNF α (D,E) and IL-6 (F,G). The data shown represent mean $\pm$ S.D. cytokine level from two independent experiments each carried out in duplicate.





E



F

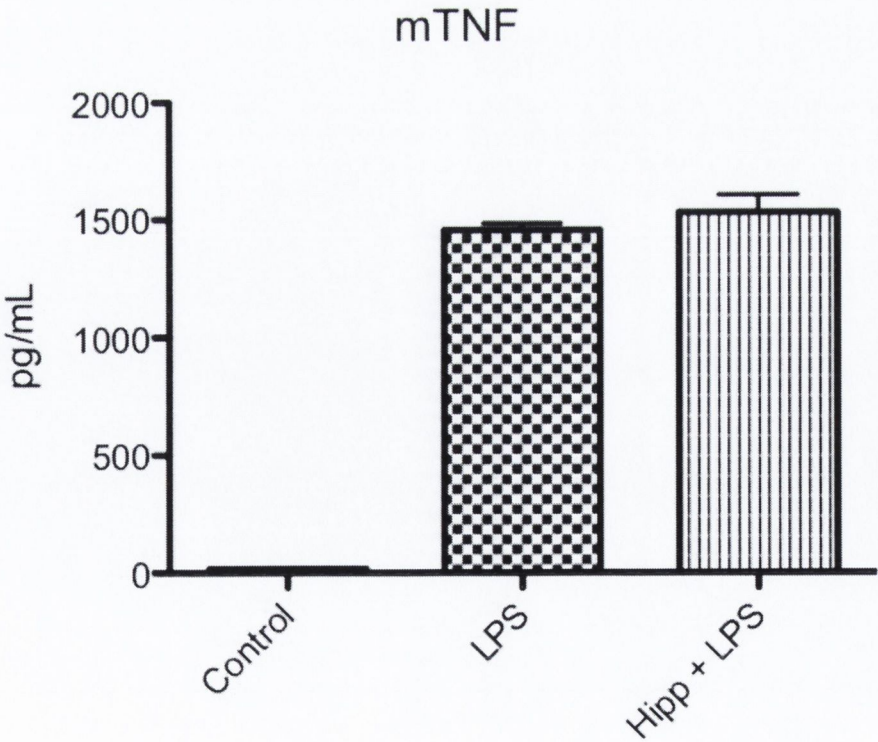
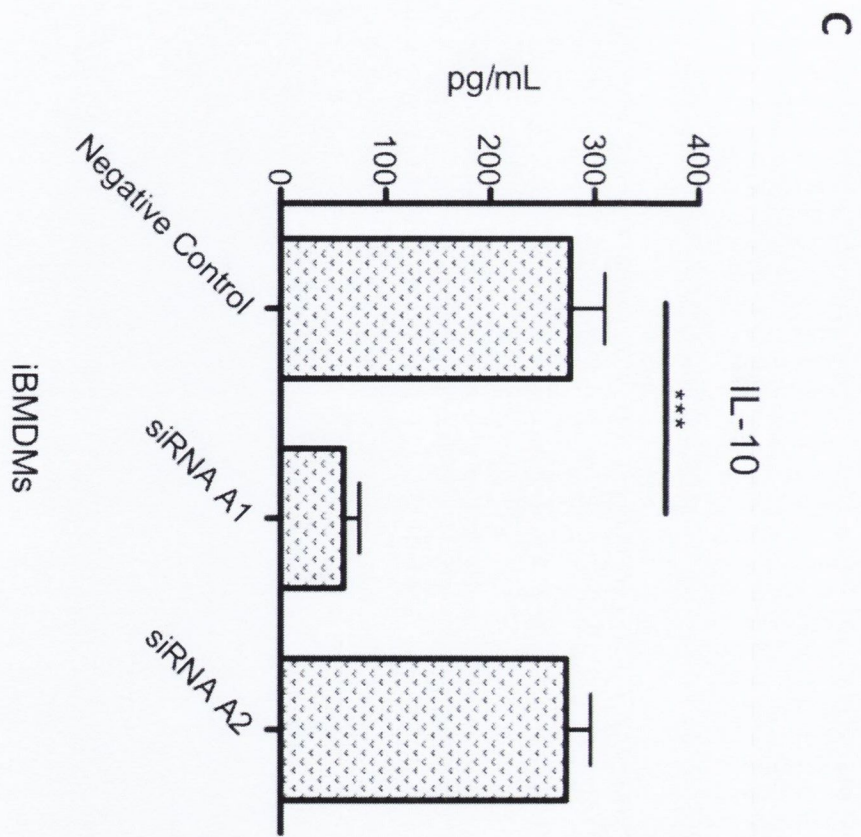
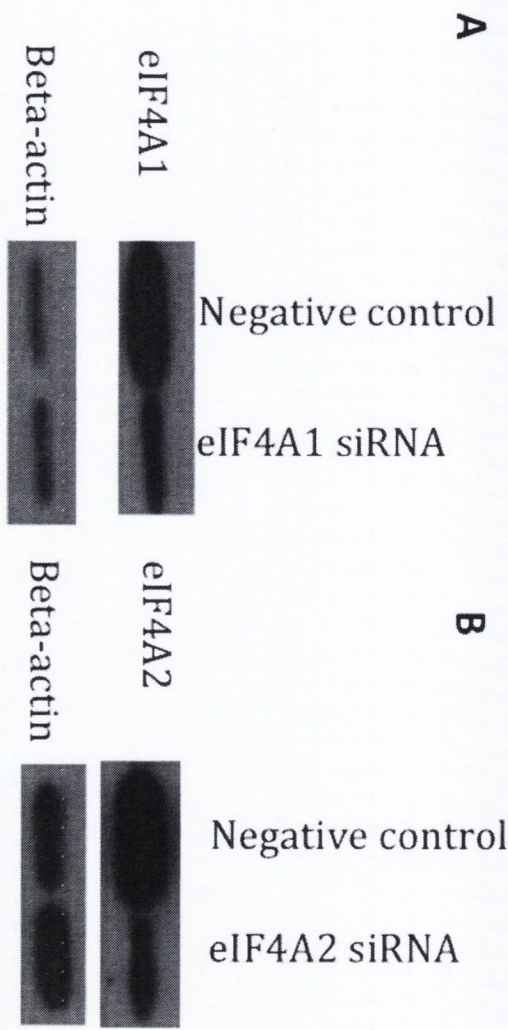


Figure 3.16 Effect of Hippuristanol and Silvesterol on LPS-induced IL-10, IL-6 and TNFα mRNA and Protein.

BMDMs were seeded at 1×10^6 /mL overnight in a 6 well plate and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. All the cells were then left untreated or, were pre-treated with 250nM Hippuristanol for 30 minutes followed by 100ng/mL LPS for 24 hours. RNA was extracted using Qiagen RNeasy kit. Reverse transcription was carried out followed by qPCR using primers specific for IL-10, IL-6 and 18s ribosomal RNA. IL-10 **(A)**, IL-6 **(C)**, and TNFα **(E)** expression were normalised to 18s ribosomal RNA expression and are presented as fold change relative to expression in un-treated cells. The data shown represent mean relative fold change value $\pm$ S.D. from three independent experiments. Supernatants were analyzed by ELISA for IL-10 **(B)**, IL-6 **(D)** and and TNFα **(F)**. The data shown represent mean $\pm$ S.D. cytokine level from three independent experiments; ** $p \leq 0.001$ (two-tailed un-paired t-test).



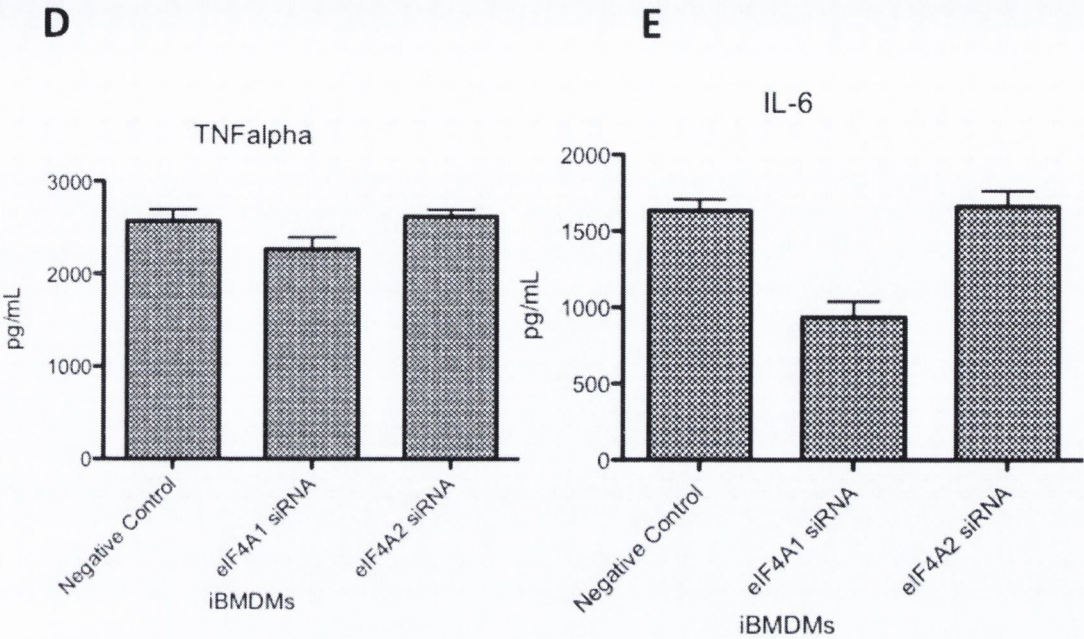


Figure 3.17 eIF4A1 mediated regulation of IL-10 translation.

iBMDM were seeded at $2 \times 10^5/\text{mL}$ in a 6 well plate and incubated overnight at 37°C . The following day media was removed, cells were washed with warm PBS and anti-biotic free media was added. All the cells were then left untreated or, were treated with lipofectamine, or lipofectamine + 50nM scramble, or lipofectamine + 50nM eIF4A1 siRNA, or lipofectamine + 50nM eIF4A2 siRNA. The cells were then left for 72 hours at 37°C . After 72 hours media was taken off, cells were washed with warm PBS and fresh media was added. The cells were then left untreated or, were treated with 100ng/mL LPS for 24 hours. Cells were lysed in sample loading buffer and lysates were Western blotted with anti-eIF4A1 (**A**), anti-eIF4A2 (**B**) and anti- β -actin antibodies (**A and B**). The blot is representative of 3 independent experiments. The supernatants were used to perform ELISA for IL-10 (**C**), TNFalpha (**D**), and IL-6 (**E**). The data shown represent mean $\pm$ S.D. cytokine level from three independent experiments; *** $p \leq 0.0001$ (two-tailed un-paired t-test).

3.2.18 Analysis of eIF4A1 interaction with IL-10

I next investigated if IL-10 mRNA interacted with eIF4A1 protein. An RNA-immunoprecipitation (IP) assay was employed to test this. As shown in Figure 3.18 A eIF4A1 was successfully immunoprecipitated. As the protein concentration was not normalized and since LPS induces eIF4A1, more eIF4A1 was present in lysates and more eIF4A1 was pulled down. As shown in Figure 3.18 B, it was observed that IL-10 mRNA interacted with eIF4A1 protein and that this interaction increased 5 fold in response to LPS.

3.2.19 The effects of Hippuristanol on IL-10 and TNF α production

I next explored the effect of Hippuristanol on eIF4A1 recruitment to IL-10 mRNA, using TNF α as a control. As shown in Figure 3.19 A, Hippuristanol again boosted IL-10 mRNA induction by LPS but repressed IL-10 protein production. There was again a boost in TNF α mRNA production (Figure 3.19 B) with no effect on TNF α protein. Samples from this experiment were used for RNA-IP to detect interaction between eIF4A1 and IL-10 or TNF α . TNF α was used as a negative control. Figure 3.19 C and D demonstrate that eIF4A1 was present in the lysate used for RNA-IP and that it was successfully immunoprecipitated.

Figure 3.19 D demonstrates that IL-10 mRNA interacted with eIF4A1 and that this interaction was not affected with Hippuristanol treatment. This suggests that IL-10

translation was inhibited, potentially, due to the lost ATPase or helicase activity of eIF4A1. On the other hand, as shown in Figure 3.19 E, the TNF α – eIF4A1 interaction was minimal and it was not affected with LPS or Hippuristanol + LPS. Figure 3.19 D and E show negative controls on the right for the RNA-IP where rabbit IgG was used instead of eIF4A1 and no interaction was observed. The experiment was not repeated with Silvesterol due to limited availability of the compound.

3.2.20 Inhibitory effects of the IL-10 5'UTR on IL-10 translation

As shown previously, the IL-10 5'UTR had a predicted capacity of forming secondary structures. Next, it was investigated if the IL-10 5'UTR was capable of forming secondary structure that could inhibit translation. To test this hypothesis, the IL-10 5'UTR (ACATTTAGAGACTTGCTCTTGCACTACCAAAGCCACAAGGCAGCCTTGCAGAAAAGAGAGCTCCATC) was cloned upstream of a luciferase construct in a pGL3-control vector.

Luciferase protein production was compared between the wild-type vector with no insert and the IL-10 5'UTR vector. As depicted in Figure 3.20 the IL-10 5'UTR vector produced less luciferase protein. This suggested that the IL-10 5'UTR potentially forms secondary structure and that this secondary structure restricts translation.

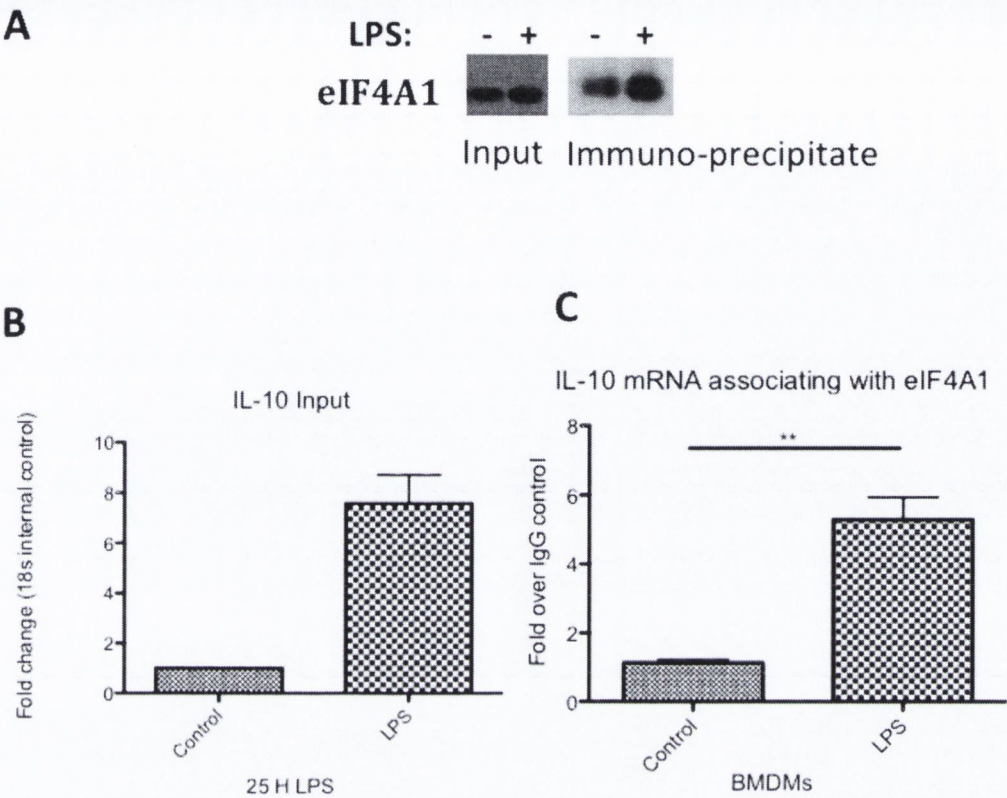
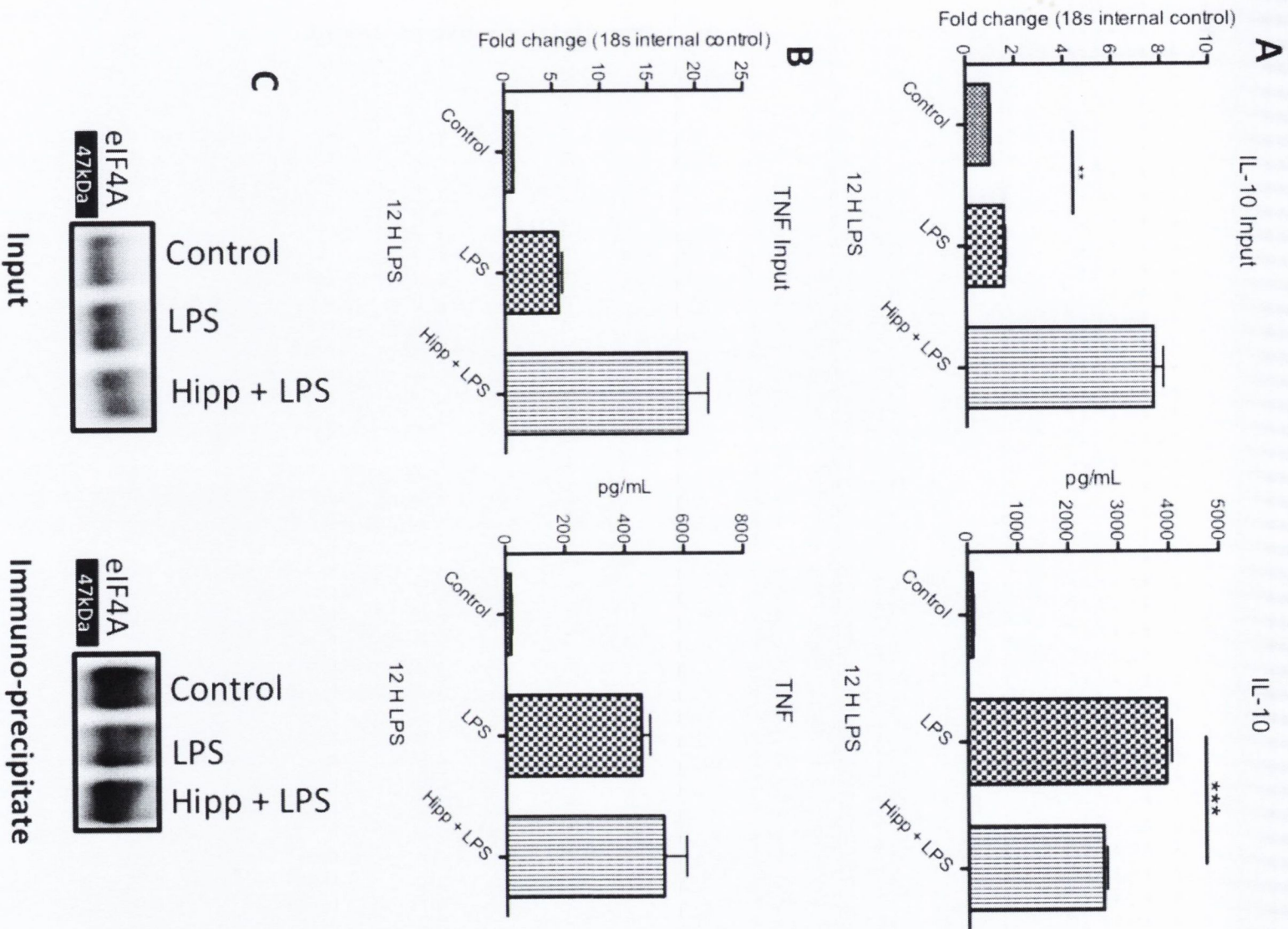


Figure 3.18 eIF4A1 protein and IL-10 mRNA interaction in response to LPS.

BMDM were seeded at 1×10^6 /mL in a 10 cm dishes and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh media was added. All the cells were then left untreated or, were treated with LPS for 24 hours. After 24 hours cells were scraped and pelleted. Following lysis a small sample (50µl) was stored for RNA extraction (input) and a small fraction (20µl) was stored for protein analysis. The remaining lysates were pre-cleared using 25µl Protein A Sepharose CL-4B beads for 1 hour. The lysates were then incubated with anti-eIF4A1 antibody or rabbit IgG coupled to Protein A Sepharose CL-4B for 2 hours at 4°C. Samples were washed three times in 1 ml of lysis buffer and resuspended in 120µl of lysis buffer. 20µl fraction of protein bound beads (immuno-precipitate) were resuspended in 20µl of 2X sample loading buffer. **(A)** represents the Input and IP of eIF4A1. **(B)** shows the RNA input and **(C)** shows enrichment from the input of average IL-10 mRNA bound to eIF4A1 over average IL-10 mRNA bound to IgG $\pm$ S.D. The results presented are representative of three independent experiments; ** $p \leq 0.001$ (two-tailed un-paired t-test) The RNA obtained from IP was normalised to same concentration.



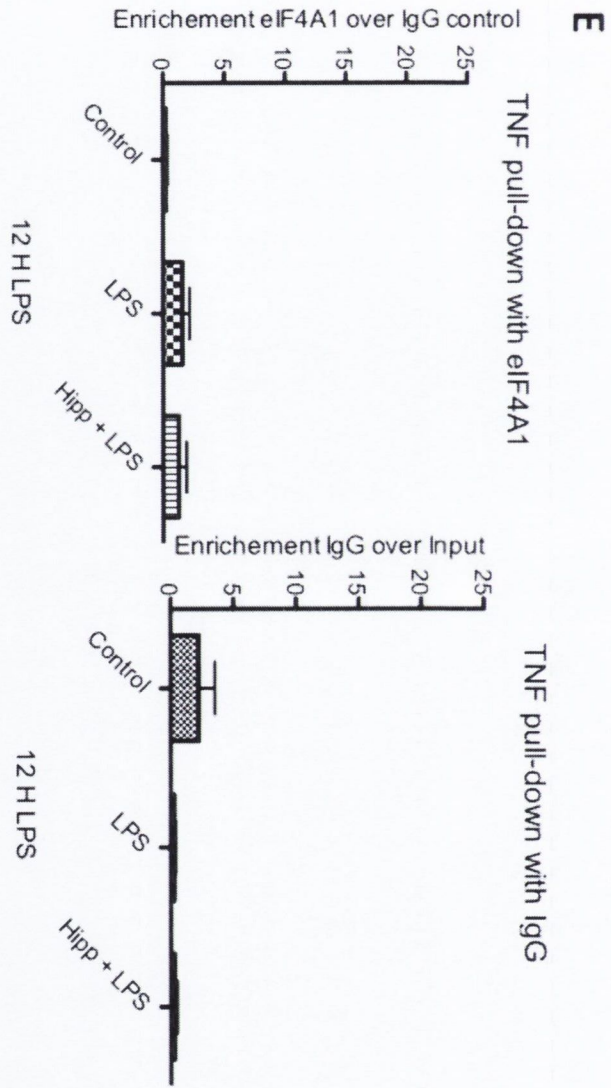
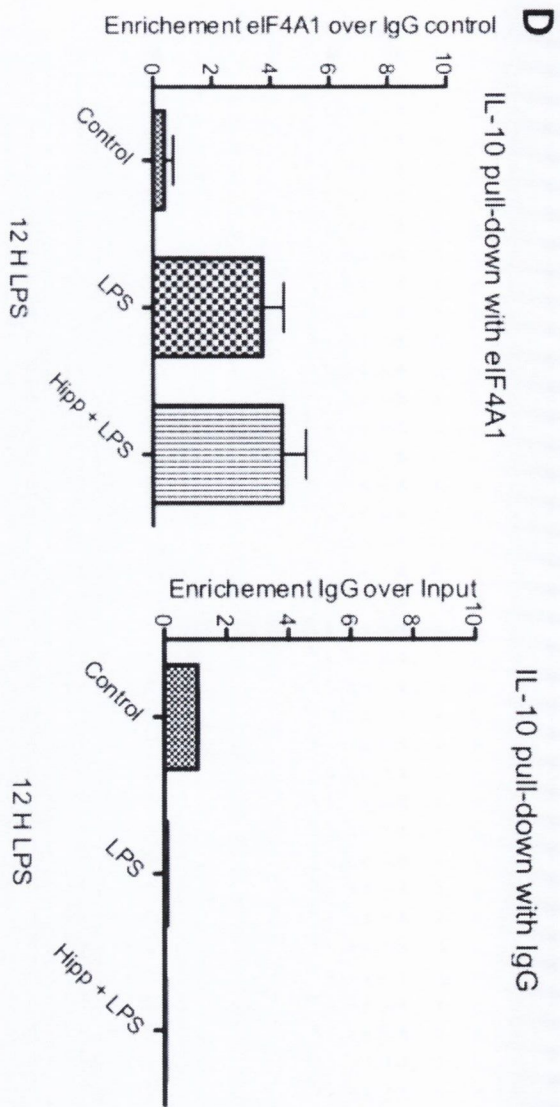


Figure 3.19 Effect of Hippuristanol on eIF4A1 interaction with IL-10, IL-6 and TNF α mRNA.

BMDM were seeded at 1×10^6 /mL in a 10 cm dishes and incubated overnight at 37°C. The following day media was removed, cells were washed with warm PBS and fresh warm media was added. All the cells were either left untreated or were treated with 250nM Hippuristanol for 0.5 hours. Following pre-treatment all the cells were either left untreated or, were treated with 100ng/mL LPS for 24 hours. After 24 hours supernatant was collected, cells were washed with cold PBS and cells were scraped and pelleted. The cells were then lysed in special lysis buffer (recipe in material and methods). Following lysis a small sample (50 μ l) was stored for RNA extraction (input) and a small fraction (20 μ l) was stored for protein analysis. The remaining lysates were pre-cleared using 25 μ l Protein A Sepharose CL-4B beads for 1 hour. The lysates were then incubated with anti-eIF4A1 antibody or rabbit IgG coupled to Protein A Sepharose CL-4B beads for 2 hours at 4°C. Samples were washed three times in 1 ml of lysis buffer and resuspended in 120 μ l of lysis buffer. 20 μ l fraction of protein bound beads (immuno-precipitate) were resuspended in 20 μ l of 2X sample loading buffer.

RNA was extracted using Qiagen RNeasy kit. Reverse transcription was carried out followed by qPCR using primers specific for IL-10, IL-6 and 18s ribosomal RNA. All mRNA expression were normalised to 18s ribosomal RNA expression and are presented as fold change relative to expression in un-treated cells. The data shown represent mean relative fold change value $\pm$ S.D. from three independent experiments. Supernatants were analyzed by ELISA. **(A)** shows IL-10 mRNA and protein, **(B)** shows TNF mRNA and protein.

eIF4A1 input and the immuno-precipitated eIF4A1 is shown in **(C)**. IL-10 **(C)**, and TNF α **(D)** pull down with eIF4A1 and Rabbit IgG under different condition is shown as enrichment over IgG $\pm$ S.D. It is representative of three independent experiments; ** $p \leq 0.001$, *** $p \leq 0.0001$ (two-tailed un-paired t-test)

Equal protein concentration was added to the beads.

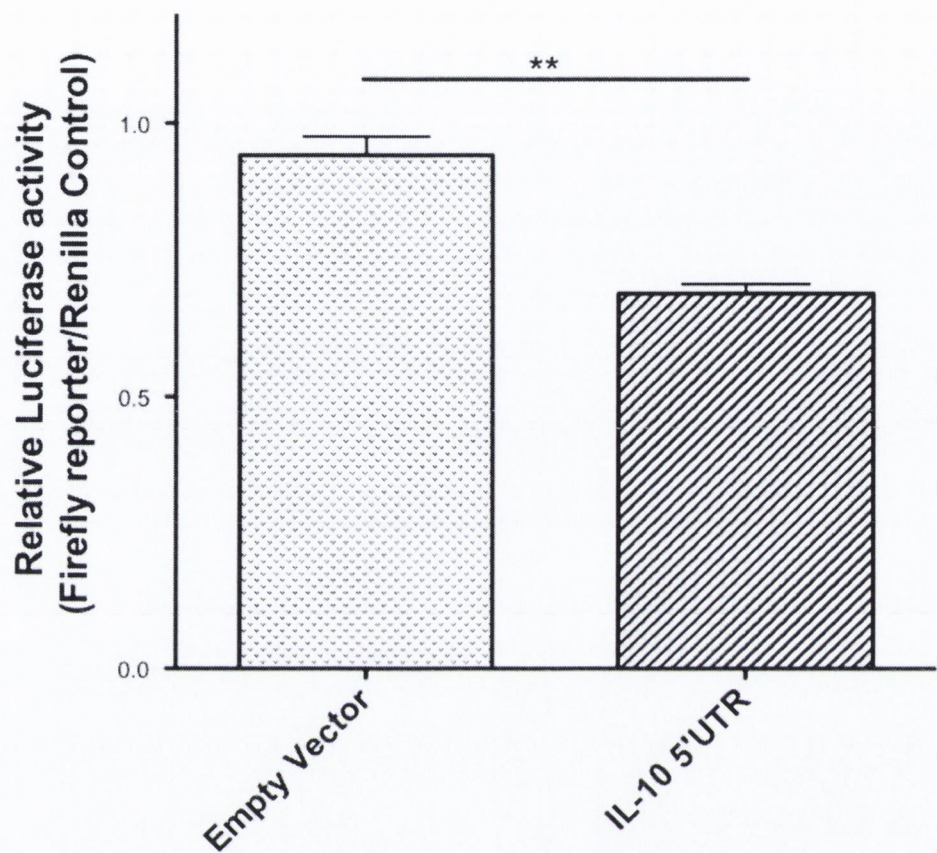


Figure 3.20 Effect of 5'UTR of IL-10 gene on translation of luciferase protein with and without LPS.

(A) HEK293 TLR4 cells were seeded at 2×10^5 /mL in a 96 well plate and incubated overnight at 37°C. The following day the cells were transfected using GeneJuice® with 200ng plasmid encoding luciferase or a fusion of the 5'UTR of the IL-10 gene upstream of luciferase, with Tk-renilla as a transfection control. The figure shows Firefly/Renilla mean $\pm$ S.D; ** $p \leq 0.001$ (two-tailed un-paired t-test).

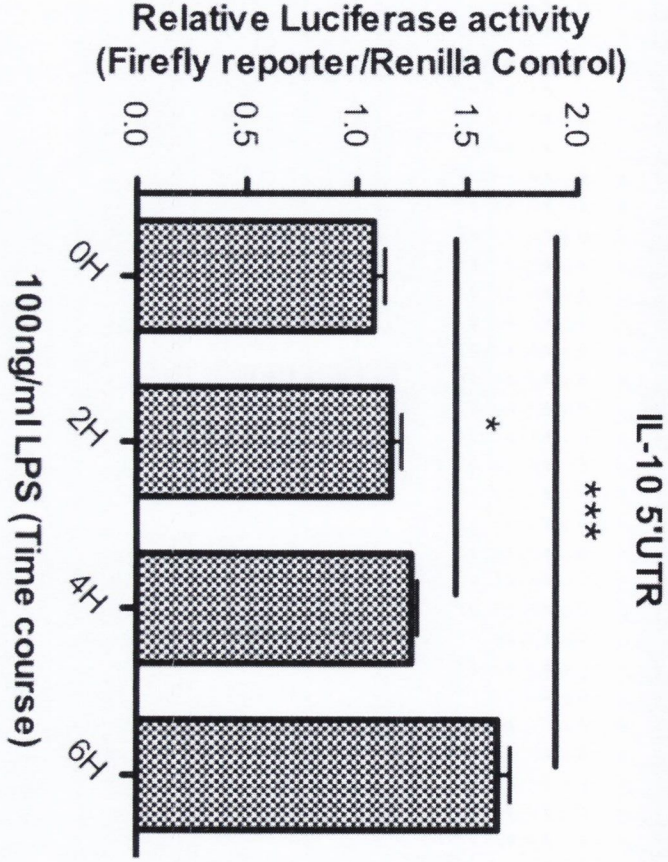
3.2.21 Effect of LPS on the IL-10 5'UTR

Next I tested whether the IL-10 5'UTR mediated repression can be lifted with LPS treatment. LPS was able to induce luciferase expression from the construct containing the IL-10 5'UTR, especially at 6 hours as shown in Figure 3.21 A. The luciferase expression was LPS-induced in a dose dependent manner, as shown in Figure 3.21 C. Figure 3.21 B and D are control samples containing luciferase-only vector and show no effect with LPS.

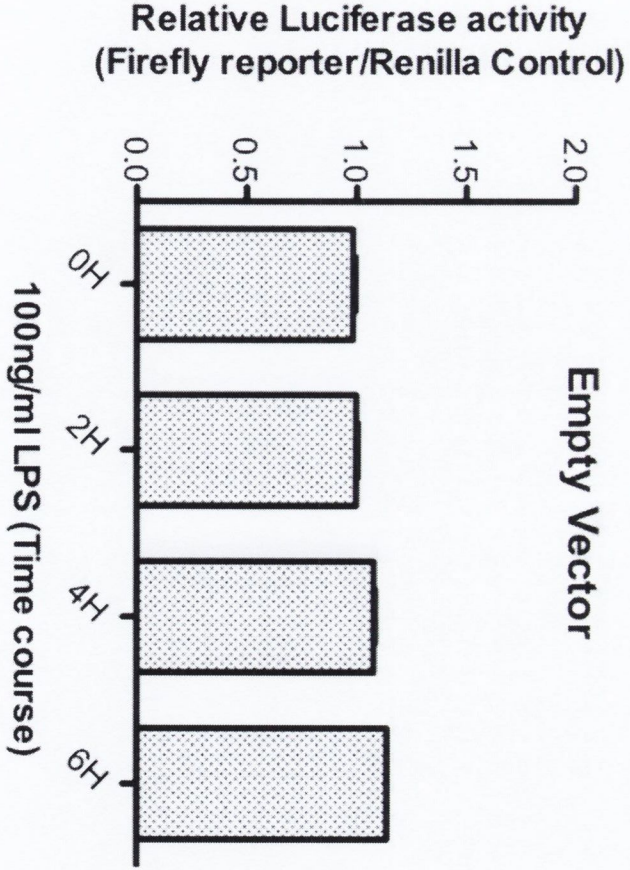
3.2.22 Effect of Silvesterol on LPS-driven IL-10 5'UTR luciferase construct

Finally I tested the effect of Silvesterol on the LPS-driven IL-10 5'UTR luciferase construct. As shown in Figure 3.22 A Silvesterol inhibited LPS-mediated luciferase expression from IL-10 5'UTR. The control construct, without IL-10 5'UTR, was unaffected (Figure 3.22 B). Access to Hippuristanol was limited and therefore we used Silvesterol for this experiment.

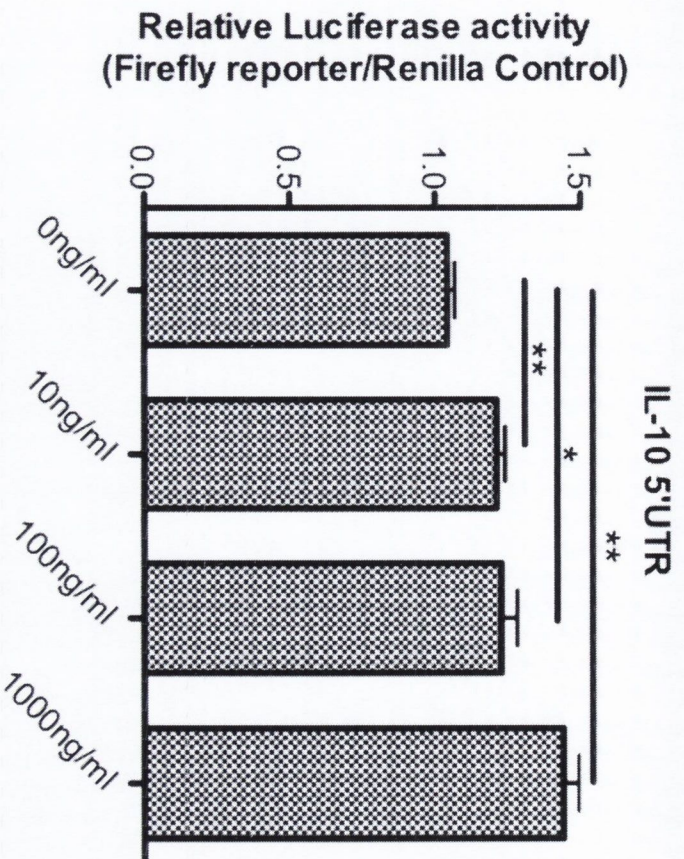
A



B



C



D

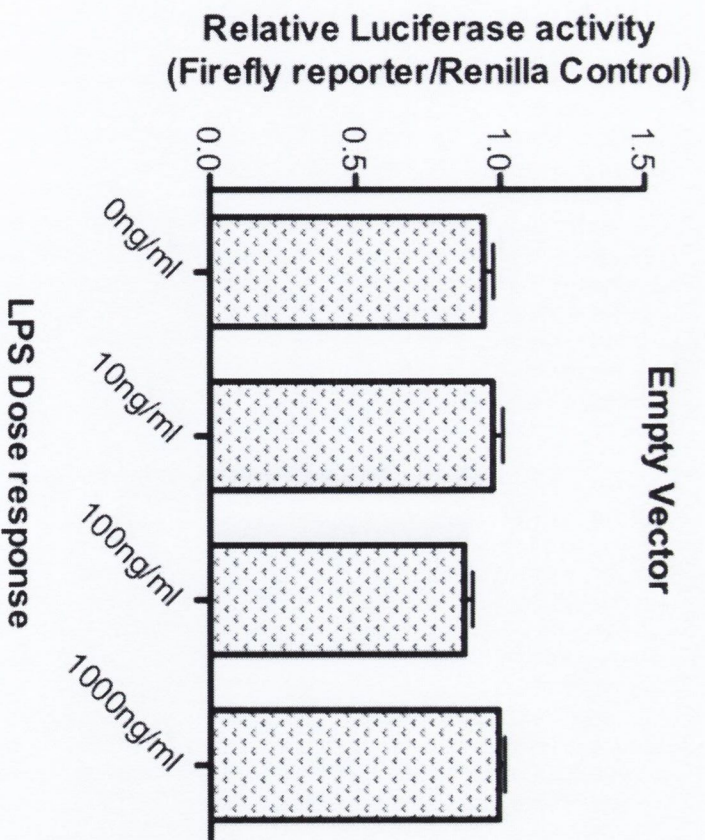
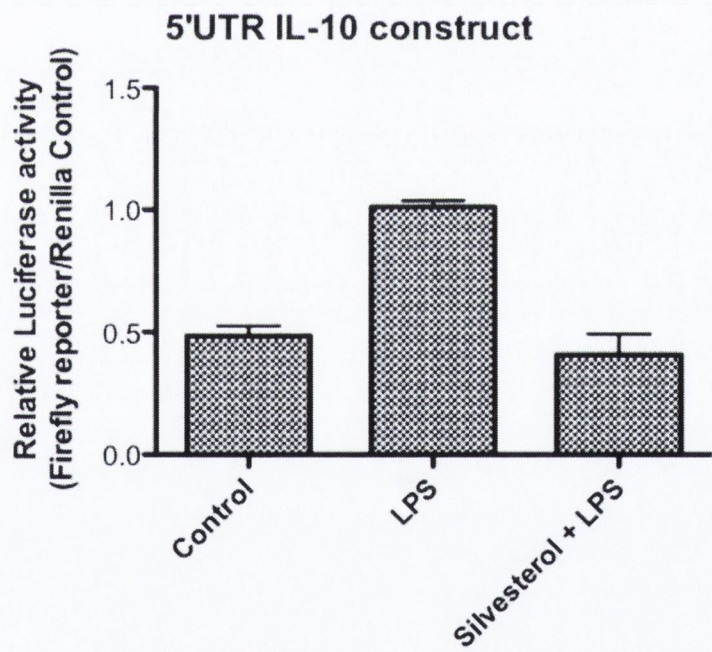


Figure 3.21 Effect of 5'UTR of IL-10 gene on translation of luciferase protein with and without LPS.

HEK293 TLR4 cells were seeded at 2×10^5 /mL in a 96 well plate and incubated overnight at 37°C. The following day the cells were transfected using GeneJuice® with 200ng plasmid encoding luciferase or a fusion of the 5'UTR of the IL-10 gene upstream of luciferase, with Tk-renilla as a transfection control. The concentration of LPS on the IL-10 5'UTR luciferase construct expression compared to untreated samples **(A)** and Luciferase-only vector construct expression compared to untreated samples **(B)**. Effect of increasing doses of LPS on the IL-10 5'UTR luciferase construct expression compared to untreated samples **(C)**. Effect of increasing doses of LPS on Luciferase-only vector construct expression compared to untreated samples **(D)**. The data shown represent mean luciferase expression $\pm$ S.D. and are representative of three independent experiments; * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$ (two-tailed un-paired t-test).

A



B

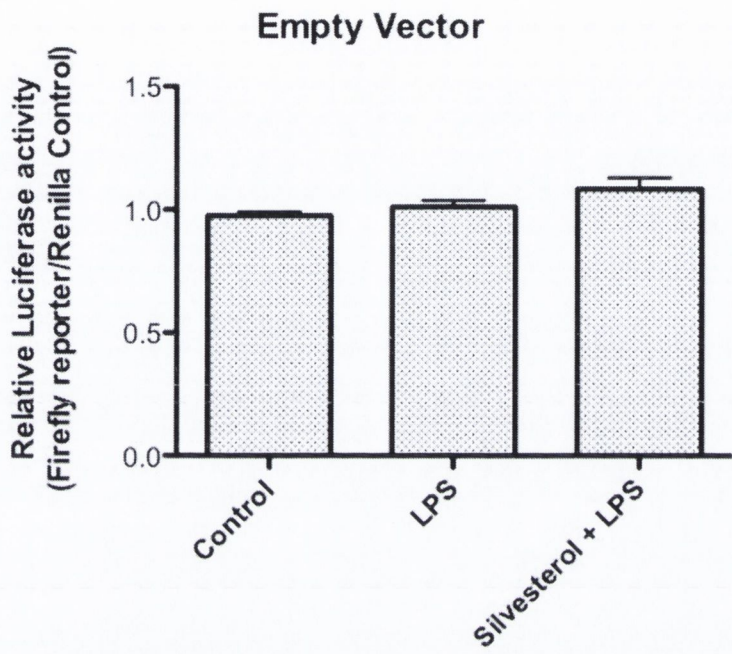


Figure 3.22 Effect of Silvesterol on translation of luciferase construct containing 5'UTR of IL-10 gene .

HEK293 TLR4 cells were seeded at 2×10^5 /mL in a 96 well plate and incubated overnight at 37°C. The following day the cells were transfected using GeneJuice® with 200ng plasmid encoding luciferase or a fusion of the 5'UTR of the IL-10 gene upstream of luciferase, with Tk-renilla as a transfection control. **(A)** compares luciferase expression from the untreated IL-10 5'UTR gene with that treated with LPS or Silvesterol + LPS. **(B)** compares luciferase expression from untreated luciferase-only vector with that treated with LPS or Silvesterol + LPS. The data shown represent mean luciferase expression $\pm$ S.D. and is representative of three independent experiments.

Chapter 4

Discussion

Chapter 4:

4.1 Discussion

4.1.1. Importance of LPS-repressed miRNA

The first line of investigation this study undertook was to examine LPS-repressed miRNA. The importance of LPS repressed miRNA has been highlighted in several studies. One such study showed that miR-107 is repressed in response to LPS leading to an increase in expression of its target cyclin-dependent kinase 6 (CDK6) (Hennessy et al., 2011a). CDK6 expression is required for adhesion of BMDM in response to LPS. Other studies have shown the importance of miRNA that are repressed following the initiation of inflammation. For instance a study on cholangiocytes showed that let-7i expression is repressed in *Cryptosporidium parvum* infected cells, thereby allowing the up-regulation of TLR-4 expression (Chen et al., 2007). Let-7i is involved in the regulation of TLR-4 protein in a MyD88/NFκB-dependent manner, where inhibition of MyD88 and NFκB blocked LPS-induced inhibition of let-7i. Upon infection with *C. parvum* let-7i is blocked which allows increased translation of TLR-4 mRNA and thereby the protein. LPS-induced TLR-4 activation leads to induction of a few hundred genes in BMDM. Genes that are induced in response to LPS have different roles. Some of these genes code for inflammatory cytokines and chemokines, whereas some others have anti-microbial and tissue repair properties, whilst there are a few that participate in more metabolic roles. Similarly it is known that LPS-stimulated BMDM induce expression of several hundred miRNA. Previous screens showed that LPS has an effect on

several hundred miRNA. These LPS-induced miRNA could participate in regulating genes for different processes.

Over the past few years numerous studies have highlighted the role that miRNA play in various cellular processes. For instance miR-125b has been shown to regulate the mitochondrial proteins BIK and MTP18 (Duroux-Richard et al., 2014), whereas miR-122 targets Pyruvate Kinase M2 (Liu et al., 2014). A number of miRNA with anti-inflammatory roles have also been identified, for example miR-149 and miR-124. miR-149 negatively regulates MyD88 (Xu et al., 2013) and miR-124 targets STAT3, thereby decreasing IL-6 and TNF α converting enzyme (TACE) (Sun et al., 2013). Furthermore, a number of miRNAs have also been associated with anti-microbial activity for instance miR-27B targets KH-type splicing regulatory protein (KSRP) and enhances iNOS mRNA stability and thereby promotes anti-microbial activity (Zhou et al., 2012). Therefore miRNA can be pro-inflammatory, anti-inflammatory, anti-microbial or participate in various metabolic and tissue-repair pathways.

From my deep-sequencing screen in BMDM it was identified that miRNA could be classified into three categories, LPS-induced, LPS-repressed and LPS non-responsive. As the focus of this study was to identify targets of LPS-repressed miRNA, we only showed the LPS-repressed list; the other two lists can be found in the appendix. Mechanistically, the observation that LPS can repress miRNAs is consistent with the activating role of TLRs. Previous studies have shown that following TLR activation the expression of many proteins are induced. As miRNA

negatively regulate protein synthesis it would make sense for TLR activation to lead to a decrease in miRNA activity. In principle this serves to relieve the brakes on protein expression. In support of this data, miR-107 and miR-125b, which have been published to be LPS-repressed were present in the LPS-repressed list. In addition LPS-induced miRNA, miR-155, miR-146a and miR-21 were also detected.

We screened for LPS-repressed miRNA at a relatively early time point, 4 hours. The immune response involves an immediate response, which is mostly pro-inflammatory and deals with pathogens quickly followed by a delayed response involved in resolution of inflammation. For the immediate early response we hypothesized that most mRNA should be present basally. Once the cell is stimulated the activation signals would unblock the miRNA-mediated repression and mRNA would be translated. It was hypothesized that certain miRNAs would be present basally to keep mRNA for pro-inflammatory mediators repressed. Such miRNAs would then degrade, when LPS activates the cell, leading to increased translation. LPS might therefore rapidly induce mRNAs and repress the miRNAs that might target them. Additionally, certain miRNA could be present basally that would be degraded in response to LPS and therefore not target mRNA induced in response to LPS. This is depicted in Figure 4.1.

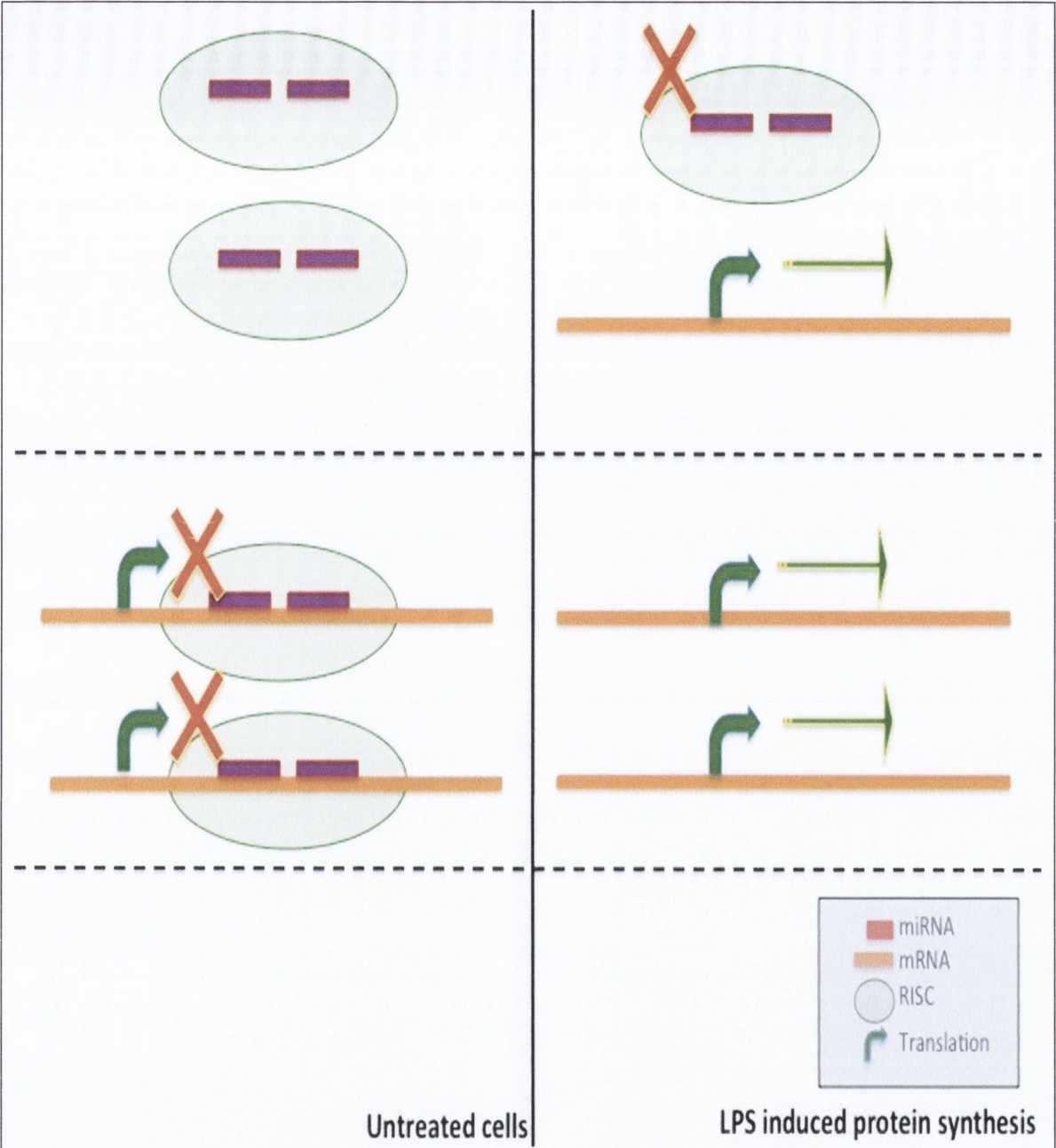


Figure 4.1 miRNA-mediated repression. The miRNA-RISC complex binds mRNA and inhibits translation initiation in untreated cells. Or miRNA-RISC complex is present basally to target mRNA induced in response to LPS. In response to extracellular stimuli such as LPS, miRNA are degraded and the translation repression is lifted allowing protein synthesis to occur. This ultimately leads to production of proteins required by the cell in response to extraceullular stimuli.

Furthermore these miRNAs that were immediately repressed by an inflammatory trigger could return to basal level at later time points and thereby be involved in negative regulation. In a study by Medzhitov and colleagues, primary response genes (PRGs) and secondary response genes (SRGs) are shown to be differentially regulated (Hargreaves et al., 2009). This study shows that many PRGs, in contrast to SRGs, have preassembled RNA polymerase II and positive histone modification basally to allow an immediate response. PRGs are basally repressed through co-repressor complexes. Once triggered PRGs are quickly transcribed. Hence PRGs would participate in immediate early response whereas SRGs would be involved in delayed responses. We hypothesized that miRNA-mediated gene regulation could be another mechanism to achieve differential regulation for PRGs and SRGs. In our hypothesis the mRNA are present basally but kept inactive via miRNAs. An example of this hypothesis is miR-98a which targets mitogen-activated protein kinase kinase 4 (MKK4), a kinase that activates JNK/stress-activated protein kinase. In TLR-triggered system, miR-98a rapidly decreased to allow the activation of the JNK/c-Jun pathway and the production of inflammatory cytokines in macrophages (Lai et al., 2013). It is also possible that certain miRNA are present basally waiting to target mRNA produced to fine tune the inflammatory response. Such miRNA would also be degraded in response to LPS to allow inflammation- such miRNA would then return to basal level to degrade mRNAs. One example of such occurring is miR-107 which is basally present and degraded in response to LPS, whilst its target CDK6 is induced in response to LPS (Hennessy et al., 2011b).

4.1.2. Targets of LPS-repressed miRNA

After identifying over 200 miRNA repressed below basal level in response to LPS, miRNA target prediction programs were used to identify putative targets for LPS-repressed miRNA. A number of target prediction programs were cumulatively used, namely Miranda, miRBase, TarBase and PicTar. All the programs use different formulae to predict miRNA-mRNA base pairing and we only shortlisted the targets that were common between various databases. This added confidence in target prediction. Furthermore, manual miRNA seed-sequence and mRNA sequence matching was also performed. In addition the criteria set to choose targets was based on 1) its immunological relevance, 2) preferably known to be up-regulated in LPS-induced responses and 3) a number of miRNA targeted the same gene. This approach again, allowed identification of more reliable targets.

A subset of LPS-repressed miRNAs targeting the eukaryotic translation initiation family members was identified. Eukaryotic translation initiation is a highly regulated process and therefore it was interesting to have made this observation. When a cell enters into an inflammatory state it actively produces cytokines, chemokines, and a lot of other inflammatory mediators. This regulatory step would therefore fine-tune the process. The inflammatory response puts the cell under stress and requires a higher than normal level of protein translation. The cell is, to an extent, in a state of growth. LPS has been known as a mitogen (Jacobs and Morrison, 1977), and here we have confirmed its role in enhancing protein translation. A number of studies have demonstrated an increased requirement of

eIF machinery to deal with cell proliferation (Donaldson et al., 1991, Mader and Sonenberg, 1995, Kleijn and Proud, 2000). Furthermore it has been shown that over-expression of translation factors leads to increased cell growth (De Benedetti and Rhoads, 1990). Hence LPS-repressed miRNAs targeting eIFs was of interest, since this process could reveal new mechanisms into regulation of immunologically relevant translation. In addition, little is known about the role of inducible eIFs in LPS action therefore I decided to pursue their role here.

Seven eIF family members came up on the screening test for miRNA-mRNA pairing; a number of them were targeted by more than one miRNA. eIF4A2 was predicted to be targeted by three miRNA, miR-98-3p, let-7c-1-3p and let-7b-3p. Let-7 family members are known for their role in cell transformation. It has been shown that transient activation of an inflammatory signal could lead to down-regulation of let-7 which allows a greater than normal expression of IL-6 (Gerard et al., 2014). Furthermore let-7 family members are also implicated in numerous inflammatory and malignant diseases. miR-98 is also a very well studied miRNA and has diverse roles ranging from regulation of tumourigenesis to inflammatory mediators. The two families, let-7 and miR-98 are often dysregulated together in various diseases and are being studied as diagnostic biomarker in ulcerative colitis (Coskun et al., 2013). Given that these miRNA families are involved in cell transformation and all were targeting eIF4A2, I decided to test their expression. I re-tested the expression of let-7c-1-3p and let-7b-3p using specific TaqMan primers and probe and observed an LPS-mediated repression.

4.1.3. The anomaly between eIF4A mRNA and Protein

Having observed the repression of let-7c-1-3p and let-7b-3p, I next analyzed the expression of eIF4A2. eIF4A2 protein was strongly induced in response to LPS at 8 and 24 hours. However eIF4A2 mRNA levels did not follow the same trend. The anomaly of reduced levels of eIF4A2 mRNA and increased eIF4A2 protein is not a unique observation to this study. It has been observed in various other circumstances, for instance in PDCD4 knock-out mice, the IL-10 mRNA was lower compared with wild-type mice, whilst the knock-outs produce more IL-10 protein (Sheedy et al., 2010).

There is some evidence that explains this phenomenon, and a number of reports have described its existence and proposed possible reasons (Ross, 1995, Jacobson and Peltz, 1996). One of the explanations is that the mRNA is degraded due to increased activity of mRNA destabilizing factors (possibly induced in response to LPS), or due to reduced transcription of mRNA, both of which could be due to increased protein stability. A different reason, and more challenging to justify is that the translation process itself negatively regulates the stability of mRNA. The former hypothesis is supported by Ross (Ross, 1997). Considering the two situations together it is possible that a negative feedback loop exists (Figure 4.2), where increased protein stability leads to reduced mRNA stability and reduced mRNA leads to increased protein stability. miRNA could be participating in this phenomenon as well. A study on NLRP3 has identified such a role for miR-223 (Haneklaus et al., 2012) in regulating NLRP3 mRNA. This study focuses on the

importance of eIF4A2 up regulation, rather than the anomaly of mRNA-protein differences, which could be an interesting aspect for future studies.

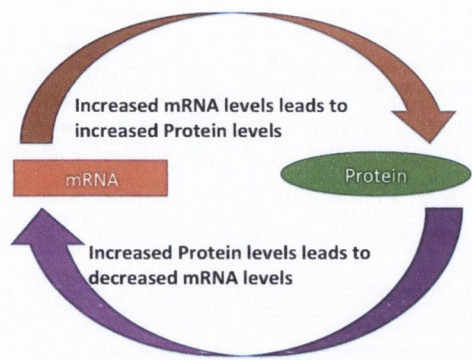


Figure 4.2. Negative feedback loop between mRNA and Protein.
Extracellular stimuli induce mRNA expression of a number of genes. This increase in mRNA leads to an increase in protein levels. Increased protein level or increased protein stability leads to a decrease in mRNA levels.

4.1.4 Regulation of eIF4A induction

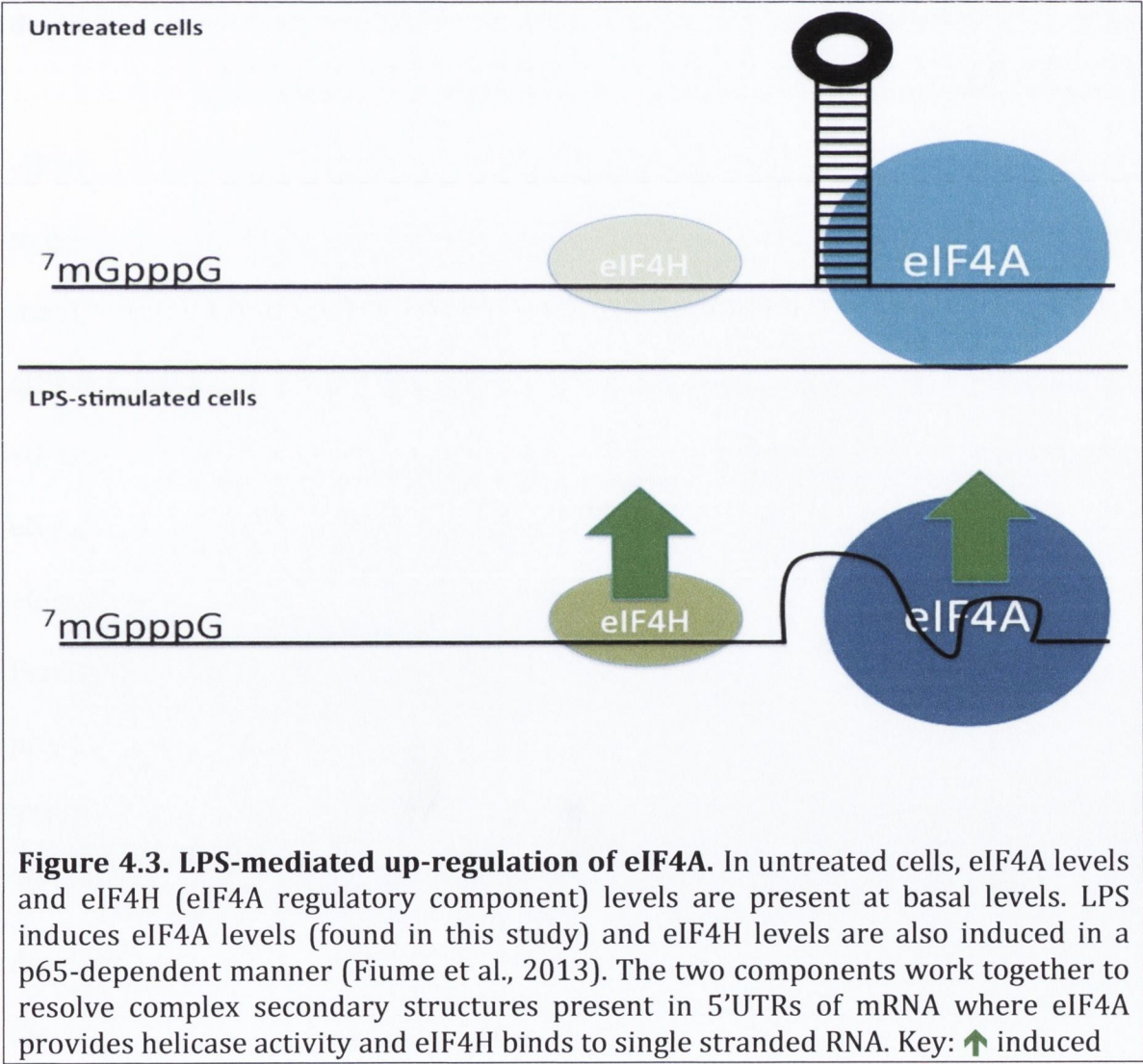
For helicase proteins such as eIF4A, up-regulation is not a sufficient readout for their increased function or activity, however an increased presence suggests an increased requirement. We therefore decided to investigate what regulated eIF4A and what the functional relevance of this up regulation might be.

eIF4A2 shares 90-95% homology with its more dominant isoform eIF4A1 (Rogers et al., 2002). Testing the eIF4A1 response to LPS revealed that eIF4A1 mRNA was up regulated up to 2 hours after LPS treatment and thereafter it was repressed. At the protein level eIF4A1, like eIF4A2, was also strongly induced at 8 and 24 hours. The two isoforms are known to be functionally indistinguishable *in-vitro* (Nielsen and Trachsel, 1988). However, eIF4A1 is the more dominant isoform present at higher levels than eIF4A2 in most human tissues (Galicia-Vazquez et al., 2012b). A few

recent studies have demonstrated that eIF4A2 is up regulated when eIF4A1 expression is blocked using siRNA. This supported the notion of eIF4A1 and eIF4A2 working interchangeably however functional analysis revealed that eIF4A2 was unable to completely relieve translation requirements in cells (Galicia-Vazquez et al., 2012a). In our study, it was found that eIF4A1 was the more dominant isoform in BMDM; and both isoforms were strongly induced with LPS.

Computational analysis of CHIP-seq data from ENCODE showed the presence of NF κ B, AP-2 α , c-Myc, IRF1, SP1, IRF3, and TAF1 binding sites in the eIF4A1 and eIF4A2 promoter. More detailed computational analysis using JASPAR, TFSEARCH and TFBIND revealed putative p50/p65 sites in eIF4A1/2 promoter. As demonstrated earlier, eIF4A1 was induced with LPS from 30-120 minutes. Although this induction was repressed with the NF κ B-inhibitors, PS1145 and parthenolide, at both mRNA and protein level, a direct role for NF κ B could not be confirmed. In addition a recent study showed that eIF4A1 and eIF4A2 promoters were not activated by p65 (Fiume et al., 2013). This further hinted at an indirect effect. PS1145 blocks NF κ B activation basally as well as blocks induction of NF κ B activation via inhibition of I κ BKinase complex. It was not tested if NF κ B effected basal levels of eIF4A; since the results show that NF κ B treatment blocked eIF4A induction and brought the levels below basal it might be interesting to test if NF κ B played a role in regulation of basal levels of eIF4A.

Fiume and colleagues recently showed that one of the regulatory components of eIF4A, eIF4H, was up regulated in cells through p65 activation (Fiume et al., 2013). p65-driven up-regulation of eIF4H suggested that when conditions change from basal to stressful the cellular translation machinery can facilitate it. In this case we show that eIF4A was induced by LPS, and this is in line with the study by Fiume and colleagues for its regulatory component eIF4H. Together both components are up-regulated to resolve complex structures present in 5'UTR of mRNA, shown in Figure 4.3.



Chapter 4: Discussion

As an RNA-dependent ATPase and a weak RNA helicase, eIF4A1 is thought to unwind secondary structures in the 5'UTR during scanning. eIF4B and eIF4H can stimulate the helicase activity during scanning and provide stability to single stranded regions by preventing re-annealing.

In mammalian cells, more than 90 % of eIF4A1 exists in an un-complexed form (Duncan and Hershey, 1983) and 10 % is part of the eIF4F complex formed by eIF4E and eIF4G. eIF4A1 free forms' role is not fully understood but its presence has been deemed important for fast recycling in the eIF4F complex.

eIF4A is the most abundant member of the eIF family and its high levels are thought to be crucial for cellular-translation-efficiency. Therefore when cellular demand for translation increases, more eIF4A is produced. eIF4H increases the affinity of eIF4A1 for RNA (Marintchev et al., 2009) and enhances unwinding activity of eIF4A1 (Rozen et al., 1990). It has been suggested that formation of the eIF4A1/eIF4H/RNA/ complex is an independent event from eIF4A1 assembly with eIF4G into the eIF4F complex (Nielsen et al., 2011) (Rozen et al., 1990). Furthermore it has been proposed that a number of eIF4A binding sites could be present to enhance the overall process of resolving complicated structures in the 5'UTR of mRNA (Nielsen et al., 2011).

A possible reason why mTOR not only lifts the repression of eIF4A, but also induces the absolute numbers of eIF4A could be that in LPS-stimulated BMDM there is an

increased requirement of eIF4A. Most of the other regulatory components of eIF4F members are regulated through mTOR, for instance eIF4E-BP, PDCD4 and S6K. PDCD4 and eIF4E-BP are degraded via mTOR signaling, thereby eIF4A and eIF4E are free to participate in the eIF4F complex. Here we show that eIF4A was also induced with LPS via mTOR. Figure 4.4 depicts the mTOR-mediated up regulation of eIF4A in LPS-induced BMDM.

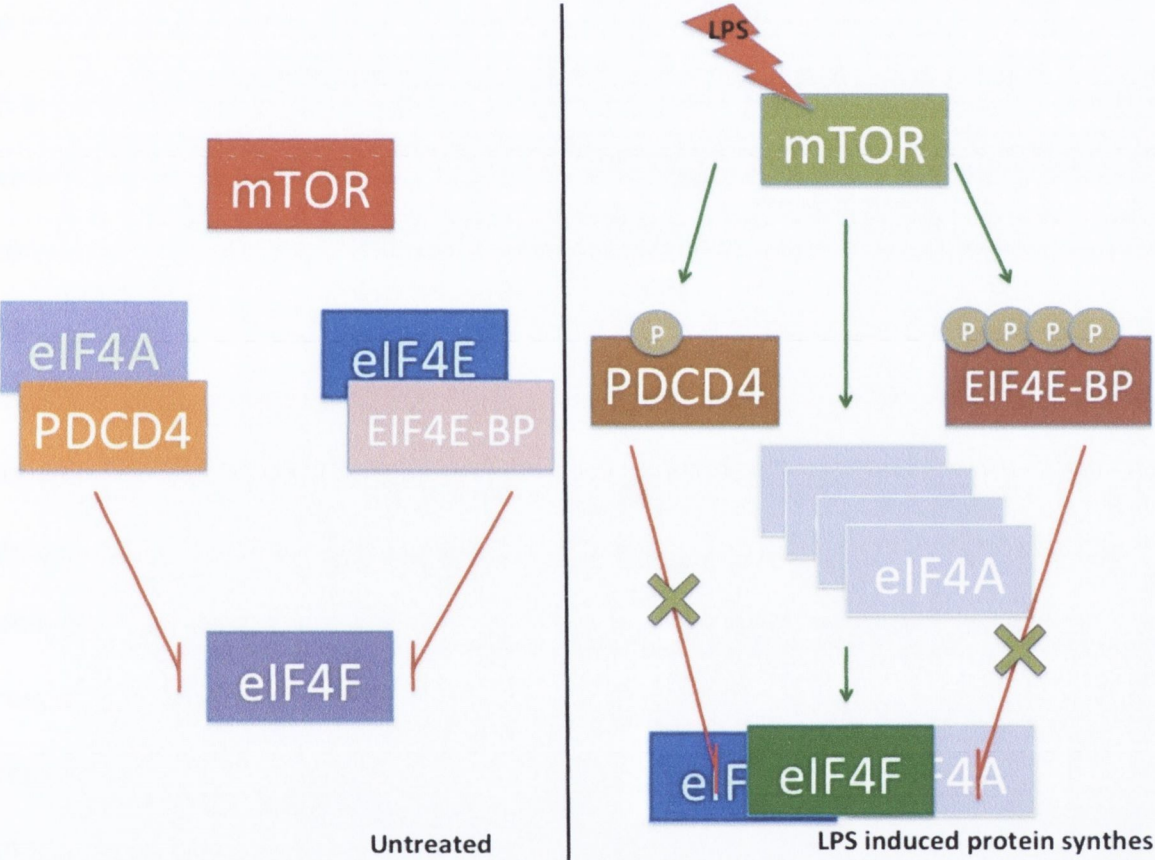


Figure 4.4. Targeting protein synthesis. The eIF4F complex binds mRNA and promotes translation initiation in response to extracellular stimuli such as LPS. In untreated BMDM, eIF4F complex activity is minimal due to limited availability of eIF4A and eIF4E, which are kept inactive by PDCD4 and eIF4E-BP. In LPS stimulated BMDM, mTOR is activated and the pathway targets two major translation inhibitors, PDCD4 and eIF4E-BP for phosphorylation. This modification blocks their actions and allows protein synthesis to occur. In addition, eIF4A is also up-regulated via mTOR activation. This ultimately supports cell growth and proliferation.

The exact mechanism how eIF4A is up-regulated in response to LPS has not been fully characterized. We predict that it could be due to increased protein translation, however it could be regulated via other mechanisms as well. For instance the protein turnover process might be regulated whereby the degradation process might be repressed in response to LPS.

4.1.5 Functional relevance of LPS-mediated eIF4A1 induction

A number of cytokines are produced in response to LPS in BMDM. TNF α and IL-6 are amongst the first cytokines produced, followed by IL-10 which is produced at a later time. As is evident from the results, TNF α and IL-10 cytokines are transcribed at the same time, however there was a delay in IL-10 translation and secretion compared to TNF α . Conversely, IL-6 transcription was slightly delayed compared to TNF α , but its translation and secretion was also immediate, similar to TNF α . The first line of defense against pathogens is primarily via innate immune cells – specifically phagocytes (BMDM and polymorphonuclear neutrophils). Once the inflammatory response is initiated, the system is brought back to homeostasis by negative regulators. Since there is now ample evidence to indicate that dysregulation of innate immunity can give rise to a range of inflammatory diseases, elaborate control mechanisms must exist to prevent its over activation. Delayed production and secretion of IL-10 could be one mechanism.

The list of transcription factors involved in the regulation of IL-10 expression is expanding, which reflects the degree of precision and complexity that the

expression of this cytokine demands. NF κ B, ERK, MAF, p38 and SP1 are amongst more than a dozen factors that have been implicated in IL-10 regulation in BMDM. Given that the expression of transcription is tightly regulated, but IL-10 and TNF α are transcribed at the same time we sought to investigate the delayed translation of IL-10 compared to TNF α and IL-6. This is depicted in Figure 4.5

Modulation of mRNA stability is an important component in the post-transcriptional regulation of several cytokines. Most cytokines have long 3'UTRs that contain ARE elements; ARE elements target mRNA for rapid degradation. Although it is known that multiple copies of potential mRNA destabilizing motifs are found in the 3'UTR of IL-10 mRNA (Powell et al., 2000) and that mRNA destabilizing factors such as tristetraprolin (TTP) target IL-10, it still does not explain the difference in timing of production of IL-10 cytokine.

We sought to look at the 5'UTR sequences to determine if that could explain the differences in translation timings. The 5'UTR of the three mRNA were explored to investigate their ability to form secondary structures. The energy requirement for the secondary structure formation is illustrated in the Figure 4.6. The sequence for the 5'UTR regions of the three cytokines were obtained from ENSEMBL and M-Fold was used to predict energy requirements for secondary structure formation. M-fold is a tool that predicts possibility of secondary structure forming for a given sequence. There are differences in lengths of the 5'UTR of the three cytokines whereby IL-6 has a relatively small 5'UTR, and IL-10 and TNF α have much longer

5'UTRs. The high-energy requirement suggests that IL-6 cannot form any secondary structure whilst IL-10 and TNF α can form complex secondary structures without requiring any energy.

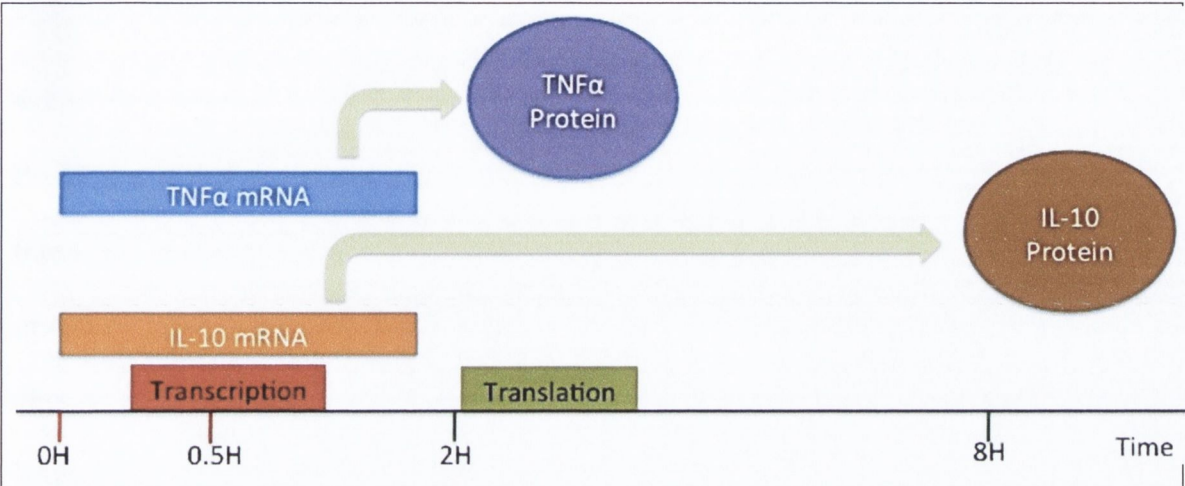


Figure 4.5. Delay in IL-10 translation compared to TNF α .

Although IL-10 transcription is tightly regulated, it is still transcribed at the same time as TNF α . Therefore the mechanistic control in delayed production must lie in translational regulation. IL-10 and TNF α are transcribed at 0.5H post LPS-stimulation. TNF α translation is immediate and it peaks from around 2H, whilst IL-10 translation is delayed and it only peaks at about 8H.

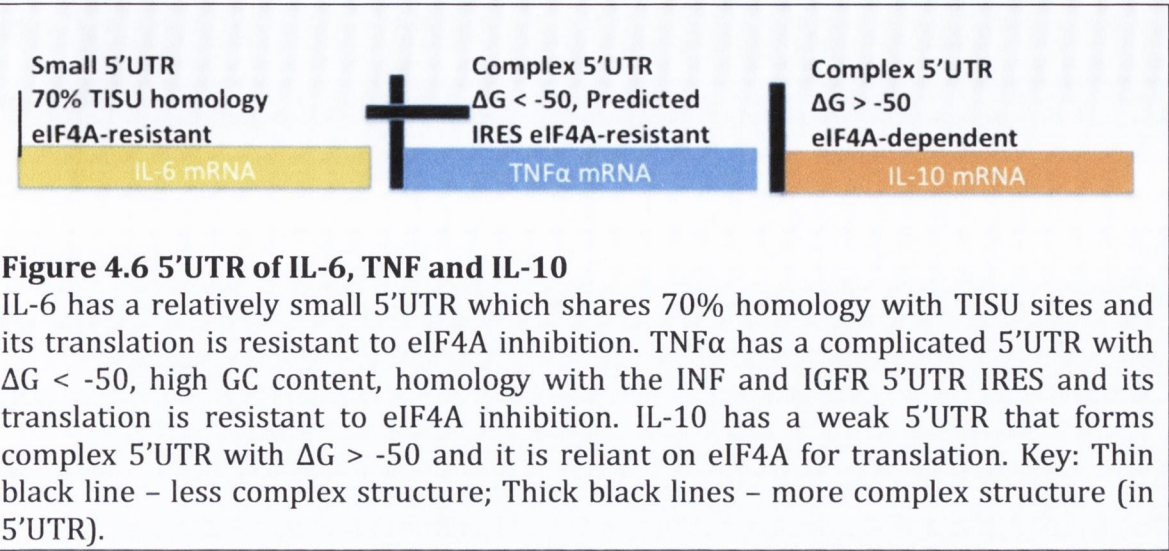
eIF4A is involved in resolving complex 5'UTR structures so that translation can initiate. Two inhibitors were used to block the activity of eIF4A to determine if they affected the translation of IL-10, TNF α and IL-6. The inhibitors had no repressive activity on IL-6 and TNF α translation and specifically blocked IL-10 translation. This was the first indication that the late induction of eIF4A could be to facilitate translation of “weak mRNA” that code for mediators of resolution of inflammation, such as IL-10. However TNF α , with its complex 5'UTR was resistant to the inhibition

of eIF4A. The effects of Hippuristanol and Silvesterol were not measured for basal levels of IL-10, IL-6 and TNF α due to limited availability of the compound. It would be interesting to see effects of these compounds at basal mRNA levels of the cytokines.

4.1.6 IRES and TISU mediated translation of TNF α and IL-6

Previously, studies have used Hippuristanol to identify translation of mRNA with internal ribosome entry sites (IRES) (Bordeleau et al., 2006, Olson et al., 2013). mRNA with IRES sites have been shown to be resistant to eIF4A inhibition. Although viral IRESs are fairly common, mammalian IRESs are rare and relatively unexplored. It was found that insulin-like receptor (INR) and insulin-like growth factor receptor-I (IGFR) are not dependent on eIF4A (Giraud et al., 2001, Spriggs et al., 2009). Their IRESs sites were first discovered using eIF4A inhibitors. The human insulin receptor (IR) 5'UTR and the IGF-1R 5'UTR are highly similar and both have been shown to contain an IRES. The 5'UTR of the two receptors is long and GC-rich, something that is also the case for the TNF α 5'UTR. We therefore predict that the TNF α 5'UTR could contain an IRES or other *cis* element and therefore its translation is resistant to Hippuristanol-mediated eIF4A inhibition. Resistance to eIF4A inhibition and presence of secondary structure in the 5'UTR of TNF α gene suggests that it may contain a *cis* acting agent. Indeed secondary structure associated with $\Delta G < -50$ have been previously reported to contain *cis* elements, such as IRES (Kaempfer, 2003, Kube et al., 1995, Matzura and Wennborg, 1996).

Conversely, IL-6 contains a relatively small 5'UTR which is not GC rich, and it is resistant to eIF4A inhibition. *In vitro* data from reconstitution experiments indicated that eIF4A is not required for 40S ribosome loading onto unstructured model mRNA templates (similar to IL-6 5'UTR) (Pestova and Kolupaeva, 2002). Translation Initiator of Short 5' UTR (TISU) sites have been identified in about 5% of protein coding mRNAs and allow translation of mRNAs without the eIF4A helicase activity. Short 5'UTR confers no complex structure formation and thereby allows leaky scanning (Elfakess et al., 2011). Genes with a TISU element have been identified to contain SAASATGGCGGC (where S can be C or G) in their 5'UTR. The IL-6 5'UTR is ~70% identical to the TISU sequence. In addition the observed resistance to eIF4A inhibition deems IL-6 5'UTR to be a candidate gene to test for a TISU site. The TISU site, like IRES, provides an advantage under certain physiological circumstances. The non-scanning nature and the short 5'UTR length in TISU genes suggest less reliance on eIF4A for fast translation (Elfakess et al., 2011). High mRNA levels of IL-6 were observed in response to Hippuristanol + LPS treatment, however the protein translation did not match high mRNA levels. Furthermore siRNA mediated knockdown of eIF4A1 had repressive activity on IL-6 cytokine production. It is therefore not conclusive if eIF4A-inhibitors had indirect effects on IL-6 mRNA regulation. I did not see a repression on IL-6 cytokine and therefore I conclude that IL-6 translation was not repressed in response to Hippuristanol. Further testing is required to identify eIF4A-mediated regulatory mechanisms affecting IL-6 mRNA. Differences in IL-6, TNF α and IL-10 5'UTR and their predicted translational requirements are demonstrated in Figure 4.6.



4.1.7 eIF4A1 mediated IL-10 regulation

Having observed eIF4A dependence for IL-10 translation using Hippuristanol and Silvesterol, eIF4A1 was then identified as a participant in IL-10 translation. Until recently the translational dogma was that eIF4A1 and eIF4A2 are functionally interchangeable and can mediate the resolution of complex 5'UTR. However a number of studies over the past few years have shown that eIF4A2 was incapable of replacing eIF4A1 functionally. Although eIF4A1 and eIF4A2 proteins have highly similar sequences and the proteins are functionally similar *in-vitro*, it has now been shown that eIF4A1 loss, and not eIF4A2, is detrimental to cell line growth (Galicia-Vazquez et al., 2012b).

Furthermore, a recent study has identified a new role for eIF4A2 in regulating miRNA-mediated mRNA repression/degradation (Meijer et al., 2013). Meijer and colleagues show that eIF4A2 was essential for miRNA to mediate its repression and

that it facilitates miRNA-mRNA binding. This study showed that without eIF4A2, miRNA cannot participate in mRNA repression. In addition Meijer and colleagues also show that eIF4A2 preferentially binds to cNOT7, a member of the CCR4-NOT complex; CCR4-NOT complex mediates mRNA degradation. A number of miRNAs, for instance miR-155, miR-21 and miR-146a participate in inflammation (Alam and O'Neill, 2011); It is possible that the strong induction of eIF4A2 observed in LPS treated BMDM is to facilitate LPS-induced miRNA repression, however further investigation is needed to verify this claim. The findings presented here are in line with these recent studies where a differential role of eIF4A1 from eIF4A2 has been confirmed, at least in case of IL-10 translation.

Not only has this work identified a role for eIF4A1 in driving IL-10 translation but direct interaction between eIF4A1 and IL-10 mRNA was also observed. This interaction increased at least five fold in response to LPS. To demonstrate the RNA-Protein interaction an assay used by Gareau et al was employed (Gareau et al., 2011). This assay is becoming increasingly popular, and uses a principle similar to CHIP. The major difference between CHIP and RNA-IP is that CHIP is used to test protein-DNA interaction where as RNA-IP is used to test protein-RNA interaction. RNA-IP experimentation was carried out mostly in non-tissue culture treated plates to prevent cell death/cell loss in scraping and re-plating cells and to prevent usage of excess MCSF. Tissue culture treated plates provide stronger adhesion to macrophages and therefore scraping is more detrimental to cell survival. Repeat experiments were carried out in tissue-culture treated plates, however no difference

was observed in results. Studies have shown effects of different plate usage on macrophages, therefore it cannot be excluded that some effects could become stronger or less strong if tissue-culture treated plates are used. Additionally the RNA-IP experiments were carried out in the presence of RNase inhibitors to allow a snap-shot of cellular activity at time of harvest. RNase inhibitors prevent degradation of mRNA by RNase and this could play a vital role in exaggerating results to greater heights than observed.

4.1.8 Hippuristanol mediated IL-10 repression

We also investigated how eIF4A-inhibitors mediated their effects. Most of the properties of these inhibitors have been shown through *in-vitro* assays and limited functional studies have been done *in-vivo*. *In-vitro* it has been shown that Hippuristanol can mediate its effect via blocking mRNA-eIF4A binding, by blocking ATPase and Helicase activity (Lindqvist et al., 2008). In vitro reconstitution experiments have shown that eIF4A was not necessary for 40S ribosome loading, and that the presence of eIF4A only enhanced this step. This was true for unstructured mRNAs (Elfakess et al., 2011) or IRES containing mRNAs (Pestova et al., 1998). However the same studies found that eIF4A helicase activity is indispensable for mRNAs containing even a modest amount of secondary structure, such as IL-10. The data presented here suggested that Hippuristanol blocked IL-10 translation in LPS stimulated BMDM, but it does not do so by blocking the eIF4A1 – IL-10 mRNA interaction, instead we predict that it does so by blocking the helicase activity. It was shown recently ATP would still be able to bind to eIF4A-NTD (main

binding site) in the presence of hippuristanol (Lindqvist et al., 2008). And it was speculated that either (i) hippuristanol interferes with proper interdomain interaction which in turn abolishes RNA binding or (ii) affects alignment of phosphate binding site for RNA (Lindqvist et al., 2008). In addition we have demonstrated that eIF4A0 mediated responses are rendered sensitive to Hippuristanol through disruption of the proteins helicase activity. Previously an eIF4A mutant has been characterized that is deficient in helicase activity but can still be incorporated into the eIF4F complex (Svitkin et al., 2001). It was shown using this mutant that translation from long unstructured or short structured 5'UTR was blocked. However mRNAs with TISU sites or short 5'UTR such as IL-6 were unaffected. This provides support for IL-10 but not IL-6 requiring the helicase activity. In addition it suggests that in BMDM Hippuristanol/Silvesterol do not block the mRNA-eIF4A interaction, instead they potentially mediate its effect by blocking the helicase activity. Although this hypothesis cannot be fully supported with the data presented here, and more detailed *in-vitro* experimentation would be required to confirm this claim, it is highly suggestive.

RNA-IP experiments revealed that TNF α -eIF4A1 binding is minimal in this system. Previous studies have shown that transcripts with the IRES site, out-compete cap-dependent transcripts under physiological conditions – especially under circumstances where eIF4A was being repressed via inhibitors. In fact it has also been shown that their translation was actually stimulated (Svitkin et al., 2005, Marr et al., 2007). As revealed in this study, TNF α was induced to an even higher level

with Hippuristanol treatment, giving further support to proposed IRES site in the TNF α 5'UTR. Furthermore, since Hippuristanol blocks eIF4A1 and eIF4A2, we speculate that TNF α mRNA could be degraded via miRNAs through eIF4A2. This might suggest that blocking eIF4A2 inhibited the degradation and therefore allows mRNA build up.

A strong boost in IL-6 mRNA and a boost in IL-6 protein was also observed in LPS + Hippuristanol treated BMDM. Previously it has been reported that LPS + IL-10 inhibits IL-6 by $\sim 25\%$ and that inhibition of IL-10 produced approximately 300% more IL-6 in LPS-treated macrophages compared to LPS-treated macrophages (Fiorentino et al., 1991). Furthermore, IL-10 is known to inhibit NF κ B, which limits transcription of IL-6 and TNF α (Wang et al., 1995). Findings in this study are in line with the literature, where Hippuristanol/Silvesterol-mediated inhibition of IL-10 led to an increased production of IL-6 and TNF α . Such an exaggerated induction of IL-6 in Hippuristanol + LPS treated BMDM could also be due to inhibition of AU-binding elements (e.g. AUF-1) that are involved in IL-6 mRNA degradation or due to stabilization of mRNA stabilizing proteins (e.g. Arid5a) (Paschoud et al., 2006, Masuda et al., 2013). Figure 4.7. Illustrates the proposed mechanism for increase in IL-6 and TNF α levels. It cannot be ruled out that the inhibitors have off target effects on transcriptional regulation of IL-6. It could be revealing to measure IL-6 mRNA levels following eIF4A1 siRNA knockdown.

Additionally, as mentioned in section 1.15.7, IL-10 is involved in production of TTP, DUSP1, ETV3, SBNO2 and IκBNS, which are all negative regulators of IL-6. Therefore, when IL-10 production is blocked these components might either not be produced or only low levels might be produced, hence IL-6 mRNA is not degraded and IL-6 production is not repressed.

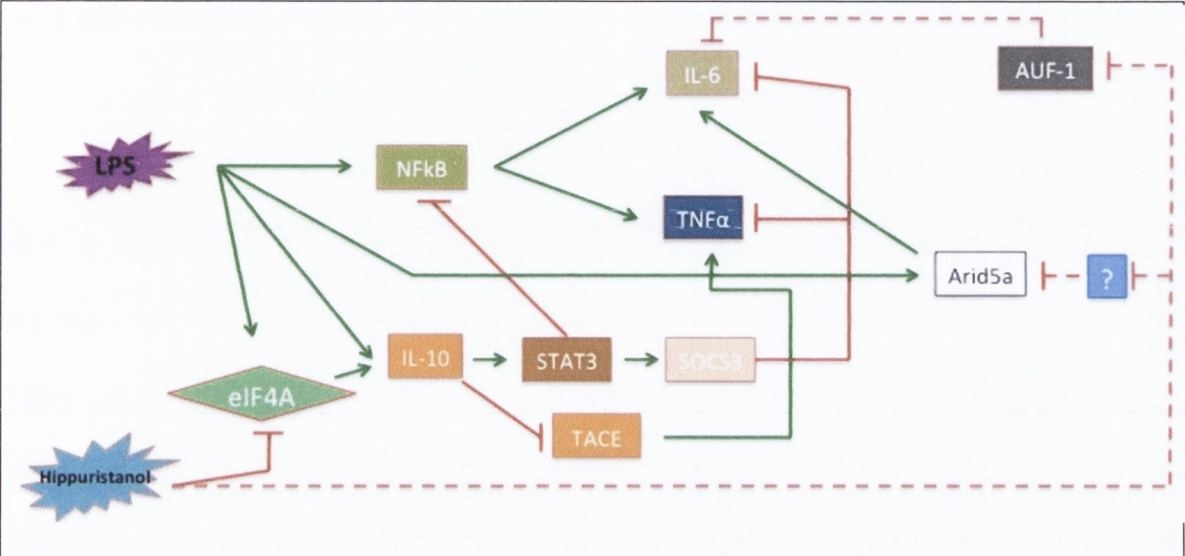


Figure 4.7. Proposed mechanism for the up-regulation of IL-6 and TNFα in Hippuristanol + LPS treated BMDM.

LPS sensing leads to IL-6 and TNFα induction via activation of NFκB. IL-10 is transcribed at a later time that restricts IL-6 and TNFα via inhibition of NFκB and through STAT3, SOCS3 and TNFα converting enzyme (TACE). The inhibition is mostly at transcriptional level. IL-6 mRNA has a half-life of 30 minutes. It is proposed that IL-6 mRNA is degraded via AUF-1. Arid5a is LPS-inducible and it is involved in stabilization of LPS-induced IL-6 mRNA. Hippuristanol-mediated inhibition of eIF4A blocks IL-10 and also possibly blocks AUF-1 production and an unknown mediator that blocks Arid5a. The overall result is that in LPS + Hippuristanol treated BMDM IL-6 and TNFα mRNA levels are not degraded and NFκB is not inactivated.

Key: ↑ Induced; -| Repressed; --, -- unconfirmed.

4.1.9 IL-10 5'UTR

To test the helicase resolving activity of eIF4A1 for the IL-10 5'UTR, the 5'UTR was cloned into a construct containing luciferase. We have confirmed that the IL-10 5'UTR was capable of repressing translation initiation and this repression was relived in response to LPS. It was also confirmed that LPS driven resolution of the IL-10 5'UTR was eIF4A-dependent, since Silvesterol inhibited this response.

4.1.10 Final conclusions and future experiments

In this study we have confirmed that IL-10 requires eIF4A1 helicase activity whereas TNF α have separate eIF4A1-independent mechanisms of translation. The results are not fully conclusive regarding the role of eIF4A1 in IL-6 translation. Furthermore the induction of eIF4A1 in response to LPS was directed towards the translation of weak mRNAs such as IL-10 and was involved in the late induced immune response. The table 4.1 summarizes the requirement for eIF4A1 and predicted TISU and IRES sites in different cytokine, eIF4A1 binding capacity and effects of different inhibitors.

We propose that the translation of pro-inflammatory cytokines such as IL-6, and TNF α is mediated through fast paced translational mechanisms in what seems to be a cap-independent process that is mediated through either IRESs or TISU sites. This requires further verification using other inhibitors for cap-dependent translation and through 5'UTR mutation studies along with bicistronic luciferase assays. In

addition, the translation of late phase cytokines such as IL-4, IL-10, $\text{INF}\alpha$ and $\text{INF}\beta$ could be more cap-dependent and rely on mTOR activation and eIF4A1. This is partially supported by our findings herein and by a recent study showing specific inhibition of cytokines involved in the resolution phase of inflammation using mTOR and eIF4F inhibitors (Ivanov and Roy, 2013).

Cytokine	ΔG	Predicted TISU	Predicted IRES	eIF4A1 binding	Hippuristanol	Silvesterol
IL-10	-12.30	$\times$	$\times$	$\checkmark$	Inhibits	Inhibits
TNF α	-51.0	$\times$	$\checkmark$	$\times$	No inhibition	No inhibition
IL-6	+0.70	$\checkmark$	$\times$	-	No inhibition	No inhibition

Table 4.1: Summary table of eIF4A1 and eIF4A2 mediated cytokine expression. IL-10 eIF4A1 helicase and IL-10 is specifically inhibited with Hippuristanol and Silvesterol. TNF α and IL-6 5'UTR analysis reveal that they may contain IRES and TISU site respectively.

This study therefore reveals that the delayed production of IL-10 in macrophages is post-transcriptionally controlled and is eIF4A1-mediated. IL-10 is produced after the initial inflammatory burst most likely due to the differences in the 5'UTR of the IL-10 transcript.

We predict that there is functional relevance of the 5'UTR in governing the delayed production of cytokines. This could be an important mechanism for differences in timings for the production of cytokines. Where, on one hand pro-inflammatory mediator, TNF α and IL-6 are produced as soon as they are transcribed and on the other hand the 5'UTR of IL-10 blocks its translation till a later time point. It could be

possible that the up-regulation of eIF4A1 observed in response to LPS is to mediate production of immunosuppressive cytokines such as IL-10.

We observed that IL-6 and TNF α (both pro-inflammatory mediators) are translated in an eIF4A-independent manner. This theme could be explored more broadly in terms of production of other cytokines. This must be a mechanism whereby cells control the timing of production of different cytokines.

I demonstrated that very low doses of Hippuristanol and Silvesterol can effectively block IL-10 translation *in vitro*. This could be followed up with *in vivo* trials of these inhibitors to test their effects on modulating IL-10 and if it can enhance bacterial and viral (e.g. LCMV) clearance and help kill tumour cells.

A number of tumours show increased serum concentrations of IL-10, and this includes lung, breast, colorectal, gastric, pancreatic, malignant melanoma, malignant glioma and hepatocellular cancers. Furthermore, an increase in IL-10 levels is also associated with negative prognostic effects in lung, colorectal, gastric, pancreatic, malignant melanoma, and hepatocellular cancers. This suggests that IL-10 is somehow mediating the survival of tumours.

Additionally, in persistent viral infections, one of the defining characteristics is the lack of functional anti-viral effector T cells. Viruses achieve this by suppressing the immune response, and one way to do this is via IL-10-mediated immune response

suppression. It is known that certain viruses can encode viral homologs of IL-10 that suppress immune responses to allow viral persistence. They do so by binding to mammalian receptors for IL-10. Viruses that do not produce homologs of IL-10 appear to preferentially induce host IL-10 production to achieve the same effect. For example in long-term lymphocytic choriomeningitis virus (LCMV) infection in mice, there was a pronounced increase in IL-10 levels during infection, and blockade of the IL-10 receptor resulted in a rapid resolution of infection (Ejrnaes et al., 2006).

Helminth parasites are known to chronically infect quarter of world's population. They survive by suppressing the immune system by inducing IL-10 and TGF β and generating regulatory T cells. Epidemiological studies in humans have showed that helminth parasite infection is negatively correlated with autoimmunity in developing countries. Auto-immunity could be a result of high levels of IL-10 that suppress the immune system, and again our results demonstrate a new mechanism to limit IL-10 production which could be further explored.

A number of studies are already underway looking at the therapeutic potential of blocking IL-10. Manipulating IL-10 has been shown to be beneficial in a number of cases. For example in *Mycobacterium avian* infected mice, the addition of blocking antibodies to the IL-10R resulted in enhanced immune response and bacterial clearance (Roque et al., 2007). Furthermore blocking antibody to IL-10R can act as an adjuvant in the induction of protective immunity upon infection with

Mycobacterium avian and importantly improved the effectiveness of chemotherapy when added late in a chronic infection (Silva et al., 2001). Also, studies to block IL-10 in cancer continue to show great promise. IL-10 has been strongly correlated with disease severity in a number of cancers (Martinez et al., 2008). A number of studies have used Hippuristanol to test its therapeutic potential for patients with different sorts of tumours. It was shown that Hippuristanol suppressed tumor growth in mice with severe combined immunodeficiency harboring tumors induced by inoculation of HTLV-1-infected T cells (Tsumuraya et al., 2011). In this study, Hippuristanol repressed the cell cycle regulators cyclin D1, cyclin D2, CDK4 and CDK6, and induced apoptosis by reducing the expression of Bcl-x(L), c-IAP2, XIAP and c-FLIP. It also suppressed I κ B α phosphorylation and depleted IKK α , IKK γ , JunB and JunD, resulting in inactivation of NF- κ B and AP-1. It is possible that this effect is partly IL-10-mediated.

Hippuristanol/Silversterol/PateamineA have been identified as potent inhibitors of eIF4A. There is much interest in targeted therapies that block eIF4F activity to assess the consequences on tumor cell growth and chemotherapy response. eIF4A1 is the most abundant translation initiation factor, present at three copies per ribosome (Galicia-Vazquez et al., 2012). In certain settings, loss of PDCD4, a tumour suppressor that sequesters eIF4A1, affects translation of selective mRNAs. As a result, PDCD4 knockout mice spontaneously develop lymphomas and PDCD4 has also been found to be an unfavourable prognostic indicator in colon cancer (Hilliard et al., 2006, Allgayer, 2010, Mudduluru et al., 2007). Hippuristanol has been used in

a number of studies to target lymphomas and other cancers, however the results from most studies are not very conclusive. However, one such study on primary effusion lymphoma (PEL), (underlying lymphoproliferative disorder due to HIV infection) used hippuristanol to inhibit the growth and invasion of PEL cells in a xenograft mouse model with ascites and diffused organ invasion (Ishikawa et al., 2013). The authors from this study underline the potential usefulness of hippuristanol in the treatment of PEL. What is interesting to note is the fact that IL-10 is the principal autocrine growth factor that promotes PEL. Rapamycin is used to treat PEL, however it has failed in long term trials. The use of this drug has become questionable because the disease comes back after an initial reduction. It was shown using mouse models for PEL that rapamycin blocks IL-10 and thereby limits PEL only at early stages and that the PEL load increases overtime and becomes resistant to rapamycin (Gasperini and Tosato, 2009). It would therefore be very interesting to test the effectiveness of hippuristanol for PEL, especially since it acts downstream of mTORC1 and has effects on IL-10. Hippuristanol is active at even lower doses compared to rapamycin to block IL-10 and we presume therefore should have fewer consequences to the host. As inhibition of mTORC1 has greater consequences for the host, as it would inhibit more processes central to cellular activity we predict that directed targeting of eIF4A would minimise such consequences.

The findings of this study are promising but need to be confirmed *in vivo* for different mouse model systems. IL-10 works primarily at the level of APCs, and therefore it is not a surprise that tumor-associated DCs and macrophages exhibit

profound phenotypic and functional alterations. One of the many roles of IL-10 is to limit antigen presentation, as mentioned in section 1.16.7 and 1.16.8. Tumor-associated DCs are defective in antigen presentation and fail to activate cytotoxic T cells (Matsuda et al., 1994). Although DC dysfunctionality could undoubtedly be due to many tumor-derived factors, the high levels of IL-10 derived from both the tumor and tumor environment cells is emerging as one of the most important immunosuppressive cytokines in cancer. Tumor-associated macrophages, for example, produce minimal amounts of the immunostimulatory cytokines TNF α and IL-12, but they constitutively produce relatively high levels of IL-10 (Martinez et al., 2008). IL-10 producing T_{Reg} cells are also present in tumours (Curiel et al., 2004). As mentioned in section 1.16.7, IL-10 plays a role in cellular differentiation and can prevent DC maturation, and inhibit DC differentiation from mononuclear cell precursors (Cirone et al., 2008). *In vitro* studies using small interfering RNA to silence IL-10 in human DCs have resulted in enhanced IL-12 production and improved cytotoxic T-cell responses to tumor epitopes (Chhabra et al., 2008). Here we show similar findings, where inhibition of IL-10 results in slight boost in TNF α and significant boost in IL-6. It would be very interesting to test this relationship in tumour-associated macrophages.

Therapies in which tumors are treated with cytotoxic therapeutics in combination with antibodies or other mechanisms to block IL-10 could prove very useful. The rationale behind this is that the antigens released from dying tumor cells can be processed and presented by DCs by blocking IL-10, which would restore their

functionality. In fact, several groups have suggested that the death of tumor cells may be precisely what induces host IL-10 production and therefore DC unresponsiveness (Hatfield et al., 2008). Other combination therapies include not only the addition of antibodies to block the immunosuppressive IL-10R, but also immunostimulatory TLR ligands to activate DCs and promote DC maturation. In small animal models, these approaches are beginning to bear fruit (Vicari et al., 2002). Continued studies in this area hold the potential to lead to the long-awaited dream of effective vaccinations against cancer.

The figure below (Figure 4.8.) demonstrates, different approaches that have been used to block IL-10 and that high levels of IL-10 are associated with tumour progression, viral and microbial persistence.

In conclusion, this study has found a novel role for eIF4A1 in the regulation of IL-10 specifically when compared to TNF α and IL-6. Furthermore, we identify a new mechanism to limit its production using very low levels of hippuristanol and silvesterol. Since it has been shown that Hippuristanol can help overcome chemoresistance in a number of lymphomas (Cencic et al., 2013) and a number of tumours are chemoresistant due to high levels of IL-10, this could be the underlying mechanism how Hippuristanol helps overcome the chemoresistance. This compound and the findings herein hold therapeutic promise.

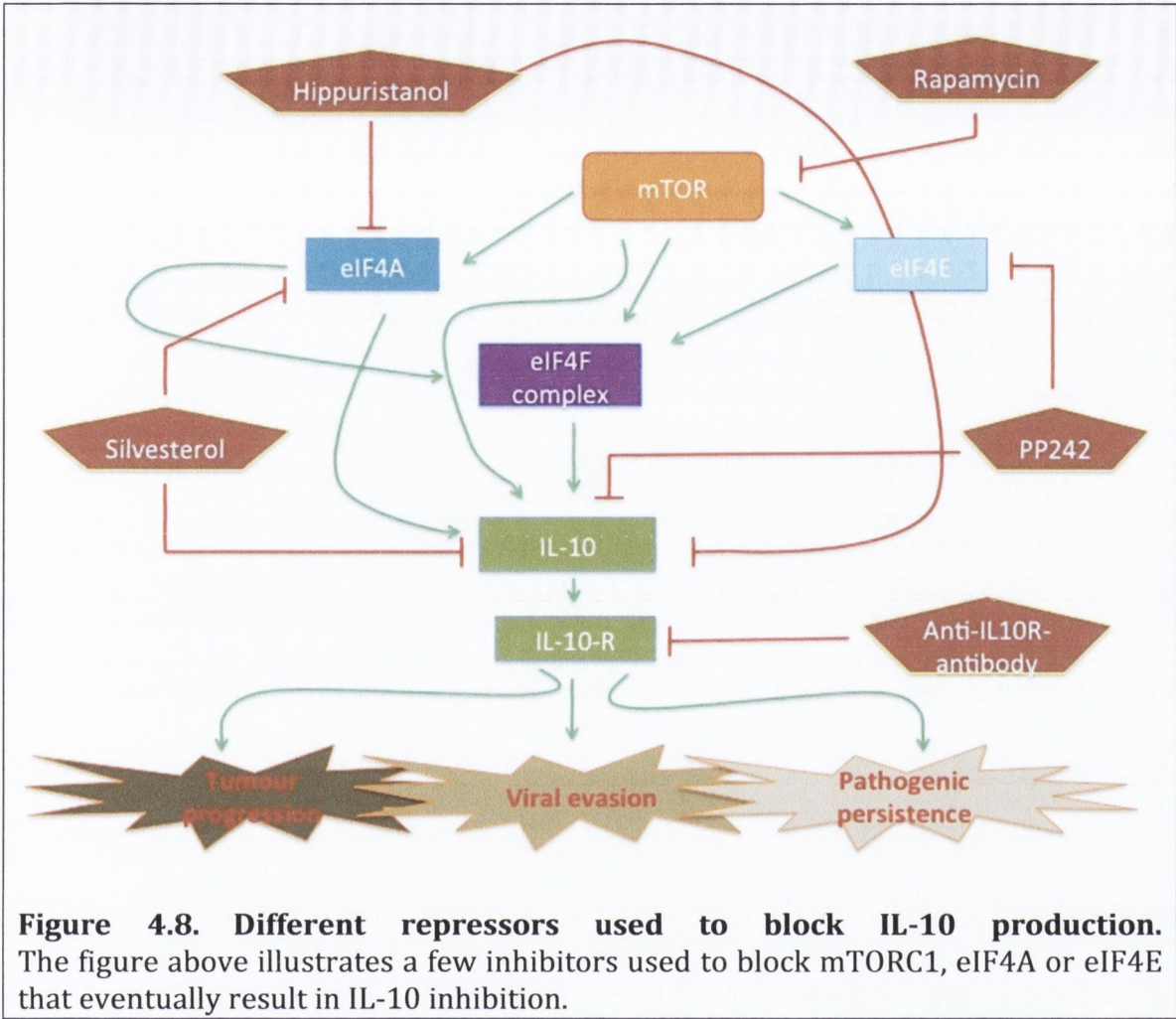


Figure 4.8. Different repressors used to block IL-10 production. The figure above illustrates a few inhibitors used to block mTORC1, eIF4A or eIF4E that eventually result in IL-10 inhibition.

Chapter 5

References

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5.1 References

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Chapter 6

Publications & Appendix

MicroRNAs and the resolution phase of inflammation in macrophages

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Toll-like receptors (TLRs) signal the presence of pathogens and tissue injury, triggering the inflammatory process in macrophages. The goal of inflammation is to resolve the injury and return the body to homeostasis. MicroRNAs are an important group of regulators of TLR signaling and several are induced by TLRs in macrophages. These TLR-induced microRNAs target signaling components in the TLR pathway, thereby producing a negative feedback loop, and they are therefore prime candidates for the initiation of repair. Importantly, their dysregulation may be important for chronic inflammation, which in turn can lead to autoimmunity and cancer, as discussed in this *Viewpoint*.

The first line of defense against pathogens is composed primarily of innate immune cells – specifically phagocytes (macrophages and polymorphonuclear neutrophils). Once the inflammatory response is initiated, the system is brought back to homeostasis by negative regulators. Since there is now ample evidence to indicate that dysregulation of innate immunity can give rise to a range of inflammatory diseases, elaborate control mechanisms must exist to prevent its overactivation. These control mechanisms are likely to be triggered after the initial activation of innate immune receptors (such as the TLRs), their job being to restore the system to homeostasis. In the case of TLR activation, a large number of such controls have been identified, ranging from decoy receptors to phosphatases to deubiquinating enzymes [1]. Recently, microRNAs (miRNAs) have emerged as key regulators of TLRs, particularly in macrophages, and it is highly likely that they fine-tune signaling in order to allow for resolution of the inflammatory process.

miRNAs are typically small (21–22 nucleotides) noncoding RNAs, the majority of which are intergenic or intronic, although a minority of miRNAs are derived from protein-coding mRNAs [2]. miRNAs form a complex with the RNA-induced silencing complex (RISC) producing miRISCs that bind to complementary 3' UTRs of target genes and thereby repress translation of mRNA, promote degradation, or stabilize the target mRNA [2]. Depending on the pathogen encountered, TLRs induce a number of miRNAs with expression of some miRNAs being decreased in response to TLRs [2].

Here, we discuss how miRNAs regulate TLRs, particularly in macrophages, a process likely to occur in the resolution phase of inflammation and speculate on the importance of miRNAs in diseases, which feature dysregulated innate immunity. We discuss three particular miRNAs – miR-155, miR-146a, and miR-21 – since these miRNAs have been strongly implicated in the regulation of TLRs in a number of cells including macrophages [3]. Interestingly, miR-155 and miR-146 are specifically present in LPS-induced macrophages, as compared with similarly activated polymorphonuclear neutrophils (PMNs), suggesting a particular role for these miRNAs in macrophages [4]. We also speculate on the potential novel therapies that target miRNAs in infection and inflammation that could be developed.

miR-155

The gene-encoding miR-155 is located on chromosome 21 in the B-cell integration cluster (BIC) [5]. BIC is highly conserved between humans and mice and is highly expressed in lymphoid organs. miR-155 expression is strongly induced in response to LPS or type I

interferons, in both monocytes and macrophages of human or mouse origin, demonstrating that this miRNA participates in the innate immune response to both bacterial and viral infection [6, 7]. Furthermore, miR-155 is highly expressed in activated B and T cells and has been shown to play a role in regulating cytokine expression in the germinal center [8]. miR-155 is induced by either the MyD88 or the TRIF pathways through LPS or poly I:C stimulation [7].

Unlike the miRNAs discussed later in this *Viewpoint*, the evidence so far presented on miR-155 function indicates that it is likely to be pro- rather than anti-inflammatory. This is because one of the roles of miR-155 in macrophages is to allow the translation of tumor necrosis factor (TNF), a key pro-inflammatory cytokine [6, 9]. In resting macrophages, the 3' UTR of TNF induces a self-repression, which is released upon LPS stimulation via the binding of miR-155. This has been shown in macrophages, where miR-155 overexpression results in increased TNF production and miR-155 deficiency results in lower levels of TNF [9]. Targeting miR-155 in macrophages would therefore limit TNF production and would be useful therapeutically in TNF-mediated disorders. An *in vivo* study has shown that B cells that overexpress miR-155 transgenically produce more TNF and the corresponding transgenic mice have an elevated susceptibility to LPS-induced septic shock [8]. miR-155-deficient B cells, on the other hand, fail to produce TNF [8].

As shown in Fig. 1, in macrophages, miR-155 is negatively regulated by IL-10, an anti-inflammatory cytokine [10]. Inhibition of miR-155 by IL-10 increases expression of Src homology2 (SH2) domain-containing inositol 5'-phosphatase 1 (SHIP1), a known target of miR-155 [11, 12]. Previously, SHIP1 has

MicroRNAs and the resolution phase of inflammation in macrophages

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Toll-like receptors (TLRs) signal the presence of pathogens and tissue injury, triggering the inflammatory process in macrophages. The goal of inflammation is to resolve the injury and return the body to homeostasis. MicroRNAs are an important group of regulators of TLR signaling and several are induced by TLRs in macrophages. These TLR-induced microRNAs target signaling components in the TLR pathway, thereby producing a negative feedback loop, and they are therefore prime candidates for the initiation of repair. Importantly, their dysregulation may be important for chronic inflammation, which in turn can lead to autoimmunity and cancer, as discussed in this *Viewpoint*.

The first line of defense against pathogens is composed primarily of innate immune cells – specifically phagocytes (macrophages and polymorphonuclear neutrophils). Once the inflammatory response is initiated, the system is brought back to homeostasis by negative regulators. Since there is now ample evidence to indicate that dysregulation of innate immunity can give rise to a range of inflammatory diseases, elaborate control mechanisms must exist to prevent its overactivation. These control mechanisms are likely to be triggered after the initial activation of innate immune receptors (such as the TLRs), their job being to restore the system to homeostasis. In the case of TLR activation, a large number of such controls have been identified, ranging from decoy receptors to phosphatases to deubiquinating enzymes [1]. Recently, microRNAs (miRNAs) have emerged as key regulators of TLRs, particularly in macrophages, and it is highly likely that they fine-tune signaling in order to allow for resolution of the inflammatory process.

miRNAs are typically small (21–22 nucleotides) noncoding RNAs, the majority of which are intergenic or intronic, although a minority of miRNAs are derived from protein-coding mRNAs [2]. miRNAs form a complex with the RNA-induced silencing complex (RISC) producing miRISCs that bind to complementary 3' UTRs of target genes and thereby repress translation of mRNA, promote degradation, or stabilize the target mRNA [2]. Depending on the pathogen encountered, TLRs induce a number of miRNAs with expression of some miRNAs being decreased in response to TLRs [2].

Here, we discuss how miRNAs regulate TLRs, particularly in macrophages, a process likely to occur in the resolution phase of inflammation and speculate on the importance of miRNAs in diseases, which feature dysregulated innate immunity. We discuss three particular miRNAs – miR-155, miR-146a, and miR-21 – since these miRNAs have been strongly implicated in the regulation of TLRs in a number of cells including macrophages [3]. Interestingly, miR-155 and miR-146 are specifically present in LPS-induced macrophages, as compared with similarly activated polymorphonuclear neutrophils (PMNs), suggesting a particular role for these miRNAs in macrophages [4]. We also speculate on the potential novel therapies that target miRNAs in infection and inflammation that could be developed.

miR-155

The gene-encoding miR-155 is located on chromosome 21 in the B-cell integration cluster (BIC) [5]. BIC is highly conserved between humans and mice and is highly expressed in lymphoid organs. miR-155 expression is strongly induced in response to LPS or type I

interferons, in both monocytes and macrophages of human or mouse origin, demonstrating that this miRNA participates in the innate immune response to both bacterial and viral infection [6, 7]. Furthermore, miR-155 is highly expressed in activated B and T cells and has been shown to play a role in regulating cytokine expression in the germinal center [8]. miR-155 is induced by either the MyD88 or the TRIF pathways through LPS or poly I:C stimulation [7].

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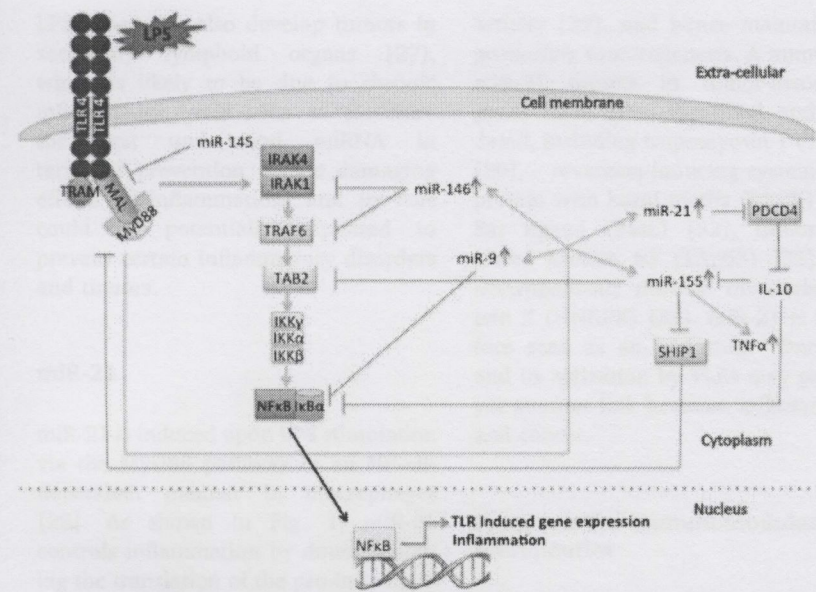


Figure 1. miRNA-mediated regulation of the NF-κB pathway in macrophages. LPS activates TLR4 and that initiates a cascade of events resulting in the induction of NF-κB. LPS stimulation also induces various miRNA that can regulate the NF-κB activation pathway: miR-146 blocks IRAK1 and TRAF6, miR-9 blocks NF-κB, miR-21 blocks PDCD4 and these three miRNA are anti-inflammatory; miR-155, on the other hand, is a pro-inflammatory miRNA, inducing TNF-α which further activates macrophages and blocks SHIP1. SHIP1 then negatively regulates the TLR-induced responses. IL-10 is an anti-inflammatory cytokine and is blocked by PDCD4. Upon miR-21 induction, PDCD4 is blocked and IL-10 blocks miR-155 and NF-κB. Hence, a negative regulatory loop forms. Overall, this figure illustrates how miRNAs work together to bring the system back to homeostasis postinflammation triggered by LPS.

been shown to function as a negative regulator of TLR-induced responses [13–15]. The action of SHIP1 is likely to be through a negative regulatory loop as the serine/threonine protein kinase AKT, which is downregulated by SHIP1, negatively regulates miR-155 [16]. Thus, after LPS stimulation, miR-155 expression increases, SHIP1 levels fall, and AKT activity increases; as AKT downregulates miR-155, the initial high miR-155 levels are brought back under control.

miR-155 KO mice have been shown to have an impaired immune response to *Salmonella typhimurium*, and these mice cannot be successfully immunized against this pathogen [17]. Further analysis revealed a defect in B- and T-cell activation, explaining the lack of immunization capacity in these mice. Furthermore, the failed T-cell response was, in part, due to the failure of DCs to present antigen and due to an altered Th1 response in which the CD4⁺ T cells had impaired cytokine production [17].

This was most likely due to the failure of DCs to functionally activate costimulatory signals and defective antigen presentation; miR-155 may be responsible for the impaired cytokine production. A second study showed that miR-155 KO mice exhibit reduced numbers of germinal centre (GC) B cells, whereas miR-155-overexpressing mice showed elevated levels [8]. This study concluded that miR-155 achieves its response partly by regulating the expression of cytokines, e.g. TNF [8]. A third study with miR-155-deficient mice revealed elevated levels of activation-induced cytidine deaminase (AID) [18]. AID is a strong mutation-causing component in the class switching process and therefore its activity needs to be tightly regulated [19]. AID initiates somatic hypermutation and is essential for class-switch recombination [19]. The gene-encoding AID contains a miR-155 binding site in its 3' UTR [8, 18]. B cells undergoing class switching express high, but controlled, levels of

miR-155; genetically modified mice with a mutation in the 3' UTR binding site for miR-155 in the AID gene that blocks miR-155 binding show increased AID levels, compared with WT cells, and increased numbers of *Myx-Igh* translocations and, as a result, have disrupted affinity maturation. miR-155 thus closely regulates AID expression in cells to prevent hypermutational activity. These *in vivo* experiments confirm that miR-155 is especially important for B-cell development and identify AID as a key target.

miR-146

miR-146 is one of the most prominent miRNAs induced by LPS in macrophages [3, 20]. Resolvin D1, an anti-inflammatory lipid mediator, also induces miR-146 [21]. miR-146 expression is NF-κB dependent and, to date, IL-1R-associated kinase 1 (IRAK1), IRAK2, and TNFR-associated factor 6 (TRAF6) have been shown to be miR-146 targets [20]. As shown in Fig. 1, these targets are components of the NF-κB pathway and control NF-κB expression. *Irak1* has been validated as a target for miR-146 in *in vivo* studies [22]. Since IRAK1 and TRAF6 are required for NF-κB activation, there is therefore a negative regulatory loop in operation whereby NF-κB activation upregulates miR-146 that, upon maturation, downregulates IRAK1 and TRAF6 and thereby represses NF-κB. IL-1β, which is produced in response to LPS, triggers miR-146 production, which blocks NF-κB, and thereby participates in a negative regulatory loop modulating LPS-induced signals [23]. Furthermore, overexpression of miR-146 results in a decrease in various chemokines and cytokines, including CXCL8, CCL5 [23], IL-6, CXCL8 [24, 25], and IL-1β itself [26], and thereby prevents overactivation of inflammation and brings the system back to homeostasis.

Within 6 months of birth, miR-146a KO mice develop a spontaneous autoimmune-like disorder that leads to death [27]. These KO mice exhibit loss of immunological tolerance and their macrophages are hyper-responsive to

LPS. The mice also develop tumors in secondary lymphoid organs [27], which is likely to be due to chronic inflammation. miR-146a is therefore the best understood miRNA in terms of prevention of the damaging effects of inflammation, and its role could be potentially exploited to prevent certain inflammatory disorders and tumors.

miR-21

miR-21 is induced upon LPS stimulation via the MyD88 pathway in an NF-κB-dependent manner in macrophages [28]. As shown in Fig. 1, miR-21 controls inflammation by downregulating the translation of the pro-inflammatory tumor suppressor programmed cell death 4 (PDCD4) [28], an inhibitor of IL-10 production. Hence, miR-21 promotes IL-10 production upon LPS stimulation by regulating PDCD4. IL-10 is an anti-inflammatory cytokine that blocks NF-κB and allows the system to go back to a homeostatic state. miR-21 could therefore be another key miRNA in the resolution of inflammation.

miR-21 regulates NF-κB in a cell-specific manner. As shown in Fig. 1, miR-21 forms a negative regulatory loop in innate immune cells that keeps inflammation in check by limiting NF-κB expression through the upregulation of IL-10; IL-10 represses NF-κB. In contrast, in tumor cells, miR-21 downregulates phosphatase and tensin homologue (PTEN) and activates AKT, thereby maintaining/increasing NF-κB

activity [29], and hence maintaining/promoting tumorigenesis. A number of miR-21 targets in tumor-associated genes have been identified and validated, including tropomyosin 1 (TPM1) [30], reversion-inducing-cysteine-rich protein with kazal motifs (RECK) [31], Fas ligand (FasL) [32], tumor-associated protein 63 (TAP63) [33], and heterogeneous nuclear ribonucleoprotein K (HNRPK) [33]. miR-21 is therefore seen as an important “Oncomir” and its activation by TLRs may provide yet another link between inflammation and cancer.

Prospects for immunomodulatory therapeutics

Given the level of research activity in the field of miRNAs, there is hope that new diagnostics or therapeutics might emerge for infectious and inflammatory diseases. The current best prospect is for hepatitis C virus (HCV) [34, 35]. The 5' UTR of the HCV genome contains sequences essential for its replication including two binding sites for miR-122. The HCV has conveniently made use of liver-abundant miR-122 to facilitate its replication and translation [36–38]. A miR-122 antagomir, that specifically silences miR-122, has been found to inhibit replication of HCV genotypes 1a and 2a [37–39]. Furthermore, experimental data generated using HVC-infected chimpanzees demonstrate that the miR-122 antisense locked nucleic acid (LNA) SPC3649 is able to clear both the HCV 1a and the

1b genotypes [40]. These data hold much promise for novel anti-HCV therapies. In the case of HCV-induced inflammation, if the target site for miR-155 in the TNF 3' UTR was to be blocked, this could provide a new strategy to limit TNF expression and TNF-associated activities. Another approach could be to specifically boost the effect that miR-21 has on PDCD4 and thus also generate an anti-inflammatory effect. These types of studies are worth pursuing, since targeting both miR-155 and miR-122 would effectively boost the resolution of inflammation.

A second example where the targeting of miRNAs regulated by TLRs might hold promise is in myelodysplastic syndrome (MDS). MDS results from the ineffective production of myeloid cells from stem cells in the BM and arises at the stage of primitive CD34⁺ hematopoietic stem/progenitor cells due to ineffective hematopoiesis. One of the most common forms is the 5q-syndrome, which results in the deletion of a segment on chromosome 5, long-arm position 32 (5q32) [41–43]. The commonly deleted region at 5q32 contains 40 genes and a number of miRNAs, including miR-145 and miR-146a. Starczynowski et al. [41] found that 5q-MDS individuals had low levels of miR-145 and miR-146a, thereby confirming their deletion [41]. A key target for miR-145 is known to be the adapter Mal, which is required for signaling by TLR2 and, especially, TLR4 [42]. As mentioned in the miR-146 section, miR-146 targets IRAK1 and TRAF6. The knockdown of miR-145 and miR-146a or, in particular, the enforced

Table 1. In vivo-verified mouse models investigating microRNA effects

Targets	Technique	Expression	Phenotype	Reference
miR-155	bic/miR-155 KO	Thymus, spleen	Increased lung airway remodeling, defective adaptive immunity, impaired DC antigen presentation	[18]
miR-155	bic/miR-155 KO	Thymus, spleen	Reduced germinal centre B cells, defective B and T cells	[8]
miR-155	miR-155 site KO in AID gene	Thymus, spleen	Impaired affinity maturation, deregulated AID and elevated CSR expression in splenic B cells	[20]
miR-146	miR-146 KO	Lymphoid organs	Healthy birth, develop autoimmune-like disorder within 6 months	[28]
miR-21	miR-21 KO/LNA-antimiR-21	Fibroblasts	No obvious phenotype	[44]
miR-21	Synthetic antagomir	Fibroblasts	Inhibits interstitial fibrosis and attenuates cardiac dysfunction	[45]

expression of TRAF6 in hematopoietic stem/progenitor cells transplanted into mice results in thrombocytosis, neutropenia, and megakaryocytic dysplasia [41]. These changes lead to the induction/overexpression of pro-inflammatory cytokines, such as IL-6, leading to chronic inflammation, which again appears to promote tumorigenesis in this disease. Other studies, e.g. [43], have failed to find a correlation between 5q-MDS and downregulation of miR-145–miR-146a, however; hence further analysis is needed. Nonetheless, blockade of the Mal/TRAF6 pathway could prove to be therapeutically useful in MDS.

Conclusions

Clearly, the targeting of miRNAs for therapeutic purposes is at an early stage; however, given the roles of miR-146a, miR-155, and miR-21 in the control of inflammation, and, in particular, in macrophage function, they remain of interest for future drug development. An important consideration is in vivo validation, and Table 1 summarizes this aspect for these miRNAs. As summarized in Table 1, deletion of miR-155, miR-146, and miR-21 has serious consequences in mice, e.g. autoimmune disease. These miRNAs therefore seem to regulate important functions and their deletion leads to numerous immune dysfunctions, e.g. miR-155 KO mice have defective DCs. Ultimately, the hope is that the extensive knowledge that is emerging on these important fine-tuners of inflammation might be brought to bear on the complex processes in the resolution of inflammation, and from there possibly to cancer, where dysregulation of inflammation plays an important role.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: AID: activation-induced cytidine diamine · BIC: B-cell integration cluster · IRAK1: IL-1R-associated kinase · MDS: myelodysplastic syndrome · miRNA: microRNA · PDCD4: programmed cell death 4 · SHIP1: Src homology2 (SH2) domain-containing inositol 5'-phosphatase 1 · TRAF6: TNFR-associated factor 6

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gene	Control1		Control2		Control3		LPS_04h1		LPS_04h2		LPS_04h3		LPS_24h1		LPS_24h2		LPS_24h3		LPS_28h1		LPS_28h2		LPS_28h3	
	DESeq		DESeq		DESeq		DESeq		DESeq		DESeq		DESeq		DESeq		DESeq		DESeq		DESeq		DESeq	
miR-27b-3p	228972.8		192568.9		196929.2		2111199.9		216334.6		181682		369117.1		353413.5		316209.3		413286		375665.3		294493.3	
miR-22-3p	208226.5		191449.3		235862.3		210810.9		215036.1		218897.1		288285.6		315642.5		255363.2		293621.9		320389.6		252284.8	
miR-27a-3p	207667.7		175276.8		174820.8		192771.6		198784.3		163441.2		347189.7		333606.2		297030.9		388453.7		354776.6		276417.1	
mmu-let-7a-5p	153290.1		119459.1		146548		144912.9		98552.8		178067.8		151460.2		111841.1		176215.3		162513.4		103724.4		187431.6	
mmu-let-7f-5p	142421.7		112060.6		139229.8		135207.3		92232.1		163959.5		141439.2		108007		153477.8		148492.8		98096.7		166110.2	
miR-21-5p	89912.5		107018.6		99340.5		103449.9		125611.4		98064.3		224780.9		222095.1		180702.9		227021.6		210142		195550.8	
miR-26a-5p	50325.5		54798.2		49575.9		44954.4		54956		52067.7		34675.4		34205.4		42060.8		32151.7		34832.8		38275.9	
miR-191-5p	47740.7		37044.5		29977.2		48160.5		41903.7		42034.7		42827.6		21793.8		46172.2		52362.2		31728.5		43871.3	
miR-146a-5p	42740.2		39012.4		55863.4		47229.3		32456.1		62661.7		53692.5		45602.5		83438.3		64946.2		44983.5		100070.2	
mmu-let-7c-5p	33823.2		25625.4		24107.3		30974.6		22711.4		37660.7		28093.6		20465		31728.4		33016		22191.4		32134	
miR-181a-5p	31871.7		24838.7		23897.1		30948.7		23473		25181.4		29346.6		26833.8		29213.1		32858.9		28546.4		31096.5	
miR-30a-5p	31830.6		29465.1		34207.3		27617.6		27186.9		31646.7		26684.3		29995.3		28135.7		29999.7		29518.4		28814.5	
miR-10a-5p	31670.2		20560.4		18515.1		21617.9		24408		16803.6		22489.7		19836.1		13919.3		23826.6		21077.4		13789.6	
miR-10b-5p	29008.9		18829.1		16921.3		19868.7		22419.1		15440.3		20538.2		18165.3		12797.1		21771.9		19285.7		12650.5	
mmu-let-7i-5p	27059.3		24163.4		20496.6		30309.8		26299.4		25385.4		48008.6		37899.4		38075.5		50598.5		38854		39685.5	
miR-30d-5p	26321		22072.4		27507.2		23287		20000.2		26428.7		21445.6		22511.4		22302.6		24358.4		23144.6		22823.5	
miR-142-5p	25476.6		31254.2		34846.3		26405.9		31562.6		28558.1		29228.9		39358.1		31085.4		26301		34042		30790.3	
mmu-let-7e-5p	22350.1		15930.1		21870.4		21978.6		13086.7		31906.1		24932.6		14593.6		40840.9		29495.8		15313.7		41356.7	
miR-16-5p	19299.2		33864.6		20837.6		19564.5		29629.6		23240.7		24172		25029.4		25675.6		22275.8		27484		24352.9	
mmu-let-7b-5p	13484.8		10509.1		7362.1		11853.3		9748.2		12898.2		9362.2		7077.8		9868.9		11750		8033.4		9340.8	
miR-222-3p	11808.4		11103.5		18918.8		25724.6		18544.8		65048.5		6439.7		5459.2		10816.1		11167.6		7878		14527	
miR-30e-5p	8764.1		10151.3		10363.8		7428.3		9779.1		8479.2		8142.1		10328.7		8533.3		8672.9		9288.9		8764.1	
miR-378-3p	8082.1		7077.2		6474		6803.6		6814.8		7488.7		8503.1		7430.1		8842.1		8027.5		7451.1		8292.7	
miR-125a-5p	7124		4316.8		4981.8		8307.7		5496.1		9666.9		15939.2		4644.1		18940.7		20058.4		7070.3		18599.1	
mmu-let-7d-5p	7009.4		7036.9		5865.7		6273.2		6309.9		8710.4		6835.6		5841.9		8715.9		8068.1		5920.1		9813.8	
miR-92a-3p	6992.2		4918.9		5649.3		8057.8		5397.5		8984.4		7045		3882.8		7769.3		8331.2		5177.9		7995.6	
miR-221-3p	6414.3		6477.1		6246.4		6688.1		7795		9832.1		10066.1		12289.4		13524.5		11592.4		13453.1		14325.4	
mmu-let-7g-5p	6300.6		5143.9		5844.8		5665		4991.1		6815.7		6990.9		6178.4		8554.8		8177.1		5655		8709.5	

gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-23b-3p	5633.9	5259.4	5294.3	5410.4	5777.7	5834.6	4496.7	4342.1	4706.8	3910.1	4339.4	4701.9
miR-151-3p	5367.4	3531.9	2444.3	4872.9	3073.6	2460	3887.2	2743.3	3204.9	4043.4	3410.5	3194.5
miR-186-5p	5015.8	5569.1	5425.8	3795.5	5973.7	5122.9	5750.2	6514.4	6115.1	5627.6	6535.6	6395
miR-99b-5p	4689.2	2548.9	3432.6	4646.5	2977.3	4311.8	5333.6	2120.6	8015.3	6176.7	2899.2	7941
miR-146b-5p	4501	4984.3	6377.3	7463.7	9445.3	7330.3	7512	6865.7	14353	10143.5	7514.6	14098.8
miR-103-3p	4301.3	4772.7	3550.9	4132.6	4119.1	3878.2	4901.1	4712.1	4063.7	4660.4	5031.4	4433.6
miR-23a-3p	4279.4	4538	4690.2	3951.1	4947.2	5320	4232.2	4197.5	4594.6	3550.2	4204.4	4664.7
miR-423-5p	4233.5	2600.8	4322	3342.9	1411.4	7369	3648.1	1105.4	6340.7	4882.4	1616.9	6321.5
miR-25-3p	4170.5	3574.2	2593.9	4354.2	3453.8	2684.3	4273.5	4068	2662.4	3774.2	4589.1	2777.6
miR-101a-3p	3481.8	3218.3	3239.8	3390	3825.6	1887.4	4264.5	6849.9	2324	3486.3	5759.7	2472.2
miR-148a-3p	3417.8	1769.8	2861.2	3250.9	2106.5	2170.3	3276	2190.1	2146.7	2887.1	2380.9	2034.9
miR-26b-5p	3217.2	4332.1	4151.5	2854.9	3945.6	4389.7	3449.9	3784.8	4676	3200.4	3748.6	4254.7
miR-107-3p	3206.7	3535.7	2616.9	3229.7	3049.8	2768.5	3781.2	3676.7	3046.8	3485.8	3967.6	3293.7
miR-125b-5p	3042.4	2283.4	2557	2786.5	2636.4	4127.2	1772.9	1259.7	2323.5	2007.5	1666.6	2451.7
miR-140-3p	2892.4	2277.6	2799.9	2623.9	2183.7	2095.5	2469.9	2437.8	1905.4	2334	2457.4	2001.6
miR-99a-5p	2865.6	3287.6	2022.5	2727.6	3975.3	1924	2096.8	3110.8	1800.7	2245.8	3047.7	2133.4
miR-148b-3p	2728.1	1998.7	1918.8	2371.6	2121.9	1678.2	2069.2	1769.3	1811.8	2163.7	1933.6	1770.4
miR-423-3p	2687	1729.4	3073.4	2131.1	1778.6	3014.8	1380.2	1470.4	2288.6	1706.9	1355.3	2197.8
miR-29a-3p	2683.2	3705	4682.5	2793.6	4071.6	3504.3	3607.8	5583.1	3659.1	2790.8	5063.2	3608.3
miR-100-5p	2663.1	3076	1878.4	2567.3	3723.5	1802.6	1939.3	2817.9	1689.7	2060.2	2819.8	1999.3
miR-24-3p	2497.9	3939.7	4101.4	2793.6	3013	4567.5	3149.4	2876.8	5144.7	2980.3	3153.4	4815.6
miR-340-5p	2462.5	2883.6	1817.2	2531.9	2661.3	1101.3	2923.1	3672.1	1362.9	2308.1	3141.5	1585.5
miR-151-5p	2438.7	2435.4	1351.6	2319.7	2493.8	1410.9	2261.6	1963.2	1746.7	2314.2	2262.8	1683.2
miR-29c-3p	2412.9	3389.5	4183.5	2482.4	3671.2	3131.5	3159.4	4937	3239.2	2475.4	4390	3183.1
miR-101b-3p	2226.6	2233.4	2311.3	2364.5	2691	1388.5	3268.1	4639.5	2024.6	2825.2	4066.3	2143.3
miR-532-5p	2165.5	1517.8	1757.3	2046.3	1764.3	1698.6	2684.5	2790.5	2328.1	2372.5	2916.1	2340.3
miR-181b-5p	1894.2	1679.4	1618.8	1824.7	1668.1	2026.5	1672.2	1591.7	1931.6	1898.5	1707.3	1985.6
miR-3970	1825.4	1000.3	1889.6	1796.4	1305.7	1800	2663.9	2083	3610.8	3203.9	1844.3	3492.3

gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-223-3p	1805.4	3660.8	4560.7	1904.8	3850.6	2995.5	1441.1	2368.7	1503	1094.5	1761.9	1355.8
miR-181d-5p	1599	1427.4	1375.9	1555.9	1377	1756.1	1373.3	1292.2	1651.9	1597.9	1390	1690.1
miR-93-5p	1591.4	2000.6	1497	1522.9	1696.6	1719.5	1741.1	1989.6	1760	1753	2055.8	1681.7
miR-301a-3p	1566.6	1979.5	1282	1383.8	1827.3	1214.8	1193.1	1264.8	740.2	1257.2	1207.8	698.8
miR-30c-5p	1534.1	2179.5	2146.4	1563	1656.2	2257.1	1065.9	1116.6	1757.1	1043.3	1060.9	1706.7
miR-301b-3p	1489.2	1871.7	1189.4	1332	1725.1	1142.7	1153.9	1197.3	682	1199.9	1136.4	635.1
miR-192-5p	1436.6	1021.5	1292.4	2039.2	1368.7	1231	1562	1914	1084.4	1471.7	1793.6	1169.4
miR-484	1391.7	1656.3	2770	1539.4	1701.3	2586.5	1382.8	1606.4	2324	1316	1537.5	2579.8
miR-125b-2-3p	1339.2	1069.6	991.8	1075	1322.3	989.4	1100.8	1343.5	1079.2	1102.6	1587.6	1088.3
miR-98-5p	1297.2	1148.4	1090.6	1214.1	1089.5	1287.5	1303.8	1245.5	1214.1	1424	1253.5	1274.8
miR-30b-5p	1288.6	1921.8	1846.4	1546.5	1898.6	1714.8	1304.9	1728.7	1428	1119.8	1573.7	1460.4
miR-21-3p	1238.9	694.5	2036.4	2696.9	2651.8	1572	8075.4	12053.3	6410.5	7146.4	10534	7067.2
miR-3107-5p	1228.4	584.8	286.7	1230.6	472.9	491.6	832.7	320.8	337.8	889.7	455.7	407.7
miR-486-5p	1228.4	584.8	286.7	1230.6	472.9	491.6	832.7	320.8	337.8	889.7	455.7	407.7
miR-351-5p	1167.3	850.3	1366.9	999.6	752.1	889.5	570.3	453.2	578.5	644.8	491	598
miR-130b-3p	1096.6	1248.5	733.6	1133.9	1082.3	803.8	1284.8	1092.8	943.1	1183.2	1215.3	1005.7
miR-182-5p	1088	713.7	659.1	1148.1	666.5	626.5	753.2	578.6	570.4	764.5	593.2	513.8
miR-24-2-5p	1085.1	1123.4	1520.7	938.3	1075.2	1042.8	918.5	1108	725.1	824.8	911	870.8
miR-342-3p	1068.9	1415.8	1134.4	961.8	1472	1529.6	982.1	838.5	1300.7	1012.9	1134.9	1471
miR-1839-5p	1065.1	983	772.5	1077.4	954	879.1	1421	1367.3	1094.9	1388	1259	1248.2
miR-142-3p	1051.7	1664	1575.7	836.9	1944.9	839.9	1717.8	2444.4	881.5	1323.6	2123.3	864
miR-322-5p	1044.1	1011.9	1147.7	855.8	1112	1047.5	1191	1225.2	1186.2	1139.6	1224.2	1220.9
mmu-let-7d-3p	948.5	1067.6	1405.2	1223.5	715.2	1852.8	394.9	380.7	823.3	438	347.5	770
miR-15a-5p	913.2	1313.9	1207.5	907.6	1403.1	1073.6	1248.2	1677.5	1354.8	1117.8	1675.5	1367.2
miR-221-5p	786.1	573.3	789.9	1129.2	1600.3	2256	765.9	615.7	720.4	1067.6	931.3	1155.8
miR-101c	772.8	819.5	1048.8	742.6	1003.9	554.3	1000.7	1846	732	806.6	1511.7	794.3
miR-15b-5p	687.8	861.8	508.8	603.5	977.8	585.2	614.8	626.8	478	598.7	637.4	474.4
miR-744-5p	686.8	592.5	753.7	641.2	588.1	1012.4	278.3	210.1	430.9	359.4	222.4	437.3

miR-210-3p	606.6	240.5	314.6	671.9	267.3	380.7	1730.5	1224.2	1675.7	1863	1353.8	1499.1
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-3096b-5p	599.9	175.1	257.5	584.6	147.3	159.5	86.9	68	114	88.7	88.9	95.5
miR-425-5p	592.2	806	803.9	754.4	706.9	722.7	659.3	575.6	775.7	620.5	617.1	784.4
miR-28-5p	558.8	723.3	494.8	591.7	684.3	460.7	830.5	903.9	701.2	778.2	825.1	660.1
miR-30e-3p	555.9	398.2	255.4	457.3	364.7	265.1	253.3	193.4	154.7	246.9	215	160.7
miR-3096-5p	516.8	127	242.9	525.7	106.9	137.5	56.7	41.6	94.8	56.3	57.6	75
miR-378b	516.8	540.6	469.8	485.6	474	453.4	736.7	747.6	685.5	657	790.3	643.4
miR-30a-3p	509.1	367.4	227.6	424.3	332.7	247.4	233.7	175.6	136.6	227.6	192.6	142.5
miR-27b-5p	490	446.3	476	438.5	398	435.1	495.6	275.1	366.3	554.6	380.8	412.3
miR-421-3p	449	429	419.7	379.6	446.7	442.9	460.6	434.5	470.4	466.4	472.1	412.3
miR-5099	427.9	582.9	506.7	572.9	710.5	680.4	673.1	1064.8	1201.9	764.5	1309.6	1396.8
miR-652-3p	413.6	517.5	425.2	431.4	419.4	486.9	425.1	393.4	674.5	411.6	448.8	607.8
miR-5109	371.6	257.8	306.2	518.6	105.7	208.1	66.8	65	92.5	60.3	61.1	97.8
miR-19b-3p	357.3	731	470.5	202.7	610.7	386.5	413.4	779.1	283.7	350.3	675.2	415.3
miR-320-3p	349.6	311.6	212.3	370.1	266.1	313.8	307.4	146.7	272.1	344.7	201.1	253.9
miR-19a-3p	340.1	700.2	456.6	209.8	584.5	373.4	393.8	743.1	267.5	334.6	656.8	393.3
miR-92b-3p	318.1	221.2	198.4	346.5	203.2	258.9	364.7	271.5	404.1	420.8	328.6	429.7
miR-1843b-5p	315.2	286.6	286.7	337.1	337.4	197.2	304.2	333	265.1	272.7	302.8	262.2
miR-106b-3p	312.4	350.1	174.7	261.7	244.7	230.1	341.3	334.5	222.1	298.6	359.4	226.6
miR-1843-5p	309.5	284.7	281.2	332.4	330.3	189.3	303.2	331.9	259.9	267.2	301.8	257.7
miR-328-3p	277	211.6	236.6	285.3	174.6	506.2	163.2	139.1	376.8	173.4	155.9	372.9
miR-501-3p	273.2	355.9	171.2	238.1	348.1	236.9	277.2	261.4	231.4	266.7	317.2	222.1
miR-339-5p	269.4	215.5	198.4	266.4	228.1	177.8	77.4	97.4	87.2	77.1	95.8	70.5
miR-511-3p	269.4	263.5	127.4	249.9	248.3	138.1	245.4	247.7	121.5	239.8	263.6	121.3
miR-27a-5p	268.4	140.4	222	89.6	63	135.4	190.8	136.5	150.6	152.1	143.5	184.2
miR-1198-5p	236.9	286.6	240.1	228.7	262.6	367.6	282	222.8	427.4	290.5	293.4	441.8
miR-181a-1-3p	232.1	219.3	155.2	238.1	243.6	234.8	87.5	89.3	76.8	76	75.5	71.2
miR-223-5p	230.2	311.6	212.3	96.7	118.8	162.6	381.1	219.8	381.4	295	196.1	366.1

miR-674-3p	229.3	202	271.4	252.2	222.2	377.6	152.6	145.7	224.4	142.5	130.1	223.6
miR-511-5p	219.7	203.9	91.9	235.7	230.5	91.5	273.5	314.2	94.8	260.6	298.4	102.3
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-340-3p	218.7	290.5	233.8	228.7	254.2	275.6	159.5	186.3	211.1	156.1	174.7	185.7
miR-872-5p	207.3	253.9	258.2	188.6	249.5	250	207.2	221.8	210.5	203.3	224.4	209.9
miR-34a-5p	206.3	203.9	245.7	148.5	193.7	304.9	172.3	243.1	264	195.2	244.2	238
miR-130b-5p	203.5	240.5	92.6	252.2	200.8	198.7	187.6	198.5	127.3	192.1	173.3	127.3
miR-342-5p	199.6	265.5	157.3	198	241.2	317.4	170.7	100.5	226.8	157.7	115.7	217.5
miR-3964	199.6	361.7	308.3	252.2	313.7	186.7	650.3	1290.7	582.6	506.9	1061.4	575.2
miR-361-5p	197.7	246.2	193.5	157.9	305.3	226.4	199.3	217.7	241.3	183	245.7	210.7
miR-30d-3p	184.4	140.4	192.1	141.4	192.5	132.8	169.6	237.5	106.4	153.1	217.4	113.7
miR-339-3p	179.6	203.9	226.2	148.5	224.5	183	121.9	133	152.9	107	119.6	154.6
miR-106b-5p	176.7	315.5	174.7	209.8	327.9	162.1	239	374.1	157	204.3	290.9	194.8
miR-28c	170	192.4	144.1	162.7	141.4	99.9	275.1	344.1	264.6	278.8	297.4	243.3
miR-671-3p	169.1	90.4	116.2	132	67.7	122.4	53	25.4	45.9	74.5	29.3	50.8
miR-664-5p	161.4	125	136.4	183.9	109.3	193.5	129.9	75.6	159.3	128.8	96.3	158.4
miR-222-5p	151.9	125	162.9	726.1	678.4	728	85.3	83.2	81.4	166.3	182.7	143.2
miR-326-3p	150	121.2	251.9	136.7	179.4	170	102.8	171	114.5	95.3	140.5	112.9
miR-181c-5p	144.2	105.8	119	139.1	95	90.5	120.3	149.2	111.1	127.2	137.5	116
miR-5097	138.5	148.1	148.2	134.4	218.6	65.9	210.9	235.5	93	191.1	250.2	114.4
miR-17-5p	133.7	205.8	137.1	136.7	221	197.7	142.6	147.2	151.8	140.4	184.2	164.5
miR-322-3p	128	130.8	133.6	103.7	109.3	165.8	76.3	53.3	111.1	81.6	67.5	104.6
miR-128-3p	124.2	211.6	353.6	165	174.6	292.3	164.3	227.9	294.2	143.5	196.6	340.3
miR-29b-3p	122.3	157.7	198.4	157.9	213.9	95.2	159	330.9	116.9	138.9	233.8	103.1
miR-99b-3p	120.4	71.2	110	181.5	124.7	132.8	658.3	307.1	598.9	667.7	358.4	659.4
miR-1981-5p	116.5	86.6	48	87.2	67.7	96.7	61.5	34.5	59.3	56.8	41.2	50
miR-338-3p	115.6	146.2	146.2	103.7	168.7	111.4	124	167.5	122.1	96.8	172.8	128.1
miR-3968	112.7	138.5	119.7	207.5	115.2	84.2	175.4	219.3	91.3	113.6	186.2	75
miR-361-3p	105.1	111.6	72.4	75.4	79.6	117.7	106	118.8	119.2	129.8	137	119.7

miR-152-3p	99.3	119.3	71	91.9	108.1	62.8	94.3	81.7	81.4	83.6	77.4	75
miR-148b-5p	97.4	127	86.3	75.4	104.6	81.1	102.3	126.4	96.5	135.9	110.7	113.7
miR-330-5p	97.4	94.3	108.6	56.6	101	95.2	92.8	117.8	98.8	83.6	121.1	96.3
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-28-3p	94.6	76.9	104.4	91.9	66.5	119.8	145.8	95.9	132	128.8	108.7	131.1
miR-183-5p	91.7	75	79.3	94.3	76	73.2	78.4	57.9	72.1	71	84.4	58.4
miR-140-5p	87.9	96.2	93.3	80.2	79.6	77.4	87.5	92.4	79.1	74.5	92.8	81.9
miR-99a-3p	87.9	80.8	66.1	96.7	90.3	41.3	72.1	104	47.1	70.5	96.8	43.2
miR-152-5p	85	55.8	42.5	56.6	51.1	29.8	47.7	44.2	20.4	56.3	46.2	22.7
miR-374-5p	80.2	65.4	83.5	87.2	85.5	68.5	81.1	78.2	66.3	62.4	68	61.4
miR-574-3p	74.5	61.6	138.5	73.1	54.7	221.2	46.6	21.8	150	48.7	28.3	134.9
miR-3068-3p	73.6	100	77.9	75.4	92.7	54.4	54.1	58.4	55.8	68.9	56.6	46.2
miR-132-3p	70.7	119.3	103.7	115.5	143.8	185.1	34.5	35	86.1	36.5	43.2	93.2
miR-350-5p	68.8	59.6	64	61.3	59.4	84.2	56.2	58.9	76.2	66.4	58.6	78.8
miR-720	67.8	55.8	42.5	122.6	49.9	61.2	55.1	32.5	76.8	72.5	51.1	53.1
miR-450b-5p	66.9	61.6	58.5	63.7	67.7	43.4	52.5	54.8	24.4	47.1	38.2	23.5
miR-22-5p	65	44.2	59.9	44.8	55.8	90.5	117.1	156.8	157.6	130.8	167.3	180.4
miR-20a-5p	63	113.5	84.2	58.9	125.9	95.2	76.3	101.5	86.6	76	103.3	81.1
miR-1249-3p	62.1	30.8	20.9	44.8	11.9	30.3	6.4	14.7	18	12.2	12.4	13.6
miR-3057-5p	62.1	38.5	43.8	61.3	53.5	59.1	75.3	35.5	51.7	56.8	43.7	42.4
miR-30c-1-3p	58.3	53.9	60.6	42.4	32.1	61.2	40.8	33	66.3	51.2	31.3	50
miR-872-3p	58.3	61.6	50.8	40.1	70.1	39.2	50.9	61.9	31.4	40	68	40.2
miR-106a-5p	57.3	107.7	73.8	75.4	112.9	87.9	68.4	68.5	73.3	65.9	88.9	69.7
miR-5115	56.4	51.9	56.4	122.6	17.8	29.3	20.1	12.2	28.5	33	22.8	24.3
miR-125a-3p	54.4	44.2	37.6	193.3	114.1	301.2	147.3	60.9	271.5	273.8	108.2	358.5
miR-542-3p	54.4	36.6	32.7	33	40.4	37.7	47.7	42.1	32.6	55.8	38.7	36.4
miR-1964-3p	52.5	46.2	91.9	49.5	41.6	81.6	77.9	65	124.4	91.8	70	138.7
miR-17-3p	51.6	76.9	61.2	58.9	80.8	52.8	97.5	93.4	76.8	91.8	98.3	62.9
miR-5117-5p	51.6	82.7	7.7	115.5	7.1	3.7	4.8	13.2	1.2	2.5	8.9	1.5

miR-425-3p	50.6	75	46.6	49.5	58.2	31.9	62.5	45.7	43	36	45.7	48.5
miR-194-5p	46.8	53.9	66.8	51.9	38	71.1	51.4	48.7	84.3	51.7	61.1	90.2
miR-674-5p	46.8	44.2	48	56.6	57	50.2	64.1	74.1	79.1	61.8	93.8	100.8
miR-127-3p	45.9	15.4	11.8	49.5	7.1	11	18	4.6	8.1	19.8	10.4	12.9
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-500-3p	44.9	67.3	45.2	56.6	74.8	38.2	70	70.5	49.4	58.8	80.4	54.6
miR-362-3p	43.9	46.2	55.7	42.4	68.9	28.8	46.6	104	34.9	35	74.5	24.3
miR-582-3p	43.9	11.5	41.8	30.6	10.7	25.6	17	7.6	12.8	19.8	7.4	9.1
miR-1943-5p	43	28.9	31.3	30.6	54.7	44.5	56.7	35	66.9	60.3	57.6	56.1
miR-324-3p	43	57.7	52.2	47.1	47.5	27.7	59.9	108.1	68	57.8	103.8	59.9
miR-141-3p	42	38.5	59.2	16.5	52.3	34.5	67.3	110.6	77.9	50.2	108.2	68.2
miR-1843b-3p	41.1	53.9	39.7	56.6	49.9	49.2	33.4	16.2	27.3	25.9	24.3	34.9
miR-126-5p	39.2	51.9	32.7	33	39.2	30.9	33.9	40.6	35.5	31.4	46.7	32.6
miR-92a-1-5p	39.2	46.2	38.3	84.9	66.5	119.2	81.1	95.9	93.6	131.3	131.1	122.8
mmu-let-7a-1-3p	38.2	42.3	64	42.4	30.9	49.7	12.2	22.3	20.9	9.6	14.4	28.8
mmu-let-7c-2-3p	38.2	42.3	64	42.4	30.9	49.7	12.2	22.3	20.9	9.6	14.4	28.8
miR-1199-5p	37.3	40.4	37.6	54.2	38	25.6	43.5	32	33.1	26.4	41.2	25.8
miR-18a-5p	37.3	34.6	36.2	25.9	55.8	24.6	34.5	60.4	30.2	36	50.6	22.7
miR-200a-3p	37.3	36.6	60.6	16.5	53.5	33.5	68.4	107.6	72.1	49.7	104.3	67.5
miR-200b-3p	37.3	46.2	37.6	44.8	15.4	46	31.3	14.2	21.5	24.8	22.3	27.3
miR-26b-3p	37.3	73.1	82.8	33	40.4	73.2	35.5	40.1	79.1	28.4	41.2	68.2
miR-148a-5p	36.3	21.2	17.4	40.1	16.6	10.5	24.9	22.3	11.6	19.8	14.9	15.9
miR-298-5p	36.3	15.4	147.5	33	20.2	126.6	12.7	5.6	41.9	17.7	9.4	51.5
miR-1981-3p	35.3	30.8	37.6	51.9	41.6	69	30.7	12.7	40.1	27.9	25.8	40.2
miR-877-5p	33.4	46.2	25.8	40.1	38	39.7	31.8	13.2	23.3	34	22.8	28.8
miR-16-1-3p	32.5	21.2	30.6	33	23.8	12	33.9	90.9	19.8	30.9	64	23.5
miR-664-3p	32.5	25	36.2	51.9	30.9	31.4	22.3	19.8	36	27.9	19.9	33.3
miR-690	32.5	19.2	7	33	24.9	5.2	21.2	7.1	5.2	15.7	11.9	6.8
miR-210-5p	31.5	11.5	43.2	174.5	72.5	48.6	100.2	155.8	102.9	133.8	127.1	156.9

miR-130a-3p	30.6	30.8	41.1	35.4	33.3	40.3	35.5	30.5	41.3	32.4	35.7	37.1
miR-532-3p	30.6	32.7	26.4	33	41.6	34	22.8	35.5	28.5	27.4	35.7	31.8
miR-5119	29.6	11.5	8.4	25.9	7.1	1.6	8	5.1	1.7	8.6	4.5	3.8
miR-98-3p	29.6	32.7	67.5	16.5	26.1	36.6	4.2	12.7	11	13.7	6	19.7
mmu-let-7e-3p	27.7	11.5	8.4	47.1	33.3	40.3	25.4	6.1	23.3	31.9	10.9	26.5
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-155-5p	27.7	55.8	34.8	1619.6	2189.6	1794.3	3195	3029.1	3534.6	3307.8	3750.6	3558.2
miR-5121	27.7	28.9	20.2	40.1	10.7	9.9	4.8	4.6	8.1	3	7.4	5.3
miR-5107	26.7	11.5	12.5	28.3	5.9	20.9	2.7	2.5	1.2	1	1	1.5
miR-3470b	24.8	26.9	18.8	33	9.5	9.4	22.8	22.8	14	25.3	26.3	15.9
miR-150-5p	22	21.2	9.7	44.8	24.9	14.1	13.8	13.7	10.5	9.1	13.9	9.1
miR-3107-3p	22	11.5	0	35.4	9.5	28.8	22.8	10.2	3.5	18.3	11.9	34.1
miR-3473	22	13.5	4.9	37.7	10.7	4.2	6.4	4.6	1.7	4.6	3.5	3
miR-486-3p	22	11.5	0	35.4	9.5	28.8	22.8	10.2	3.5	18.3	11.9	34.1
miR-879-5p	22	23.1	27.8	18.9	23.8	9.4	13.8	18.3	11	7.6	20.4	5.3
mmu-let-7f-2-3p	21	26.9	26.4	11.8	20.2	22.5	18	19.8	11.6	11.2	12.9	12.1
miR-191-3p	21	19.2	29.9	25.9	16.6	30.3	19.1	17.8	29.7	22.3	18.9	30.3
miR-1947-5p	21	19.2	22.3	14.1	22.6	16.2	14.8	14.7	19.8	16.7	17.4	22
miR-25-5p	21	15.4	11.8	9.4	10.7	17.3	17.5	8.6	16.3	14.7	7.9	19.7
miR-3470a	21	26.9	16	33	10.7	9.4	18	18.3	11.6	18.3	23.8	15.9
miR-450a-5p	21	21.2	26.4	18.9	22.6	25.1	34.5	47.2	26.7	27.4	40.2	20.5
miR-18a-3p	20.1	21.2	16	28.3	20.2	28.2	26.5	12.7	29.1	24.3	14.9	32.6
miR-338-5p	20.1	30.8	25.8	16.5	26.1	24.6	23.3	23.3	27.9	20.8	17.9	13.6
miR-362-5p	20.1	15.4	16.7	16.5	20.2	15.2	19.6	21.8	14.5	17.7	11.9	13.6
miR-324-5p	19.1	28.9	22.3	7.1	32.1	13.1	37.1	44.2	34.9	24.8	44.2	27.3
miR-143-3p	18.1	19.2	2495.1	21.2	28.5	1701.7	27.6	21.8	1154.8	16.2	20.9	1070.1
miR-195-5p	18.1	11.5	8.4	9.4	20.2	15.7	23.3	13.2	13.4	21.8	17.4	12.9
miR-212-3p	18.1	19.2	28.5	11.8	34.5	35.6	5.3	9.6	25.6	10.1	10.4	30.3
miR-5114	18.1	23.1	12.5	2.4	14.3	13.1	16.4	9.6	12.2	18.3	10.4	11.4

miR-677-5p	18.1	23.1	13.9	25.9	14.3	9.4	4.8	14.2	11.6	11.2	17.9	11.4
miR-429-3p	17.2	25	23.7	14.1	14.3	27.2	18.6	24.9	33.1	14.7	16.4	31.8
miR-15b-3p	16.2	44.2	28.5	30.6	44	22	38.2	79.7	32	31.9	60.1	33.3
miR-33-5p	16.2	15.4	18.8	11.8	22.6	15.2	15.9	40.1	17.4	24.8	29.3	17.4
miR-345-3p	16.2	9.6	34.1	16.5	9.5	32.4	11.1	14.2	32	2	11.4	22.7
miR-93-3p	16.2	26.9	19.5	11.8	24.9	29.3	15.9	13.2	19.2	11.7	10.4	10.6
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-15a-3p	15.3	11.5	16	16.5	24.9	6.8	31.8	69.5	17.4	26.4	49.1	12.9
miR-1933-3p	15.3	11.5	12.5	4.7	11.9	9.4	8	11.2	5.2	13.7	8.4	9.1
miR-30b-3p	15.3	36.6	27.1	16.5	20.2	20.4	22.3	31.5	20.9	21.3	28.8	22
miR-5124	15.3	11.5	2.8	16.5	8.3	2.1	2.7	10.7	1.2	6.1	3.5	5.3
miR-574-5p	15.3	7.7	11.1	2.4	15.4	18.8	17	17.8	64	24.3	24.8	41.7
miR-149-5p	14.3	7.7	15.3	16.5	16.6	45.5	4.8	10.2	15.1	7.1	5	5.3
miR-28b	14.3	17.3	14.6	9.4	10.7	6.8	9.5	10.7	16.3	11.7	13.9	12.9
miR-16-2-3p	13.4	11.5	21.6	4.7	13.1	19.3	30.2	34.5	25.6	15.2	25.3	23.5
miR-24-1-5p	13.4	11.5	20.9	9.4	7.1	9.9	15.9	12.7	10.5	9.6	15.9	16.7
miR-3096-3p	13.4	34.6	7	40.1	9.5	4.2	2.7	7.6	1.7	3.5	6	0.8
miR-3096b-3p	13.4	34.6	7	40.1	8.3	4.2	2.7	6.6	1.7	2.5	6	0.8
miR-32-5p	13.4	23.1	18.1	11.8	20.2	9.4	17	52.3	14	18.8	36.2	20.5
mmu-let-7c-1-3p	12.4	3.8	5.6	21.2	8.3	21.4	1.1	2	3.5	3	1.5	5.3
miR-128-1-5p	12.4	5.8	9.7	0	10.7	7.3	14.3	15.7	14.5	13.2	17.9	15.2
miR-3082-3p	12.4	19.2	11.8	9.4	5.9	11	12.2	10.2	10.5	12.2	8.4	19.7
miR-10a-3p	11.5	17.3	18.1	11.8	11.9	12.6	15.4	12.7	7	11.2	8.9	9.9
miR-139-5p	11.5	7.7	11.1	18.9	8.3	11.5	6.4	13.2	14.5	4.1	11.4	9.1
miR-185-3p	11.5	11.5	20.2	7.1	10.7	12	5.3	8.1	21.5	10.1	13.9	16.7
miR-330-3p	11.5	5.8	9	9.4	4.8	14.6	4.8	6.6	8.7	6.6	6.5	6.1
miR-132-5p	10.5	21.2	11.1	14.1	16.6	18.3	6.4	6.1	7	7.1	7.4	5.3
miR-1843-3p	10.5	15.4	7.7	14.1	17.8	9.9	9	12.2	7	9.6	9.9	4.5
miR-350-3p	10.5	9.6	5.6	7.1	28.5	7.3	15.4	27.9	10.5	10.1	27.8	9.1

miR-497-5p	10.5	9.6	11.8	9.4	5.9	6.8	14.3	11.2	9.9	10.6	10.4	11.4
miR-5126	10.5	0	0	7.1	1.2	0	1.1	3.6	0	0.5	3	0
mmu-let-7b-3p	9.6	7.7	7	2.4	0	12.6	1.1	0	3.5	2	2	3
miR-188-5p	9.6	19.2	9	9.4	30.9	15.2	16.4	24.9	17.4	10.6	16.4	10.6
miR-29a-5p	9.6	13.5	19.5	16.5	11.9	8.9	10.1	19.3	8.1	11.7	21.8	18.2
miR-467a-5p	9.6	9.6	21.6	4.7	14.3	16.2	12.7	19.3	30.8	8.6	19.4	14.4
miR-467b-5p	9.6	9.6	21.6	4.7	14.3	16.2	12.7	19.3	30.8	8.6	19.4	14.4
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
mmu-let-7f-1-3p	8.6	15.4	16	7.1	5.9	13.1	1.6	3.6	11.6	2	1.5	6.8
miR-101a-5p	8.6	15.4	15.3	11.8	32.1	12.6	5.8	24.9	6.4	8.1	12.9	3
miR-203-3p	8.6	11.5	2.1	2.4	15.4	5.2	10.6	43.6	10.5	12.2	43.2	15.9
miR-30c-2-3p	8.6	3.8	20.2	2.4	4.8	11	7.4	5.1	11.6	10.6	4	8.3
miR-345-5p	8.6	1.9	4.9	16.5	9.5	5.2	9	12.2	9.3	8.6	13.4	5.3
miR-7a-1-3p	8.6	30.8	19.5	16.5	16.6	27.2	23.9	43.1	29.1	24.3	33.8	25.8
miR-1194	7.6	3.8	5.6	18.9	3.6	6.3	10.1	1.5	1.7	11.7	3.5	3.8
miR-122-5p	7.6	5.8	0	7.1	0	0	5.3	1	0	6.6	3	0.8
miR-181b-1-3p	7.6	5.8	4.2	7.1	9.5	8.4	4.8	3	4.7	2	2	0.8
miR-188-3p	7.6	5.8	2.8	2.4	7.1	1	3.7	9.6	2.9	2.5	5.5	1.5
miR-1948-3p	7.6	1.9	9.7	11.8	11.9	14.6	10.6	13.2	9.3	14.2	16.9	17.4
miR-212-5p	7.6	7.7	11.1	2.4	22.6	6.3	1.6	7.1	3.5	4.6	3.5	5.3
miR-2137	7.6	3.8	6.3	0	2.4	1	0	1.5	0	2	2.5	0
miR-26a-2-3p	7.6	5.8	2.1	0	2.4	1.6	2.1	2	1.7	0	2	5.3
miR-3076-3p	7.6	13.5	2.1	9.4	7.1	9.9	0.5	3.6	6.4	5.6	3.5	6.8
miR-3473d	7.6	3.8	16.7	16.5	17.8	10.5	13.3	23.3	20.4	12.2	20.4	22
miR-34a-3p	7.6	1.9	4.2	4.7	0	1.6	4.2	5.1	0	3.5	4.5	1.5
miR-669a-5p	7.6	5.8	27.8	11.8	8.3	24.1	19.1	32	51.2	20.8	29.8	57.6
miR-669p-5p	7.6	5.8	27.8	11.8	8.3	24.1	19.1	32	51.2	20.8	29.8	57.6
miR-676-3p	7.6	5.8	27.8	14.1	3.6	34.5	6.9	3	18.6	11.2	8.4	20.5
miR-744-3p	7.6	11.5	9	7.1	7.1	7.3	3.7	1	0.6	2.5	2	2.3

miR-215-5p	6.7	13.5	13.9	2.4	7.1	6.8	4.8	8.1	3.5	5.6	5.5	4.5
miR-34c-5p	6.7	5.8	71	4.7	3.6	51.2	4.8	5.6	20.9	9.6	6.5	18.9
miR-467c-5p	6.7	13.5	23	18.9	15.4	26.1	35.5	40.6	50.6	22.3	33.8	44.7
miR-542-5p	6.7	5.8	3.5	4.7	4.8	3.1	4.2	3.6	2.9	4.6	5	6.8
mmu-let-7g-3p	5.7	3.8	4.9	9.4	10.7	5.8	2.7	8.6	1.7	3	3.5	5.3
mmu-let-7i-3p	5.7	13.5	26.4	14.1	17.8	16.2	28.6	65.5	29.1	30.4	69.5	35.6
miR-146a-3p	5.7	5.8	2.1	4.7	13.1	16.7	4.2	10.2	7	14.2	15.9	8.3
miR-1948-5p	5.7	1.9	0.7	0	0	0.5	3.2	5.6	5.8	4.6	4	6.8
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-1983	5.7	11.5	9.7	9.4	5.9	9.4	5.8	6.1	9.3	5.1	7	8.3
miR-199a-3p	5.7	3.8	197.7	42.4	17.8	224.3	42.4	111.7	162.8	48.2	113.7	128.1
miR-199b-3p	5.7	3.8	197.7	42.4	17.8	224.3	42.4	111.7	162.8	48.2	113.7	128.1
miR-200c-3p	5.7	7.7	2.1	4.7	4.8	5.8	4.2	2	10.5	3	3.5	12.1
miR-331-3p	5.7	7.7	8.4	4.7	13.1	5.2	11.1	13.7	7.6	6.6	13.9	12.1
miR-3473b	5.7	7.7	6.3	14.1	3.6	2.6	4.8	5.6	4.7	5.1	6.5	4.5
miR-410-3p	5.7	5.8	2.8	11.8	1.2	1.6	3.7	0	1.2	2.5	2	1.5
miR-466a-3p	5.7	5.8	15.3	4.7	9.5	22	6.4	4.6	11.6	8.6	3.5	10.6
miR-466b-3p	5.7	5.8	15.3	4.7	9.5	22	6.4	4.6	11.6	8.6	3.5	10.6
miR-466c-3p	5.7	5.8	15.3	4.7	9.5	22	6.4	4.6	11.6	8.6	3.5	10.6
miR-466e-3p	5.7	5.8	15.3	4.7	9.5	22	6.4	4.6	11.6	8.6	3.5	10.6
miR-466o-3p	5.7	5.8	14.6	4.7	9.5	17.8	5.8	4.6	10.5	8.1	3.5	9.1
miR-466p-3p	5.7	5.8	15.3	4.7	9.5	22	6.4	4.6	11.6	8.6	3.5	10.6
miR-503-3p	5.7	9.6	5.6	4.7	5.9	10.5	10.6	13.2	11.6	13.2	14.9	8.3
miR-700-5p	5.7	7.7	4.9	0	1.2	2.6	2.7	4.6	2.9	2	2.5	2.3
miR-9-5p	5.7	3.8	2.1	2.4	17.8	2.6	5.8	19.3	1.2	9.6	15.9	3.8
miR-1195	4.8	0	1.4	14.1	2.4	3.7	2.1	3	1.2	2.5	4	2.3
miR-1306-3p	4.8	1.9	3.5	4.7	0	2.6	3.2	1	2.9	2.5	0.5	0
miR-147-3p	4.8	3.8	4.9	56.6	58.2	35	82.7	219.3	66.9	76	226.4	80.3
miR-184-3p	4.8	1.9	4.2	4.7	8.3	5.8	4.8	10.7	8.7	6.6	16.4	8.3

miR-185-5p	4.8	7.7	4.9	16.5	3.6	8.4	10.1	8.1	8.1	6.6	10.9	9.1
miR-3068-5p	4.8	7.7	13.2	9.4	13.1	5.8	2.7	1.5	2.3	4.6	3	2.3
miR-378-5p	4.8	3.8	7.7	9.4	5.9	6.8	10.1	9.6	8.7	6.6	9.4	7.6
miR-582-5p	4.8	5.8	3.5	4.7	3.6	2.1	2.1	2.5	0	2	2	1.5
miR-669f-5p	4.8	5.8	19.5	11.8	5.9	16.2	15.4	25.4	43	16.7	22.3	46.2
miR-700-3p	4.8	5.8	3.5	2.4	7.1	1.6	2.1	2	2.9	3.5	3	0.8
miR-704	4.8	1.9	0	0	1.2	1.6	1.6	1	1.7	2	1.5	0.8
miR-1198-3p	3.8	7.7	2.8	0	2.4	4.7	1.6	5.1	3.5	2	4.5	3.8
miR-1306-5p	3.8	1.9	1.4	4.7	0	4.7	1.1	1.5	4.7	2	2.5	3
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-181c-3p	3.8	1.9	7.7	2.4	2.4	3.7	3.7	5.1	4.1	4.1	5	1.5
miR-1839-3p	3.8	7.7	4.9	4.7	1.2	5.2	1.6	1	2.3	4.1	3.5	2.3
miR-219-1-3p	3.8	5.8	3.5	4.7	7.1	5.2	9	4.6	6.4	9.6	4	7.6
miR-219-5p	3.8	0	0.7	0	0	0.5	1.1	3	0.6	0.5	2.5	3
miR-29c-5p	3.8	7.7	5.6	0	3.6	2.6	2.7	5.6	2.9	6.1	6.5	3.8
miR-3060-3p	3.8	5.8	5.6	11.8	5.9	7.3	11.7	2.5	5.8	8.6	2.5	8.3
miR-3061-3p	3.8	1.9	3.5	4.7	2.4	4.2	4.8	3	3.5	3	4	0.8
miR-3109-3p	3.8	3.8	5.6	4.7	1.2	1	4.8	5.1	2.9	4.1	6.5	1.5
miR-365-3p	3.8	11.5	9.7	14.1	1.2	11	3.7	5.1	5.8	3	3.5	4.5
miR-381-3p	3.8	0	0.7	4.7	2.4	0	1.6	0.5	0.6	2	1	0
miR-3962	3.8	3.8	0	4.7	4.8	1	2.1	3.6	2.3	3	1.5	3
miR-3969	3.8	0	2.8	4.7	1.2	4.7	4.8	8.1	5.2	5.1	6.5	0.8
miR-467d-5p	3.8	9.6	23	18.9	13.1	25.6	29.2	25.4	35.5	17.2	23.8	41.7
miR-501-5p	3.8	5.8	2.1	2.4	4.8	2.6	2.7	12.7	4.1	3.5	9.9	3
miR-503-5p	3.8	19.2	7	2.4	5.9	7.8	19.1	11.7	13.4	5.1	19.9	18.9
miR-5627-3p	3.8	0	0	2.4	0	1.6	3.2	1	2.3	2.5	3	6.1
miR-669o-5p	3.8	1.9	16.7	9.4	7.1	16.7	15.9	25.9	41.9	16.7	21.3	47
miR-874-3p	3.8	0	6.3	7.1	3.6	1.6	1.6	0	0	1.5	0.5	0.8
miR-1191	2.9	11.5	7.7	9.4	3.6	5.2	10.6	11.7	15.7	10.1	12.4	14.4

miR-1196-5p	2.9	3.8	0	2.4	1.2	0.5	2.1	0.5	1.2	1.5	0.5	3
miR-1231-3p	2.9	1.9	1.4	4.7	0	5.8	1.6	0.5	0.6	2	0.5	1.5
miR-138-5p	2.9	1.9	4.2	7.1	4.8	3.7	1.1	2.5	4.7	2.5	4	1.5
miR-146b-3p	2.9	0	0	0	3.6	1	2.1	1.5	6.4	1	2	5.3
miR-186-3p	2.9	0	0.7	4.7	7.1	1.6	2.7	8.6	1.2	4.6	3.5	3
miR-190b-5p	2.9	1.9	0	0	2.4	1	0	2.5	0	0.5	3	0.8
miR-1927	2.9	0	0.7	0	1.2	0	1.1	1	1.2	1	0.5	0.8
miR-1930-5p	2.9	1.9	0	0	2.4	0.5	0	0	0	2.5	1	0
miR-1940	2.9	0	0	0	0	0	0	0	0.6	0.5	0	0
miR-29b-2-5p	2.9	1.9	2.8	2.4	3.6	1.6	3.7	1	0.6	0	1	2.3
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-301a-5p	2.9	1.9	2.8	2.4	3.6	0.5	2.7	4.1	1.2	3	3	2.3
miR-3074-5p	2.9	7.7	4.2	7.1	3.6	2.6	6.9	6.6	7.6	9.6	11.9	4.5
miR-3079-5p	2.9	0	0.7	0	4.8	1	0.5	2	0.6	2.5	0.5	0.8
miR-3103-3p	2.9	1.9	1.4	2.4	1.2	3.1	0.5	0	1.2	1.5	0	0.8
miR-33-3p	2.9	5.8	5.6	4.7	13.1	3.7	6.9	12.2	7	5.6	12.4	10.6
miR-369-3p	2.9	3.8	4.9	2.4	2.4	1	0.5	1	0.6	0.5	1	3
miR-409-3p	2.9	0	0	0	0	2.1	0	1	0	1	0	0.8
miR-466b-5p	2.9	3.8	1.4	2.4	1.2	2.1	1.6	4.6	6.4	2	6.5	4.5
miR-466e-5p	2.9	3.8	2.1	2.4	1.2	1.6	1.1	5.6	6.4	2	7.4	4.5
miR-466o-5p	2.9	3.8	1.4	2.4	1.2	2.1	1.6	4.6	6.4	2	6.5	4.5
miR-466p-5p	2.9	1.9	1.4	2.4	1.2	1	1.1	2.5	3.5	0.5	3.5	3.8
miR-467h	2.9	7.7	8.4	4.7	4.8	8.4	6.4	12.2	15.7	5.1	11.4	12.1
miR-5106	2.9	1.9	0	2.4	0	0	1.6	0	0	0	0	0
miR-708-3p	2.9	0	0	2.4	0	0	0	0.5	0	0	0.5	0
miR-760-3p	2.9	1.9	1.4	2.4	1.2	2.1	2.7	1	4.7	3	0.5	3
miR-126-3p	1.9	19.2	9.7	4.7	10.7	7.8	9.5	8.1	10.5	3	7.4	6.8
miR-141-5p	1.9	0	0	0	1.2	0	0.5	2.5	1.2	0	0	2.3
miR-1931	1.9	1.9	0	2.4	0	0.5	0	0	0	0	1	0

miR-1934-5p	1.9	5.8	0.7	4.7	3.6	4.2	1.6	1.5	1.2	3.5	1.5	5.3
miR-1955-3p	1.9	1.9	1.4	0	2.4	1.6	3.2	1	2.9	1.5	0.5	3
miR-1955-5p	1.9	0	0.7	9.4	7.1	2.6	2.7	2	2.3	4.6	2.5	0.8
miR-1968-5p	1.9	0	1.4	0	0	0	1.1	0	3.5	0.5	1	0
miR-20a-3p	1.9	3.8	2.8	0	7.1	3.1	2.1	5.1	2.3	1	1	6.1
miR-299-3p	1.9	0	0	0	0	0	0	0	0	0.5	0	0
miR-29b-1-5p	1.9	0	0	0	2.4	1.6	0.5	3	1.2	1	2.5	0.8
miR-3084-3p	1.9	0	0	2.4	2.4	2.6	1.6	2.5	2.3	1.5	3.5	2.3
miR-331-5p	1.9	0	2.8	2.4	2.4	1.6	6.4	2	8.7	4.1	3.5	6.8
miR-34b-5p	1.9	0	2.1	2.4	0	1.6	1.1	0	1.2	0.5	0.5	1.5
miR-451	1.9	0	0.7	11.8	0	1	0.5	0	0	0.5	0.5	0
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-466a-5p	1.9	1.9	1.4	2.4	1.2	1	0.5	2	2.3	0.5	3	0.8
miR-466c-5p	1.9	3.8	1.4	2.4	1.2	1.6	0.5	4.1	2.9	2	5.5	0.8
miR-466d-5p	1.9	1.9	0	0	1.2	0	1.1	0.5	2.3	1	2.5	1.5
miR-466k	1.9	1.9	0.7	0	1.2	0.5	1.6	0.5	2.9	1	3.5	2.3
miR-466n-5p	1.9	1.9	0	0	1.2	0	1.1	0.5	2.3	1	2.5	1.5
miR-5105	1.9	0	1.4	2.4	0	0.5	1.1	0.5	0.6	0.5	2.5	0
miR-5116	1.9	0	0.7	0	0	0	1.1	0.5	0.6	0	1	0.8
miR-5128	1.9	0	0	0	2.4	2.6	2.7	0.5	2.9	2	1	2.3
miR-671-5p	1.9	3.8	1.4	2.4	3.6	7.8	4.2	3	7	2.5	7.4	6.1
miR-7a-5p	1.9	25	4.2	7.1	7.1	3.1	23.9	27.4	15.1	16.7	30.3	25.8
miR-7b-5p	1.9	21.2	4.2	2.4	7.1	2.6	21.2	23.3	14.5	14.7	28.8	23.5
miR-103-2-5p	1	3.8	0.7	0	1.2	0.5	0.5	2	0	1	1	0
miR-107-5p	1	3.8	0.7	0	1.2	0.5	0.5	1	0	1	1	0
miR-1231-5p	1	0	0	0	0	0.5	0.5	0	0.6	0	0	0
miR-129-1-3p	1	0	0	4.7	0	0	0	0	0	0	0	0
miR-129-2-3p	1	0	0	4.7	0	0	0	0	0	0	0	0
miR-133a-3p	1	0	0	2.4	0	0.5	1.6	0	0	0.5	0	0

miR-135a-5p	1	0	0	0	0	0	0	0	0	0	0	0
miR-135b-5p	1	0	0	0	0	0	0	0	0	0	0	0
miR-136-3p	1	0	0.7	2.4	0	0	0.5	0.5	0	1	0.5	0
miR-136-5p	1	0	0	0	0	0	0	0	0	0	0	0
miR-138-2-3p	1	0	0	0	0	0	0	0	0	0	0	0
miR-147-5p	1	0	0.7	2.4	4.8	0.5	1.1	1.5	0	1.5	2.5	0.8
miR-153-3p	1	0	0	0	0	0	0	0.5	0	0	0	0
miR-183-3p	1	0	2.1	0	1.2	1.6	1.1	0.5	0.6	0.5	0.5	0
miR-187-3p	1	0	0	0	1.2	0.5	1.6	0.5	1.2	0	1	0.8
miR-1893	1	0	0	0	0	0	0	0	0	0	0	0
miR-192-3p	1	0	0	0	0	0	0	0	0	0	0	0
miR-1929-5p	1	0	1.4	0	0	0.5	0	1.5	1.2	0.5	0.5	0
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq
miR-193-3p	1	0	2.1	0	4.8	0	0	0	0.6	0.5	0.5	0.8
miR-1933-5p	1	0	0	0	0	0.5	1.1	0	0.6	0	0	0
miR-1953	1	0	0.7	0	0	0.5	0.5	1	0.6	1	1	0
miR-1956	1	1.9	2.1	4.7	2.4	1.6	6.9	10.2	4.7	7.1	10.4	6.1
miR-1966	1	0	0	2.4	0	0	0	0	0	0	0	0
miR-1969	1	0	1.4	0	1.2	1	1.1	1	1.2	0.5	0.5	1.5
miR-1b-3p	1	0	1.4	2.4	0	0.5	0	0	0	0	0	0
miR-200c-5p	1	0	0	0	0	0	0	0	0	0	0	0
miR-208b-3p	1	0	0	0	0	0	0	0	0	0.5	0	0
miR-215-3p	1	0	0	0	0	0	0	0.5	0	0.5	0	0
miR-23b-5p	1	0	0.7	2.4	0	0	0.5	0.5	0.6	0.5	1	0
miR-300-3p	1	0	0.7	4.7	2.4	0	1.6	0.5	0.6	2	0	0
miR-301b-5p	1	0	0	0	0	0	0	0.5	0.6	1	0	0
miR-3061-5p	1	1.9	1.4	0	1.2	1	2.1	0.5	0.6	2.5	1.5	0
miR-3064-5p	1	0	0	0	2.4	4.2	1.6	0	2.3	1	2.5	1.5
miR-3065-3p	1	0	0	0	1.2	0	0	0	0	0	0	0

miR-3066-5p	1	0	0	0	2.4	1.2	2.6	4.2	3	1.7	2.5	5	1.5
miR-3074-1-3p	1	5.8	0.7	0	0	2.4	2.1	1.6	3.6	2.3	1.5	1.5	3
miR-3076-5p	1	0	0	0	0	0	0	0.5	0	0.6	0	0	0
miR-3086-5p	1	1.9	2.8	0	0	1.2	1	2.1	1.5	1.7	0	2	1.5
miR-3092-3p	1	0	0	0	0	0	1	0	1	0.6	0.5	0	0
miR-3098-3p	1	0	2.1	0	0	0	1	0	1	1.2	0	0	0
miR-3098-5p	1	0	0.7	0	0	1.2	1.6	0	0.5	0	0	0	0
miR-31-5p	1	1.9	14.6	2.4	2.4	5.9	13.6	3.2	5.1	29.1	4.6	5.5	24.3
miR-3103-5p	1	0	0.7	4.7	4.7	1.2	0.5	3.2	0.5	1.7	2	0	1.5
miR-326-5p	1	1.9	1.4	0	0	1.2	0	0	0	0	1	0	0
miR-351-3p	1	0	0.7	0	0	1.2	0	0.5	0	0	0.5	0.5	0.8
miR-365-2-5p	1	0	0.7	0	0	2.4	3.1	1.6	1	0.6	0.5	0	0.8
miR-370-3p	1	0	0	0	0	0	0	0	0	0	0	0	0
gene	Control1 DESeq	Control2 DESeq	Control3 DESeq	LPS_04h1 DESeq	LPS_04h2 DESeq	LPS_04h3 DESeq	LPS_24h1 DESeq	LPS_24h2 DESeq	LPS_24h3 DESeq	LPS_28h1 DESeq	LPS_28h2 DESeq	LPS_28h3 DESeq	
miR-374c-3p	1	1.9	0	0	0	0	2.1	0.5	0.6	1	2.5	2.3	
miR-375-3p	1	3.8	1.4	0	0	0.5	2.1	1	1.2	0	1	0	
miR-3963	1	3.8	1.4	0	0	1.2	2.1	4.1	0	0.5	3	0	
miR-450b-3p	1	1.9	0	0	0	2.4	1	2.5	0	0.5	3	1.5	
miR-455-5p	1	0	1.4	18.9	13.1	3.7	3.7	1.5	5.8	3	3	5.3	
miR-466d-3p	1	1.9	2.1	4.7	0	3.1	2.1	2.5	3.5	1	0	2.3	
miR-466g	1	3.8	6.3	0	0	7.8	2.7	4.1	4.1	4.1	4	8.3	
miR-467a-3p	1	3.8	2.1	0	0	2.6	0.5	1.5	0.6	1	0	2.3	
miR-467b-3p	1	1.9	1.4	0	0	2.1	0.5	0	0.6	1	0	0.8	
miR-467c-3p	1	1.9	0.7	0	0	2.6	0.5	0.5	0.6	1	0	0.8	
miR-467d-3p	1	3.8	2.1	0	0	2.6	0.5	1.5	0.6	1	0	2.3	
miR-467e-5p	1	3.8	3.5	2.4	3.6	1	1.6	3.6	6.4	1	7	6.1	
miR-467f	1	1.9	0.7	0	0	2.1	0.5	0	0.6	1	0	0.8	
miR-500-5p	1	1.9	0	0	0	0	0	3	1.2	1.5	2	0.8	
miR-5100	1	0	0	2.4	0	0	1.6	0	0.6	1	1.5	0	

[illegible]