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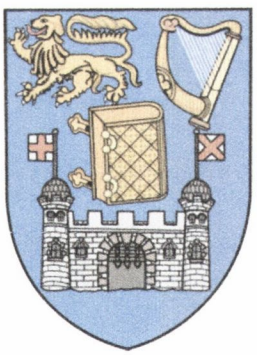
Regulation of the Immunomodulatory cytokine IL-10 by Twist2

Thesis submitted to the
University of Dublin
For the
Degree of Doctor of Philosophy

By
Mirjam W.M. van den Bosch, MSc



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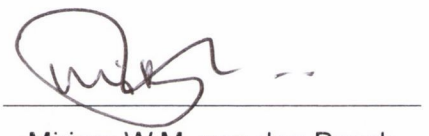

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ABBREVIATIONS

Abbreviation	Term
ACIN1	Apoptotic chromatin condensation inducer 1
AKT	Protein Kinase B
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AP-1	activator protein-1
APC	antigen-presenting cells
APS	ammonium persulfate
ARE	adenosine- and uridine rich elements
ATF1	activating transcription factor 1
ATP	adenosine 5'-triphosphate
β-TRCP	F-box/WD repeat-containing protein 1A
BCAP	B-cell adaptor for PI3K
bHLH	basic Helix Loop Helix
BMDM	bone marrow derived macrophage
BSA	bovine serum albumin
°C	degrees centigrade
C-terminal	carboxy-terminal
C57	C57 black 6/J (mouse line)
cAMP	3'5'-adenosine cyclic monophosphate
CARD	Caspase activation and recruitment domain
CaMK	Calmodulin-dependent protein kinase
CBP	CREB binding protein
cDNA	complementary DNA
C/EBP	CCAAT/enhancer-binding protein
CLR	calcitonin- like receptor
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding proteins
CSIF	cytokine synthesis inhibitory factor
Ct	threshold cycle
Da	Dalton
DAMP	damage associated molecular patterns
DC-sign	dendritic cell-specific ICAM3 grabbing non-integrin
DD	death domain
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
DTwist	Drosophila Twist
ECL	enhanced chemiluminescence reagent
EDTA	ethylenediaminetetraacetic acid

EGF	epidermal growth factor
eIF	eukaryotic initiation factor
ELK	ETS domain-containing protein
ERK	extracellular signal regulated kinase
FCS	foetal calf serum
FDA	U.S. *Food and Drug Administration*
Fgfr	fibroblast growth factor receptor-2
FKBP12	FK506-binding protein 12
FOXP3	forkhead box p3
FRB	FKBP12-rapamycin-binding
g	gram
G-protein	guanine nucleotide-binding proteins
GDP	guenosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GRB2	growth factor receptor-bound protein-2
GSK3	Glycogen synthase kinase 3
GST	glutathione S-transferase
GTP	guanosine 5-triphosphate
h	hour
HIF-1α	hypoxia-inducible factor 1α
HIV	human immunodeficiency virus
hToll	human target of rapamycin
HRP	horseradish peroxidase
HSCT	hematopoietic stem cell transplantation
IBD	inflammatory bowel disease
IBTK1	inhibitor of Bruton Agammaglobulinemia tyrosine kinase 1
IFN	interferon
IGF-1	Insulin-like growth factor 1
IKK	IκB kinase
IL	interleukin
IL-1R	IL-1 receptor
IL-10R	IL-10 receptor
IP	immunoprecipitation
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
IRS1	insulin receptor substrate 1
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
k	kilo
KO	knock out
L	litre
LAMP1	lysosomal-associated membrane protein

LKB1	liver kinase B1
LPS	lipopolysaccharide
LRR	leucine rich repeat
m	milli
M	molar
MAVS	mirochondrial antiviral-signalling protein
Maf	musculoaponeurotic fibrosarcoma
c-Maf	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)
Mal	MyD88 adaptor like
MAP	myelin associated protein
MAPK	mitogen activated protein kinase
MAPKAPK/MK	MAPK activated protein kinase
MAPKK/MEK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MARE	Maf recognition elements
MCF	Michigan Cancer Foundation
MEF	mouse embryonic fibroblast
MEF2	myocyte enhancer factor-2
MEKK	MEK kinase
mLST8	SEC13 protein 8
min	minute
miRNA	microRNA
MK	MAPKAP kinases
MNK	MAP kinase-interacting kinases
mRNA	messenger RNA
MSK	mitogen and stress activated protein kinase
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex
MyD88	myeloid differentiation primary response
gene (88)	
n	nano
NBD	nucleotide-binding domain
N-terminal	amino-terminal
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
NLK	NEMO like kinase
NLR	NOD-like receptor
Nlrp3	PYD domans-containing protein 3
Nod-1	nucleostide-binding oligomerization domain
OTU domain,	ubiquitin aldehyde binding
P65	RelA
P100	NF-κB2
P105	NF-κB1

PA	phosphatidic acids
PAGE	polyacrylamide gel electrophoresis
PAMP	Pathogen –associated molecular pattern
PBS	phosphate buffered saline
PBX	pre-B-cell leukemia transcription factor
PC	phosphatidycholine
PCR	polymerase chain reaction
PDCD4	programmed cell death protein 4
PDK	phosphoinositide-dependent kinase
PI3K	phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKB	protein kinase B (aka Akt)
PKC	protein kinase C
PLD	phospholipase
PMA	12-O-tetradecanoylphorbol-13-acetate
Pol	polymerase
PRAS	proline-rich AKT substrate
PREP	PBX-regulating protein
Pri	primary
PRR	Pattern recoginatition receptors
PTEN	Phosphatase and tensin homolog
qPCR	quantitative polymerase chainreaction
Raptor	regulatory-associated protein of mTOR
RH	Rel homology
Rictor	rapamycin-insensitive companion of mTOR
RIG-I	retinoic acid-inducible gene 1
RIP	receptor interacting protein
RLR	Retinoic acid-induceble gene I – like receptors
RNA	ribonucleic acid
rRNA	ribosomal RNA
RPOL	RNA Polymerase II
RSK	ribosomal S6 kinase
RT-PCR	reverse transcriptase PCR
Runx	Runt-related transcription factor
s	second
S6K1	S6 kinase 1
SAPK	p38 kDa stress activated protein kinase
SARM	Sterile a and HEAT-Armadillo motif
SDS	sodium dodecyl sulphate
SEC13	Protein SEC13 homolog
SHIP	protein interaction of Scr homology 2 domain- containing inositol phospatase
siRNA	small interfering RNA
SLE	systemic lupus erythematosus
snRNA	small nuclear RNA

SOC3	Suppressor of cytokine signalling 3
SOS	son of sevenless
SP	specific protein
STAT	signal transducer and activator of transcription
TAB	TAK1 binding protein
TAK1	Transforming growth factor β (TGF-β)-activated kinase 1
TANK	TRAF family member-associated NFKB activator
TBK	TANK-binding kinase 1
Th	T-helper cells
TBS	tris buffered saline
TBST	tris buffered saline/TWEEN
TEMED	tetramethylethylenediamine
TF	transcription factor
TIR	Toll-Interleukin 1 Receptor
TK	tyrosine kinase
TLR	Toll-like receptor
TMEM	transmembrane protein
TNF	tumour necrosis factor
TOR	target of rapamycin
Tpl	tumor progression locus
TRAF	tumor necrosis factor receptor-associated factor
tRNA	transfer RNA
TRADD	tumor necrosis factor receptor type 1-associated death domain protein
TRIF	TIR-domain-containing adapter-inducing interferon-β
TTP	tristetrapolin
UTR	untranslated regions
UV	ultraviolet
V	volts
v/v	volume to volume
w/v	weight to volume
μ	micro
x g	units of gravity
YB	Y-Box binding protein

ABSTRACT

Dysregulation of cytokines can lead to infectious and inflammatory diseases. The anti-inflammatory cytokine IL-10 is known to control pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12. The regulation of IL-10 is complex and it has been shown previously that the human tumour suppressor PDCD4 is a negative regulator of IL-10 production. Following LPS stimulation, PDCD4 acts as a molecular switch whereby its degradation results in increased IL-10 production. In this study, I have examined in detail the regulation of PDCD4 in LPS- treated macrophages. I have shown that the mTOR pathway and proteosomal degradation are involved in LPS- induced PDCD4 degradation using rapamycin, an mTOR inhibitor, or the proteosomal inhibitors MG132, both of which block this response. Inhibition of PDCD4 degradation by rapamycin also decreased IL-10 and c-Maf expression, a transcription factor critical for IL-10 induction. I have also found evidence through immunoprecipitation, of interaction between PDCD4 and Twist2. Through chromatin immunoprecipitation (ChIP) and oligonucleotide pull down assays, I have demonstrated a new regulatory role for PDCD4 and Twist2 in LPS-induced IL-10 production by showing increased binding of Twist2 to the c-Maf promoter. The PDCD4-Twist2 complex is inhibitory for LPS-induced c-Maf and IL-10 expression. A new mechanism in the complex regulation of the anti-inflammatory cytokine IL-10 has therefore been discovered.

CHAPTER 1

INTRODUCTION

1.1 The immune response

1.1.1 The immune system

The immune system is a highly evolved biological process within an organism to protect against diseases caused by a wide variety of pathogens such as viruses, bacteria and parasitic worms. The cells of the immune system recognise and clear the body of pathogens, causing minimal damage. An efficient and specific response against a variety of pathogens is required. The immune system has historically been divided into two parts, innate and adaptive immunity. Following infection or injury these two systems coordinate distinct phases of the immune response: initial detection, inflammation, pathogen clearance and finally a resolution phase.

When bacteria, viruses and other infectious agents enter the body, the cells of the innate immune system react first. Cells such as dendritic cells or macrophages can present antigen to antigen-specific lymphocytes to identify the invading pathogen. This leads to production of a specific immune response, which includes the production of antibodies to eliminate the invading pathogen, and cell-mediated immunity. This adaptive immune response is slower than the innate immune response, however it is capable of developing an 'immunological memory', so that it can react faster to subsequent pathogen attack (Iwasaki and Medzhitov, 2010).

1.1.2 The innate immune response

The ability to transduce signals from the environment is important to control diverse cellular functions including growth, division, and differentiation. In the immune system, intracellular signalling is required to allow a cell to mount the correct response once it

has encountered an invading microorganism. The principle cells of the innate immune system, such as macrophages and dendritic cells, have the ability to mount the correct response after recognising specific classes of molecules derived from the pathogen (pathogen-associated molecular patterns or PAMPs) (Janeway, 1989b, Medzhitov and Janeway, 1997). These cells have a series of receptors referred to as pattern recognition receptors (PRRs), to recognise the different pathogens. These receptors enable the immune system to differentiate infectious non-self from non-infectious self. The signalling pathways activated by the PRRs in the innate immune response result in recruitment of more cells of the immune system from the lymphoid organs to the site of infection to limit further damage. The innate immune response will eventually lead to the induction of the adaptive immune response through the activation of B- and T-cells by antigen-presenting cells (APC).

1.1.3 PRRs

Triggering of the innate immune response starts by the binding of specific PAMPs to PRRs on the innate immune cell. An example of a PAMP is lipopolysaccharide (LPS), a component that is found on the cell wall of gram-negative bacteria. PRRs can be expressed on cell surfaces, in intracellular compartments, or as secreted receptors released into the tissue fluids and bloodstream (Medzhitov and Janeway, 1997). In the innate immune system, macrophages and dendritic cells express a variety of PRRs, to recognise different PAMPs. Each PRR recognises a particular PAMP, for example the PRR Toll-like receptor 4 (TLR4) recognises LPS (Poltorak et al., 1998) (Figure 1.1). Usually the recognition of a PAMP by a PRR is one of the first signs of an infection (Janeway, 1989b). However, PRRs can also respond to damage associated molecular patterns (DAMPs), endogenous molecules released from damaged cells (Janeway, 1989a). When stimulated, PRRs activate intracellular

signalling cascades that ultimately induce the required cellular response against the particular pathogen. Such responses include phagocytosis, apoptosis and the production of pro- and anti-inflammatory cytokines. PPRs can be divided into different groups based upon the type of response they induce but also based upon their subcellular location and the range of PAMPs they recognise. Four different classes of PRRs that are the best-understood are: Toll-like receptors (TLRs), Nod-like receptors (NLRs), C-type lectin receptors (CLRs), and CARD-containing helicases such as the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (Palsson-McDermott and O'Neill, 2007, Kawai and Akira, 2010). TLRs are the most studied family of PRRs. They sense a diverse range of PAMPs and will be described in detail below, as they are a major focus of this thesis.

NLRs are a family of intracellular receptors and contain a leucine rich repeat (LRR) domain, a nucleotide-binding domain (NBD) and a caspase-1 activation and recruitment domain (CARD) or PYRIN domain (PYD) (Fritz et al., 2006). Their key role is to recognise both intracellular microbial motifs and danger motifs like DAMPs. NLRs can be divided into, the nucleotide-binding oligomerization domain 1 (Nod-1) like group and the NACHT, LRR and PYD domain-containing protein 3 (Nlrp-3) like group. Nod-1 like receptors are known to recognise bacterial PAMPs in the cytoplasm, and activate early-phase nuclear factor κB (NF-κB), whereas Nlrp3 receptors recognise a wide range of PAMPs such as LPS and fungal zymosan in the cytoplasm resulting in the activation of the proteolytic enzyme caspase-1. Caspase-1 is responsible for cleavage of pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) into their active forms (Creagh and O'Neill, 2006). IL-1 and IL-18 are potent pro-inflammatory cytokines known to be involved in pathogen clearance.

RLRs or RIG-I-like helicases are located intracellularly and recognise RNA from viral infections. There are three RLRs, namely RIG-I, MDA-5 and LGP-2, which sense

dsRNA. However, LGP-2 lacks the CARD domain, which is necessary to induce a cellular response. LGP-2 is needed for effective RIG-I and MDA-5-mediated antiviral responses (Schlee, 2013, Satoh et al., 2010). In 2006, it was discovered that RIG-I receptors signal through mitochondrial antiviral-signalling protein (MAVS), a central player in the activation of the TBK1 complex via a process requiring TRAF3-dependent formation of K63-linked ubiquitin chains. The TBK1 complex phosphorylates the transcription factors interferon regulatory factor 3 (IRF3) and IRF7, leading to induction of IFN-α and IFN-β (Saha et al., 2006), in a similar pathway to that seen in TLR signalling.

CLRs are a family of PRR, which bind to carbohydrate through their carbohydrate motif in a calcium-dependent manner. The prototypes of this group are Dectin-1, Dectin-2 and dendritic cell-specific ICAM3 grabbing non-integrin (DC-sign), which recognise zymosan from fungal infections. Dectin-1 is the key receptor for β-glucan, a polysaccharide found in fungi (Dennehy and Brown, 2007, Saijo et al., 2007). The CLRs can be found on both the cell membrane and in the cytoplasm. Signalling through CLRs leads to the production of cytokines and chemokines. Recent studies have demonstrated that CLRs play an important role in the development of ant-inflammatory responses and the maintenance of host immune homeostasis (Yan 2013). It has also been found that Dectin-1 regulates IL-10 production through the same mitogen and stress activated protein kinase (MSK) induced in the LPS response to limit inflammatory cytokine production (Elcombe et al., 2013).

TLRs are the key immune signalling receptors investigated in this study, and thus will be discussed in further detail in the following section.

1.2 TLRs

As stated above, the most studied group of PRRs in recent years is the TLRs. This thesis concerns the action of a particular TLR, TLR4, which senses LPS. The TLRs are all transmembrane receptors and are front-line sensors of infection. They are therefore initiators of innate immunity and are also a critical link between innate and adaptive immunity. This is because they are involved in antigen processing and presentation, co-stimulation of T-cells (Th1, Th2 and T-reg cells) and B-cells. They are also involved in the activation of natural killer (NK) cells. Through TLR activation, multiple genes are induced resulting in the production of cytokines and chemokines, which direct the destruction of invading pathogens. TLRs are central components of the innate response and are mainly expressed on macrophages and dendritic cells.

1.2.1 Discovery and diversity of TLRs

The TLRs were identified after the discovery of Toll. Toll was originally identified in the fruit fly *Drosophila*, named by Nusslein-Volhard in 1985 (Anderson et al., 1985, St Johnston and Nusslein-Volhard, 1992). The protein was established as an essential receptor in the dorso-ventral patterning in developing embryos (Hashimoto et al., 1988). The sequence of the Toll protein in the fruit fly is highly homologous to the human Type 1 IL-1 receptor and the first human form of *Drosophila* Toll protein was found in 1997. In 1997 and 1998, several reports were published on a human homologue of Toll (hToll) and information about its function in humans was uncoverd. Firstly, hToll was shown to signal activation of adaptive immunity via induction of the co-stimulatory molecule B7.1. It was also revealed that hToll is a type I transmembrane protein with an extracellular domain consisting of a leucine-rich repeat (LRR) domain, and a cytoplasmic domain homologous to the cytoplasmic

domain of the human interleukin (IL)-1 receptor, which was termed the Toll-IL1 receptor resistance (TIR) domain (Medzhitov et al., 1997). In this paper, Medzhitov et al. (1997) also showed that Drosophila Toll and the IL-1 receptor signal through the NF-κB pathway. They showed that a constitutively active mutant of human Toll transfected into human cell lines could induce the activation of NF-κB and the expression of NF-κB-controlled genes for the inflammatory cytokines IL-1, IL-6 and IL-8. The expression of the co-stimulatory molecule B7.1, which is required for the activation of naive T cells, was also induced, resulting in activation of the adaptive immune system. In 1998 another paper was published describing a family of human receptors structurally related to Drosophila Toll (Rock et al., 1998). This paper predicted the structural alignment of Toll-homology domains from various insect and human TLRs, the vertebrate IL-1 receptor and MyD88 factors by sequence-tagged site database analysis (Rock et al., 1998).

In total, thirteen TLRs have been identified to date in mice and ten in humans (Siednienko and Miggin, 2009). They each recognise different PAMPs derived from various microbial pathogens, including viruses, bacteria and fungi (Figure 1.1) (Akira et al., 2006). TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are located at the cell membrane, and sense bacterial ligands such as bacterial cell wall lipopeptides (TLR1, TLR2 and TLR6) (Alexopoulou et al., 2002, Takeuchi et al., 2001), flagellin (TLR5) (Hayashi et al., 2001) and LPS (TLR4) (Poltorak et al., 1998, Shimazu et al., 1999). TLR3, TLR7, TLR8 and TLR9 are located intracellularly, in the endosomes and lysosomes, and are specialized to recognise nucleic acids such as double-stranded RNA (TLR3) (Alexopoulou et al., 2001), single-stranded RNA (TLR7 and TLR8) (Heil et al., 2004, Hemmi et al., 2000) and unmethylated CpG sequences in DNA (TLR9) (Hemmi et al., 2002). The ligand for TLR10 remains unclear although the sequence of this receptor is known to be most similar to TLR1, and therefore TLR10 might heterodimerize with TLR2. It cannot be found in mice which makes study difficult,

although it does have structural homology with TLR1 and TLR2 (Nyman et al., 2008). TLR11, TLR12 and TLR13 are only found in mice. Mouse TLR11 has been shown to detect a component of uropathogenic bacteria (Zhang et al., 2004) and in cooperation with TLR12 to bind to the Toxoplasma gondii profilin protein (Andrade et al., 2013, Koblansky et al., 2013). TLR13 has recently been shown to recognize bacterial ribosomal RNA (rRNA) (Oldenburg et al., 2012, Hidmark et al., 2012).

TLR4 is located on the cell membrane. Following binding to LPS, TLR4 can activate signalling at the membrane. Unlike other plasma membrane TLRs, following activation, TLR4 can be internalised to endosomes from which it can also signal. TLRs can recognise PAMPs through either direct interaction or intermediate PAMP-binding molecules. For example, TLR4 needs MD2 and CD14, an intermediate PAMP-binding molecule, to recognise LPS (Kim et al., 2007).

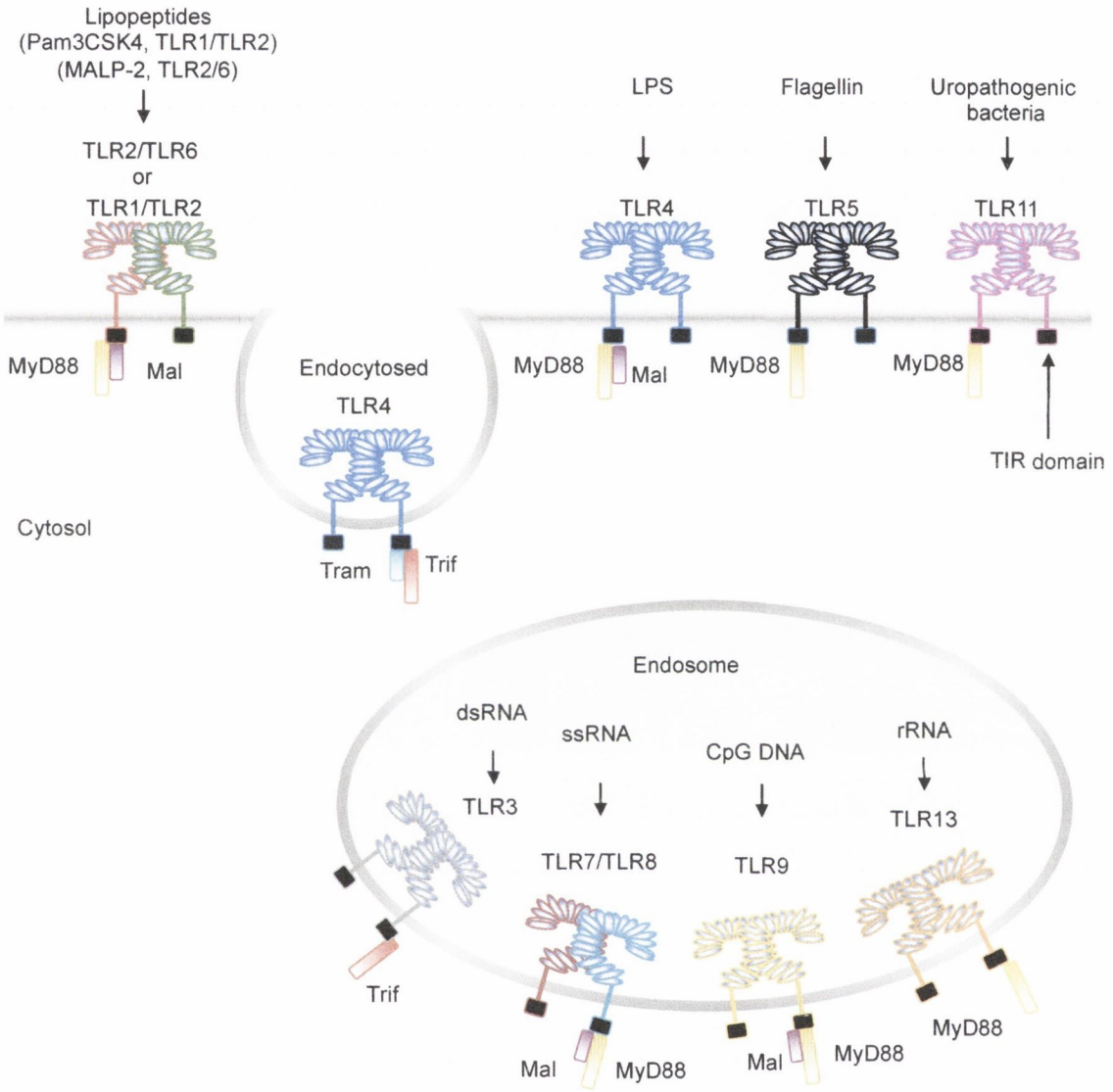
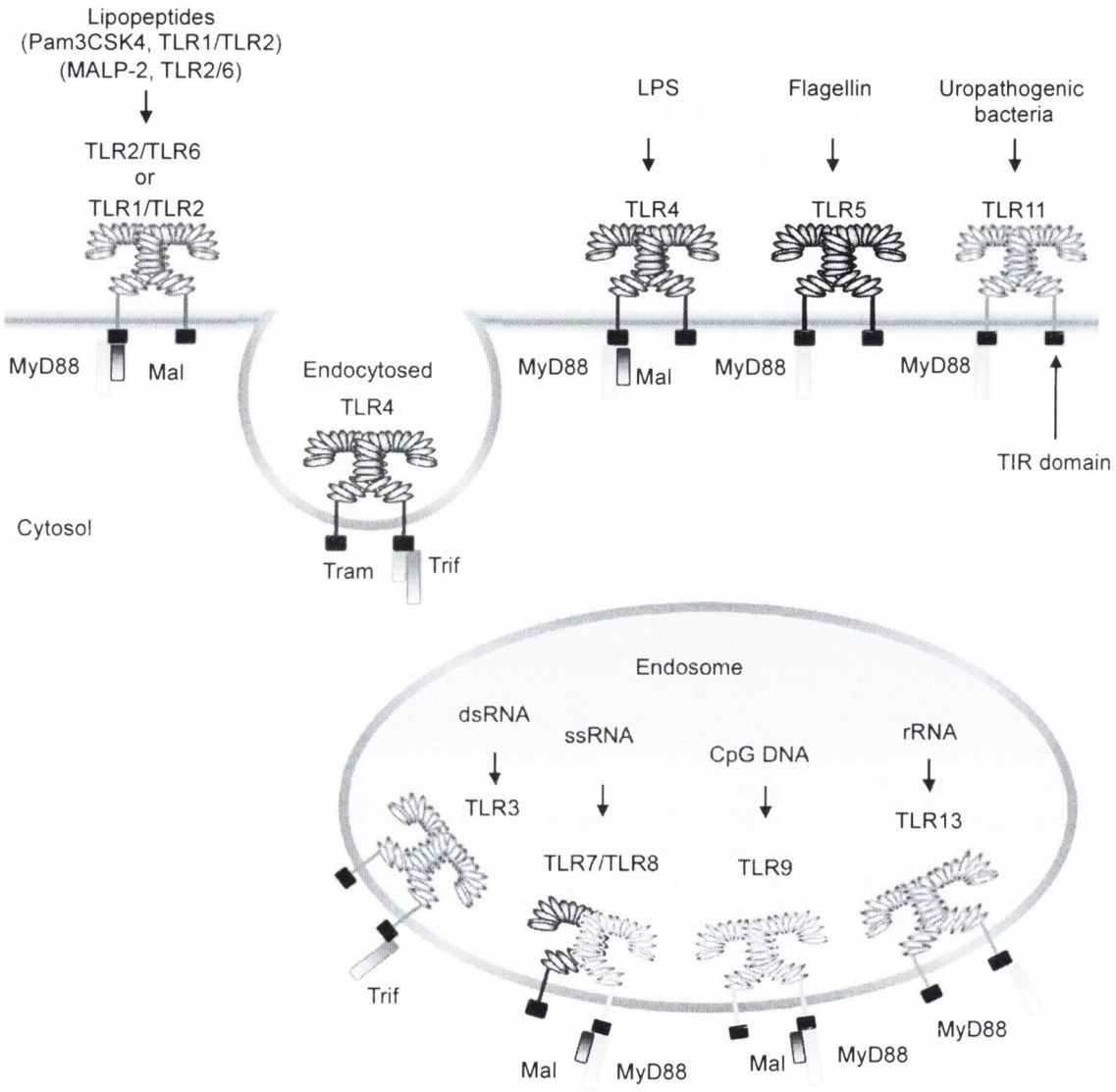


Figure 1.1 – The TLRs with their stimuli and adaptor molecules.

TLR5, TLR11 and TLR4 and the heterodimers TLR1/TLR2 or TLR2/TLR6 bind to their respective ligands at the cell surface, whereas TLR3, TLR7/8, TLR9 and TLR13 are located in the endosome where they sense microbial and host-derived nucleic acids. TLR4 can localize to both the plasma membrane and the endosomes. Most of the TLRs signal via the adaptor molecule MyD88, however TLR3 signals via TRIF. TLR4 is unusual in that it signals via both MyD88 and TRIF.



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1.2.2 TLR signalling pathways

1.2.2.1 Early events in TLR signalling – adaptor molecules.

Following ligand binding, TLRs dimerize and undergo conformational changes required for the recruitment of TIR domain-containing adaptor molecules. These include Myeloid differentiation primary response gene 88 (MyD88), the first member of the TLR adaptor molecules to be discovered (Wesche et al., 1997, Muzio et al., 1997), TIR domain-containing adaptor-inducing interferon-beta (TRIF) (Oshiumi et al., 2003, Yamamoto et al., 2002b), MyD88 adaptor like (Mal), Sterile α and HEAT-Armadillo motif (SARM), a negative regulator of TRIF (O'Neill et al., 2003a, Carty et al., 2006), and the more recently discovered B-cell adaptor for PI3K (BCAP) (Troutman et al., 2012).

1.2.2.1.1 MyD88

It was discovered that MyD88 knockout mice were completely resistant to endotoxic shock but macrophages stimulated with LPS and Poly I:C still induced residual activation of NF-κB, JNK and p38 in a MyD88-independent pathway (Shimada et al., 1999). Therefore, TLR signalling can be either MyD88-dependent or TRIF-dependent. The MyD88-dependent signalling pathway is used by all TLRs except TLR3 and is responsible for NF-κB and mitogen-activated protein kinase (MAPK) activation, which controls the induction of cytokines and chemokines (Shimada et al., 1999, Yamamoto et al., 2002a).

1.2.2.1.2 Mal

In the case of TLR4 and TLR2, a second TIR domain-containing adaptor protein, Mal (also known as TIRAP), assists in the recruitment of MyD88 to the TLR. It was originally shown not to be required for signalling from endosomal compartments (Fitzgerald et al., 2001, Horng et al., 2001, Yamamoto et al., 2002a). Mal acts as an electronegative bridging adaptor protein for MyD88 to bring it into the signalling complex after activation of TLR2 and TLR4 (Dunne et al., 2003). However, it was unclear how MyD88-dependent responses are initiated by endosomal TLRs and how the Myddosome, a complex of MyD88 with kinases of the IRAK family, can assemble from multiple locations to control pro-inflammatory cytokine expression. A recent study discovered that Mal is in fact an adaptor molecule playing a role in bacterial detection at the plasma membrane and virus detection by TLR9 in endosomes (Bonham et al., 2014). Evidence was predicted for Mal being a bridging adaptor for all TLRs that signal via MyD88. A role in TLR9 signalling had been previously missed because Mal is required for physiological ligand concentrations. Mal binds to lipids that direct it to different organelles, allowing it to function in multiple compartments of the cell (Bonham et al., 2014). This was discovered using confocal microscopy and live time-lapse microscopy in primary bone marrow-derived macrophages (BMDM) expressing a GFP-tagged lipid-binding domain of Mal (Bonham et al., 2014).

1.2.2.1.3 TRIF

TLR3 does not signal via MyD88 but instead uses the TRIF-dependent signalling pathway. TRIF is also known as TICAM1, a third TOR domain that was characterized between 2002 and 2003 (Yamamoto et al., 2002b, Hoebe et al., 2003, Yamamoto et al., 2003a). Downstream of TLR3, TRIF is required for the activation of the

TBK1/IRF3 pathway, which is necessary for the induction of IFN-β and IFN-inducible genes. In addition, TLR3 activates the NF-κB and MAPK pathways, leading to the production of cytokines such as IL-12p35 (Adachi et al., 1998, Kawai et al., 1999). TLR4 can also signal via TRIF to activate the TBK1/IRF3 pathway, however recruitment of TRIF to TLR4 also requires the TRIF-related adaptor protein TRAM (Yamamoto et al., 2003b, Fitzgerald et al., 2003).

1.2.2.1.4 TRAM

TRIF-related adaptor molecule (TRAM), also known as TICAM2, is unique among TIR-containing adaptors as it is only required for TLR4 signalling. It is specific in its recruitment to endosomes following TLR4 ligation. Mal and TRAM have been described as 'bridging adaptors' responsible for recruiting MyD88 and TRIF to the activated TLR2 and TLR4 complexes. TRAM is transiently phosphorylated by PKCε on Ser16 in an LPS-dependent manner. This was found through a mutation in the Ser16 phosphorylation site of TRAM. It was also found that TRAM-dependent activation of IRF3 and induction of the chemokine RANTES, were attenuated in PKCε-deficient cells (Rowe et al., 2006, McGettrick et al., 2006). Recently an interaction between TRAM and TRAF6 was discovered through immunoprecipitation as well were observed to colocalize together using confocal microscopy. Using TRAM-deficient macrophages, it was found that there was a significant reduction of interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and RANTES production compared to wild-type macrophages confirming TRAM/TRAF6 activation of the inflammatory response by TLR4 (Verstak et al., 2014).

1.2.2.1.5 BCAP

BCAP has been demonstrated to serve as an adaptor linking the B-cell receptor and CD19 to activation of Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) (Inabe and Kurosaki, 2002, Okada et al., 2000). However, recently it has been demonstrated that BCAP can be linked to TLRs and PI3K through the TIR domain (Troutman et al., 2012). This paper shows that BCAP regulates activation of NF-κB and associates with Mal and MyD88. In addition BCAP is required for TLR-mediated activation of PI3K/Akt signalling (Troutman et al., 2012).

1.2.2.2 MyD88-dependent signalling downstream of TLR4

The crystal structure of the TLR4-MD2 complex shows that MD2 binds to the concave surface of the N-terminal and central LRR domains of TLR4 and is essential for recognising LPS (Kim et al., 2007). In response to TLR4-MD2-CD14 complex stimulation by LPS, MyD88 and Mal associate with the cytoplasmic TIR domain of TLR4 and allow the recruitment of the IL-1 receptor (IL-1R)-associated kinase (IRAK) 4 (IRAK4), followed by IRAK1 and IRAK2. All the IRAKs contain an N-terminal death domain (DD). This DD mediates the interaction with MyD88 (Neumann et al., 2008). IRAK4 phosphorylates both IRAK1 (on Thr209 and Thr387) and IRAK2, which leads to autophosphorylation of IRAK1 (Burns et al., 1998, Wesche et al., 2013). This allows the release of IRAK1 and IRAK2 from MyD88 (Kawagoe et al., 2008, Jiang et al., 2002). IRAK1 and IRAK2 then interact with TRAF6 and induce TRAF6 ubiquitinylation. On TRAF6, the ubiquitin chains are Lys63-linked and are proposed to act as a scaffold to recruit other signalling proteins, such as transforming growth factor β (TGF-β)-activated kinase 1 (TAK1) followed by IκB kinase (IKK) complexes downstream of TAK1 (Cao et al., 1996). Lys63-linked ubiquitinylation is not limited to

TRAF6, as other proteins such as IRAK1 in the TRAF complex may also become ubiquitinated. TRAF6 can also activate the transcription factor IRF5, leading to IFN production (Negishi et al., 2006), but this can only be found after TLR7 or TLR9 stimulation.

The interaction of K63-linked polyubiquitin chains with the TAK1 complex (via the subunits TAB2/TAB3) induces a conformational change in TAK1 and allows TAK1 to auto-phosphorylate its activation loop and become active to other substrates (Wang et al., 2001). The presence of TAK1 is essential for the activation of MEK3/6 and MEK4/7, which activate p38 MAPK and c-jun N-terminal kinases 1/2/3 (JNK1/2/3) and IKK respectively. The IKK complex then phosphorylates IκBα, which stimulates proteasomal degradation of IκBα. This releases NF-κB and allows it to translocate to the nucleus where it binds to the promoters of multiple pro-inflammatory genes (Figure 1.2). NF-κB is a vital participant in regulating the transcription of genes involved in immunity and inflammation (O'Neill et al., 2003b).

1.2.2.3 TRIF-dependent signalling downstream of TLR4

As mentioned previously, TLR3 signals via the TRIF-dependent pathway while TLR4 can use both TRIF and MyD88. Like MyD88, TRIF can also associate with the TLR TIR domain, however this leads to a recruitment of TRAF3 and TRAF family member-associated NF-κB activator (TANK). TANK forms a bridge to (TANK)-binding kinase 1 (TBK1) and IKKε, which is responsible for the phosphorylation of the transcription factors IRF3/7 (Kawai and Akira, 2010). Phosphorylation of IRF3/7 allows their translocation to the nucleus to bind the interferon-β (IFN-β) promoter, resulting in the production of IFN-β, IFN-α and subsequent induction of IFN-inducible genes (Figure 1.2) (Sato et al., 2003). TRIF-dependent signalling can also lead to the activation of

NF-κB and MAPK signalling. Activation of NF-κB in TRIF-dependent signalling is complex and may occur via several different pathways. TRIF contains three typical TRAF6-binding domains, which mediate interaction with TRAF6 (Sato et al., 2003). It is also known that TRIF contains a Rip homotypic interaction motif that mediates interaction with receptor interacting protein-1 (RIP-1), and that RIP-1 converges with the IKK complex (Meylan et al., 2004) together with Pellino-1 and Tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD). These signalling pathways ultimately lead to the phosphorylation and activation of several proteins that stimulate the production of cytokines and chemokines.

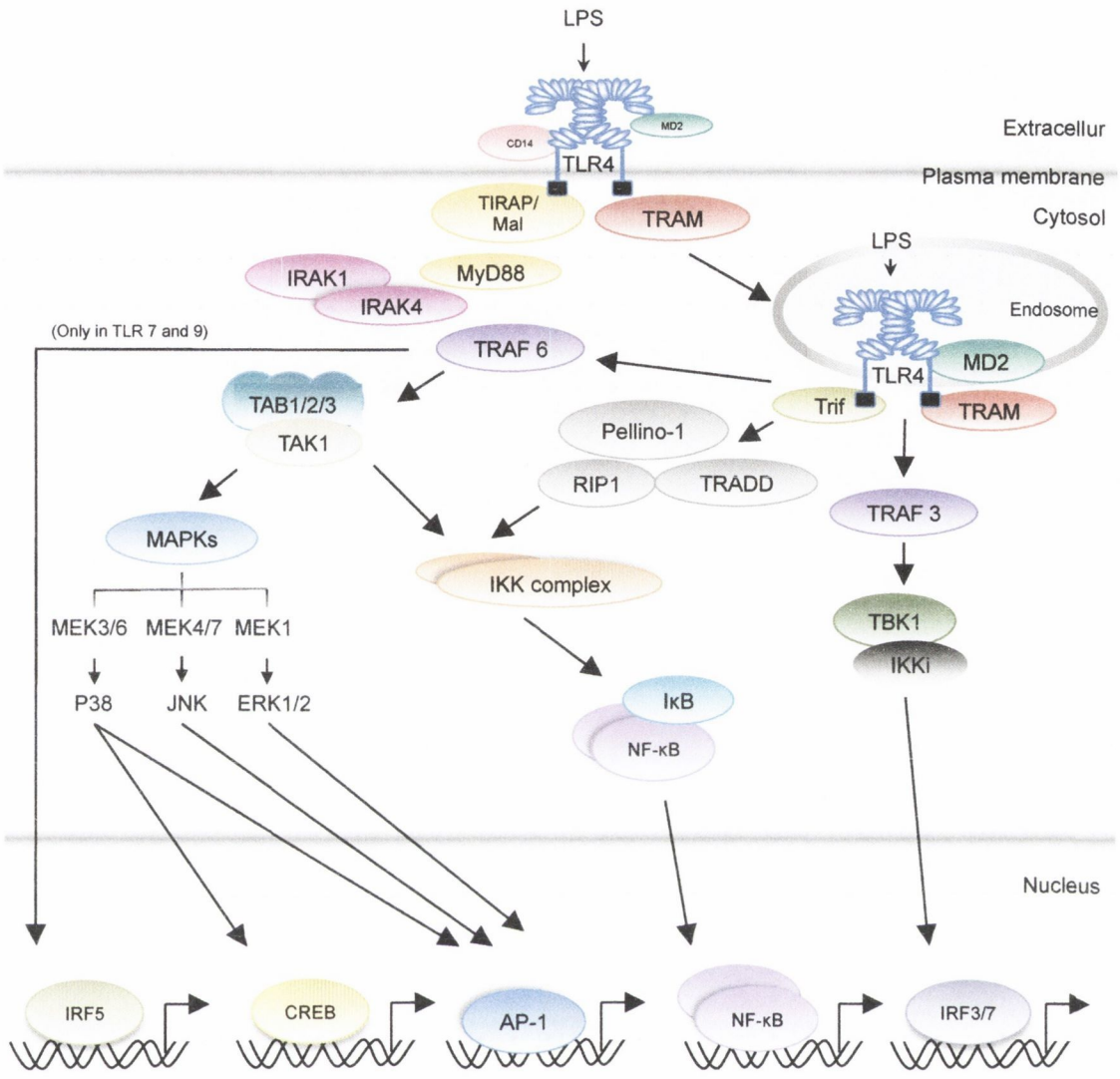


Figure 1.2 – Downstream signalling of TLR4 receptor in response to LPS.

In response to LPS the TLR4 receptor activates the MyD88-dependent and TRIF-dependent pathways. TIRAP/Mal and Tram are required for the activation of MyD88- and TRIF-dependent pathways, respectively. The activation of MyD88 leads to the recruitment of IRAK1/4 and TRAF6. TRAF6 activation recruits TAK1 and its subunits TAB2/TAB3 via K63-linked-ubiquitination. The activated TAK1 complex then activates the MAP kinase pathway, which results in the phosphorylation of the MAP kinases and activation of AP-1. Activated TAK1 can also activate the IKK complex (IKKα, IKKβ and IKKγ/Nemo), which phosphorylates IκBα. When the proteasome pathway removes IκB, this allows NF-κB to translocate to the nucleus. The activation of the TRIF-dependent pathway leads to recruitment and activation of TBK1 and IKKε, which are responsible for the phosphorylation of IRF3. TRIF also interacts with TRAF6 and RIP1, which mediate NF-κB activation. Both of these pathways lead to cytokine and chemokine production.

1.2.3 MAPK

Several MAPKs can be activated by TLRs. The MAPK cascades are key regulators of many cellular processes including survival, proliferation and differentiation. When regulation of a MAPK cascade is disturbed there can be serious consequences, for instance, mutations that constitutively activate extracellular signal regulated kinase (ERK1/2) result in cancer, while p38 MAPK has been implicated in autoimmune disorders (Cook et al., 2004). In mammalian cells, fourteen different MAPKs have been described and are divided into four major groups: ERK1 and 2; p38 MAPKs; JNKs; and atypical MAPKs such as ERK5, ERK3 and ERK8 (Raman et al., 2007). The most extensively studied MAPKs are ERK1/2, p38 kinases and JNK (Figure 1.3).

1.2.3.1 ERK1/2

ERK1 and ERK2 are activated in response to growth factors and cytokines (Hoshi et al., 1989, Ray and Sturgill, 1987). The ERK1/2 cascade is known as the classical mitogen kinase cascade and consists of the MAPKKKs: A-Raf, B-Raf, Raf-1 and c-Mos. Tpl2, which is also a MAPKKK, can activate this cascade in response to TLR signalling, followed by the MAPKKs, MEK1, MEK2 and the MAPKs, ERK1 and ERK2 (Macdonald et al., 1993, Kyriakis et al., 1992, Hallberg et al., 1994). Cell surface receptors can activate signals to the MAPKKKs through different isoforms of the small GTP-binding protein Ras (Castellano and Downward, 2011). When ERK1/2 are activated they are able to phosphorylate various substrates including nuclear substrates (MEF-2, c-Fos, c-Myc, STAT3), membrane proteins, cytoskeletal proteins and several MAPKAP kinases (MKs) including ribosomal S6 kinase (RSK), MSK and MAP kinase-interacting kinases (MNK) (Raman et al., 2007). ERK1/2 have general functions such as regulation of the cell cycle, cell differentiation, cell death,

development and tumorgenesis (Kim and Choi, 2010). However, macrophage specific functions have also been described. For example, the inhibition of ERK1/2 can reduce the production of pro-inflammatory cytokines such as TNF (Loniewski et al., 2007).

1.2.3.2 p38 MAPK

The 38 kDa stress activated protein kinase (SAPK), now known as p38α, was discovered in 1993 as a protein that was tyrosine phosphorylated in response to LPS in macrophages (Han et al., 1993). The p38 MAPK kinases are strongly activated by stress stimuli such as osmotic shock, ionizing radiation or cytokine stimulation (Han et al., 1994, Rouse et al., 1994, Freshney et al., 1994). Soon after the cloning of p38 α (Lee et al., 1994), three other isoforms were identified: p38β (Jiang et al., 1996), p38γ (Lechner et al., 1996) and p38δ (Goedert et al., 1997, Wang et al., 1997). Several MAPKKKs exist for the p38 MAPK cascade, including MEKK1-4, MLK2 and MLK3, ASK1, and TAK1. The MAPKKK required depends on cell type and stimulus-specific use. Once activated, the MAPKKKs can in turn activate the MAPKKs MEK3 and MEK6 (Enslen et al., 2000, Moriguchi et al., 1996). These MAPKKs can activate the different isoforms of p38 via phosphorylation of the TxY motif (corresponding to Thr180-Tyr182 in p38α) (Kim et al., 2008). MEK3 and MEK6 are highly specific for p38 activation as they do not activate ERK1, ERK2 or JNK (Enslen et al., 2000). Activated p38 kinase has been shown to phosphorylate and activate MK2 and 3 as well as MSK1 and 2. Many other substrates also exist for p38 such as the transcription factors ETS domain-containing protein (ELK1) (Cruzalegui et al., 1999) and SAP-1 (Janknecht and Hunter, 1997). In innate immunity, an important function of p38α kinase is to promote the production of IL-12, IL-18 and TNF-α by monocytes (Kang et al., 2008). However, this production was not related to other major signalling

pathways. This is because p38α deficiency in macrophages did not affect LPS-induced activation of other major signalling pathways (NF-κB, Jnk, and Erk). However, it has been shown to have a small inhibitory effect on LPS-induced AP-1 activity, and it significantly inhibited LPS-induced C/EBP-β and CREB activation (Kang et al., 2008). In the same study it was shown in vivo that p38α is involved in sepsis (Kang et al., 2008). Therefore, p38 is an important player in inflammatory responses

1.2.3.3 JNK

The c-Jun N-terminal Kinase (JNK) family, was independently cloned by two groups (Derijard et al., 1994, Kyriakis et al., 1994). JNK1, the founding member of the JNK family, was identified as a kinase which could be activated by both Ras and UV stresses to act on the c-Jun activation domain to potentiate its transcriptional activity (Hibi et al., 1993). In total, three JNK genes, each encoding multiple splice forms, have been identified. Their major gene products, known as JNK1, JNK2 and JNK3, have been shown to be crucial in a number of cell processes including regulated apoptosis and stress responses. They are also involved in the activation of the activation protein 1 (AP-1) transcription factor leading to production of the pro-inflammatory cytokine RANTES, IL-8 and GM-CSF (Bogoyevitch and Kobe 2006). It also has been demonstrated that PKB, also known as Akt, can phosphorylate JNK, however there is conflicting evidence as to the consequences this has for JNK activity (Shao et al., 2006, Kawamori et al., 2006). The differing consequences may be due to variation in signalling between cell types.

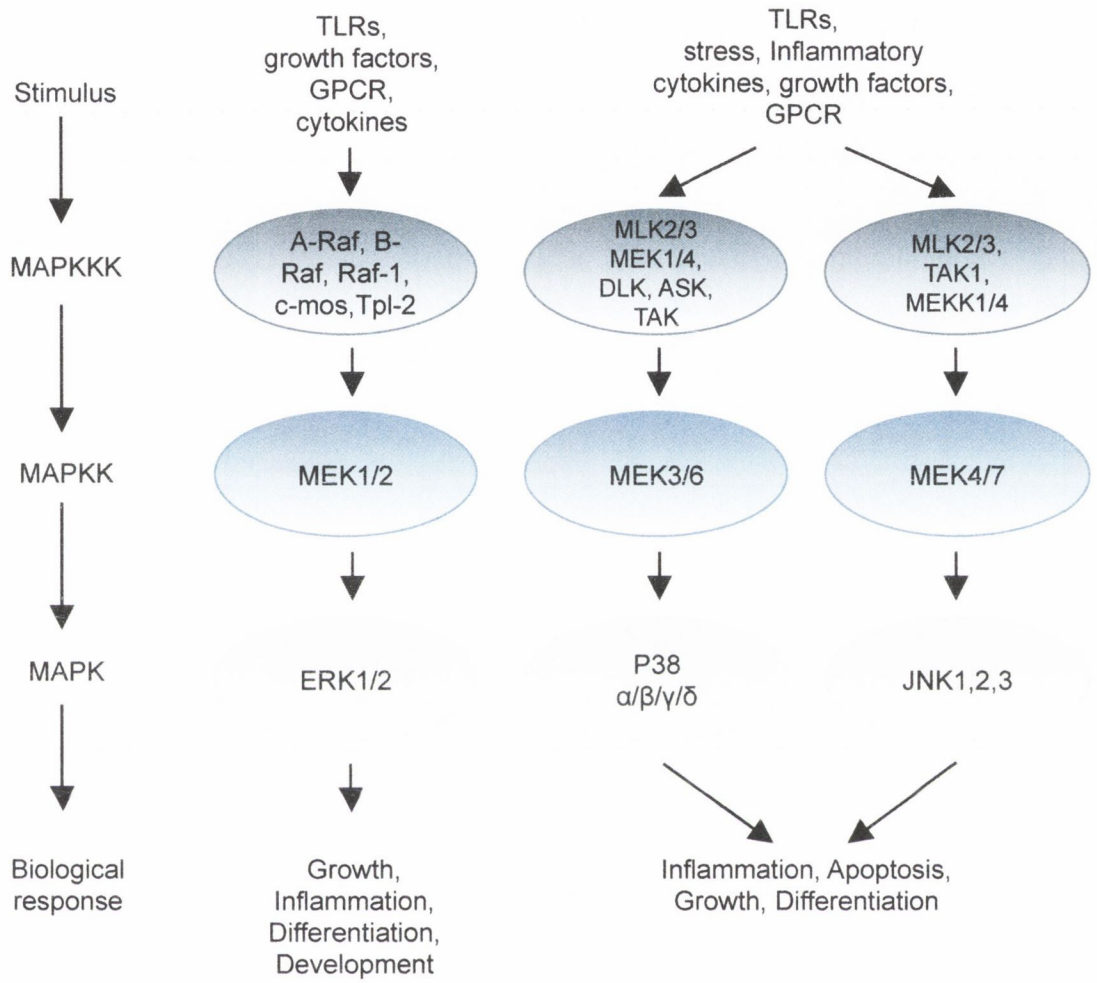


Figure 1.3 The most extensively studied MAPKs.

After stimulation, the MAPKKK can activate the MAPKK, which in turn can activate MAPK. Every MAPK pathway can activate different biological processes in the cell. Activation of TLRs, growth factors, G protein coupled receptors (GPCRs) and cytokines can activate the Raf proteins while TLRs activate Tpl-2, resulting in the activation of MEK1/2 and ERK1/2. The growth factors, GPCRs and cytokines, as well as stress, can also activate the MLKs, TAK and MEKK1/4 proteins resulting in the activation of MEK3/6 and p38 proteins or MEK4/7 and JNK proteins.

1.2.4 Transcription factors in the immune response

The inflammatory response is complex and tightly regulated at different levels of control. An important manner of regulation is controlling the transcription of pro-inflammatory or anti-inflammatory genes after TLR signalling. This transcriptional response is regulated by transcription factors, which recognise different motifs located in the upstream (5') promoter region of a gene. Binding of a transcription factor to a motif on a gene upregulates the rate of transcription of that gene, thus increasing its messenger RNA (mRNA) levels and protein production. In the immune response are some key examples of well documented transcription factors, including NF-κB, cyclic AMP response element binding proteins (CREB), glucocorticoid receptors, signal transducers and activators of transcription (STAT), AP-1, IRFs and c-Maf.

1.2.4.1 NF-κB

The main pro-inflammatory transcription factor activated by TLRs is NF-κB. There are five members of the NF-κB family: RelA (p65), RelB, c-Rel, and precursor proteins NF-κB1 (p105) and NF-κB2 (p100), which are processed into p50 and p52 respectively (Oeckinghaus et al., 2011). NF-κB transcription factors have (A) a highly conserved DNA binding and dimerization region and (B) a Rel homology (RH) domain (Hoffmann et al., 1999). NF-κB proteins can form heterodimers or homodimers in the cytoplasm, with the most common form being the p65/p50 heterodimer (Wietek and O'Neill, 2007). Normally, NF-κB dimers are in an inactive form in the cytoplasm and are bound to inhibitory IκB protein isoforms; IκBα, IκBβ and IκBγ (Li and Stark, 2002). Following TLR activation, signal transduction pathways lead to the activation of IKKs and degradation of the IκB proteins through phosphorylation of two specific serine residues (Ser32 and Ser36) on the IκB proteins (Ghosh et al., 1998). IκB proteins are recognised by the E3 ligase TrCP-containing ubiquitin ligase complex which then

catalyses the formation of Lys48-linked polyubiquitin chains at positions K21 and K22, thus targeting IκBα for degradation by the 26S proteasome (Karin and Ben-Neriah, 2000). The destruction of IκBα releases the NF-κB dimer to go to the nucleus where it binds to the sequence (5'-GGGACTTCC-3'), found upstream of the promoters of over 500 genes including for IL-6, IL-12, IL-1β, IL-2 and TNF-α. The expression of these genes leads to an inflammatory response. However, many other genes are regulated by NF-κB apart from pro-inflammatory cytokines (Karin and Ben-Neriah, 2000) For instance, it has been described that NF-κB p65 subunit as well as homodimers of NF-κB p50 binds to a κB site upstream of the *IL10* promoter in macrophages after LPS response suggesting a role in IL-10 expression (Cao et al., 2005, Cao et al., 2006).

1.2.4.2 IRFs

The IRFs are another group of transcription factors that can regulate pro-inflammatory cytokine transcription when certain family members are activated by TLRs. However, the IRFs also coordinate the expression of type I IFNs as well as chemokines (Juang et al., 1998). Viral infections detected by TLR3, TLR7/8, TLR9 and TLR4 can activate IRF3 and IRF7 (Doyle et al., 2002, Fitzgerald et al., 2003). In contrast, the activation of IRF5 is much more restricted whereby only certain viruses, including herpes simple virus type 1, have been shown to activate IRF5 (Barnes et al., 2002). IRF3 and IRF7 are activated via the TRIF-dependent pathway. TRIF promotes TBK1 activation leading to the phosphorylation of IRF3. Upon phosphorylation IRF3 forms homodimers and enter the nucleus where it can bind to the interferon sensitive response element (ISRE –GAAANNGAAG) upstream of promoters of interferon response genes. IRF3 can induce IFN-β and RANTES (regulated on activation normal T cell expressed and secreted) (Fitzgerald et al., 2003). TLR7/8 and TLR9 activate IRAK proteins and TRAF-6 leading to the activation of IRF5 and IRF7, which results in the production of IFN-α mRNA, in a MyD88 dependent manner (Hemmi et

al., 2002). The other IRF family members such as IRF1, IRF4 and IRF8 are not directly involved in TLR signalling but play diverse roles in immunity. For example, IRF1 induces other genes involved in an inflammatory response such as caspase-1 (cleaves pro-IL1β). IRF4 is involved in T-cell, B-cell and dendritic cell development and IRF8 controls T helper responses (Honda and Taniguchi, 2006). Although these IRFs are not directly activated by TLRs, they are activated by TLR-induced interferons.

1.2.4.3 CREB

CREB, a subfamily member of b-zip transcription factors, was the first transcription factor shown to be regulated by phosphorylation of Ser133 by the cAMP-dependent protein kinase PKA (Yamamoto et al., 1988). Several other kinases including MSKs, through p38 (Arthur and Cohen, 2000, Arthur et al., 2004) and Calmodulin-dependent protein kinase (CaMK) (Matthews et al., 1994, Sun et al., 1994) have also been shown to be able to phosphorylate CREB. Before activation, CREB is already bound to cyclic AMP response element (CRE) in the promoters of multiple target genes. However, CREB still needs to be activated before it has full transcriptional activity. Phosphorylation of CREB on Ser133 creates a binding site for the co-activator proteins CREB binding protein (CBP) and p300 (Radhakrishnan et al., 1997). Recruitment of CBP or p300 results in the transcriptional activation of CREB. CREB activity has been shown to regulate many genes including cyclin D2, known as the first cell cycle protein to be induced following stimulation through the T-cell receptor, B-cell receptor or cytokines. It was discovered that the regulation of cyclin D2 promoter by IL-2 is PI3K and CREB dependent. A CREB S133A mutant was also shown to reduce IL-2 induction of cyclin D2 promoter activity (White et al., 2006).

CREB also regulates anti-inflammatory IL-10 transcription after activation of TLR4 by LPS via p38 (Naqvi et al., 2014).

1.3 IL-10 and its negative regulation of the immune response

Inappropriate or uncontrolled production of cytokines through TLR signalling can lead to conditions such as septic shock. LPS is a potent inducer of the pro-inflammatory response, but is also able to induce multiple negative feedback regulators of inflammation, notably the immune-inhibitory cytokine interleukin-10 (IL-10), which is a key focus for this study.

1.3.1 IL-10 discovery and function

IL-10 is a potent immune modulatory cytokine and has been shown to be an essential and diverse coordinator of the immune response. IL-10 primarily operates as an anti-inflammatory cytokine in many model systems. IL-10 was first described in the late 1980s as cytokine synthesis inhibitory factor (CSIF), and was found in a mouse system (Fiorentino et al., 1989). Through the observation that two different strains of mice responded differently to *Leishmania major* (a parasite) infection the scientists at DNAX, a research institute, isolated functionally distinct T helper (Th)-1 and Th2 clones. Thereby, they provided evidence that these two subsets were mutually inhibitory. Knowledge of this inhibition led to the discovery that Th2 cells make IL-10 to suppress Th1 cell cytokine production (Fiorentino et al., 1989). However, various immune cells including Th2 cells, regulatory T cells, B cells, macrophages, mast cells, eosinophils and dendritic cells can also secrete IL-10. IL-10 has multiple mechanisms of action including suppression of antigen presentation, suppression of cytokine production and inhibition of inflammatory mediators (Figure 1.4). IL-10 can negatively regulate TLR-induced gene expression by decreasing the production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12 (Murray, 2006). This rapid

response is critical for the acute early pro-inflammatory response to pathogens, which is then limited by IL-10.

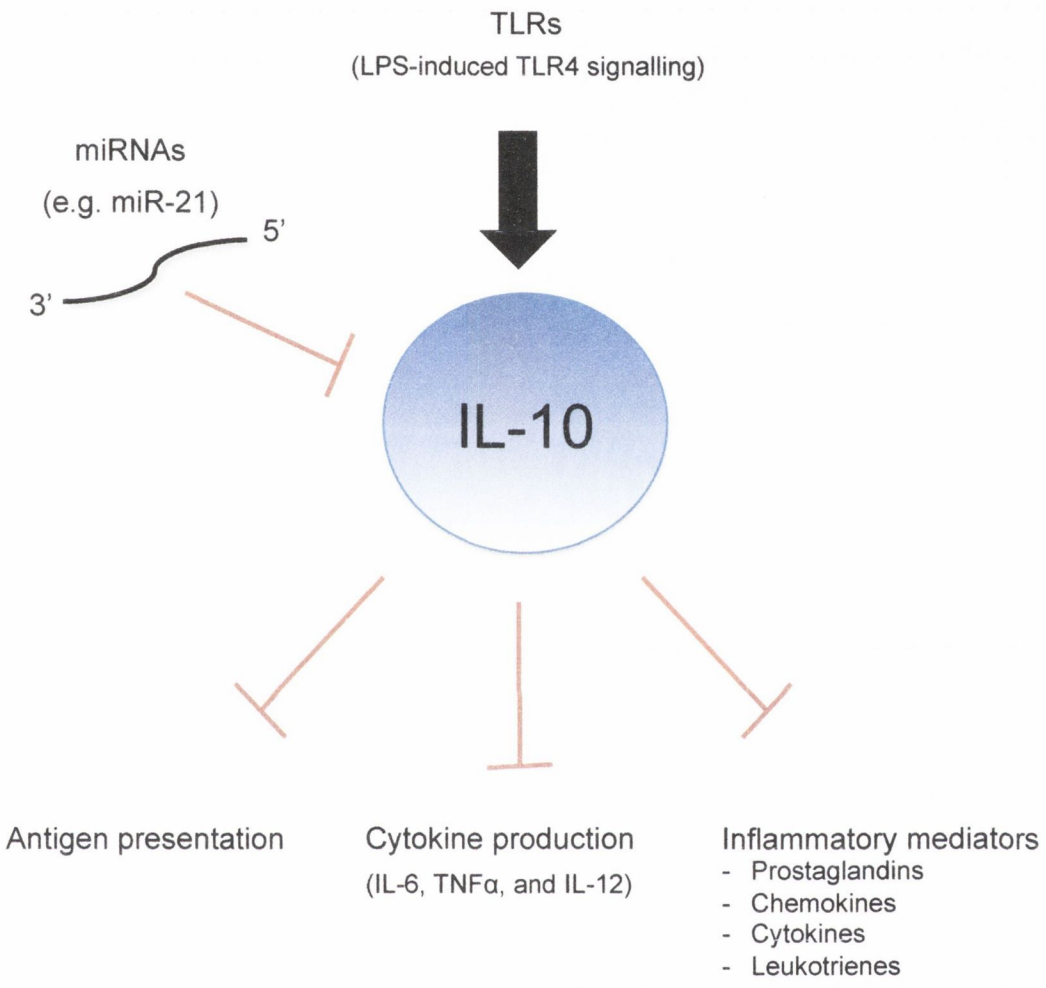


Figure 1.4 – IL-10 has multiple mechanisms of action.

IL-10 can be regulated in many ways, e.g. via miRNAs and TLR signalling. IL-10 has multiple mechanisms of action such as suppression of antigen presentation and suppression of cytokine and inflammatory mediator production.

1.3.2 IL-10 in inflammatory diseases

Activation of IL-10 induction is a critical feature of IL-10 mediated resolution of inflammation as it has been shown that IL-10 has a crucial role in negatively regulating inflammation by controlling gene expression of a variety of pro-inflammatory cytokines. Studies in chronic inflammatory diseases such as inflammatory bowel disease (IBD) have shown, using IL-10-deficient mice, that IL-10 does not act as a general inhibitor of all TLR-induced gene expression (Berg et al., 1995, Kuhn et al., 1993, Rennick et al., 1995). IL-10-deficient mice originally revealed the importance of the IL-10 cytokine in gut homeostasis and in the prevention of uncontrolled inflammation (Kuhn et al., 1993). These IL-10-deficient mice spontaneously developed intestinal inflammation characterized by lesions affecting the small and large intestine (Kuhn et al., 1993, Berg et al., 1996). Similarly, the genetic loss of IL-10R or IL-10 in humans led to IBD and colitis (Glocker et al., 2010). Treatment of IL-10-deficient mice with exogenous IL-10 prevented disease development in young mice but could not fully reverse established disease in adult mice. Intestinal inflammation in the IL-10-deficient mice was mediated by Th1 cells (Davidson et al., 1996). Furthermore, uncontrolled generation of IFN-γ-producing Th1 cells enhanced the disease in IL-10 deficient mice (Berg et al., 1996). Mice with IL-10-deficient B-cells failed to recover from disease in an experimental autoimmune encephalomyelitis (EAE) model, a primary animal model of multiple sclerosis (MS) (Fillatreau et al., 2002). Lampropoulou et al. (2008) showed that TLR signalling in B-cells is required for the B-cell-mediated IL-10 production to inhibit the disease. However, secondary stimulation of the B-cells via B cell receptor (BCR) and CD40 was also required (Lampropoulou et al., 2008).

The dysregulation of IL-10 and the IL-10R1 and IL-10R2 has been linked to various immunological diseases, such as asthma, infectious disorders, rheumatoid arthritis,

systemic lupus erythematosus (SLE), transplant rejection and cancer (Asadullah et al., 2003, Iyer and Cheng, 2012). Therefore, control of IL-10 must be specific in order to regulate its roles in inflammation but also inflammatory diseases.

1.3.3 IL-10 as a therapeutic

The consequences of impaired IL-10 function can lead to numerous diseases. Different animal disease models, those for including IBD, rheumatoid arthritis, psoriasis and systemic lupus erythematosus, have been used to study the effects of IL-10 (Iyer and Cheng, 2012). In addition, there have been different attempts to improve the symptoms of inflammation in IL-10- and IL-10 receptor-deficient patients, including treatments with immunosuppressive drugs (corticosteroids, azathioprine and methotrexate), immunomodulatory agents (anti-TNF-α monoclonal antibody, and thalidomide) and surgical interventions. However thus far, complete disease clearance is a rare event (Asadullah et al., 2003). Both IL-10 overexpression (in lymphoma and SLE) (Llorente et al., 1997, Grondal et al., 1999) as well as IL-10 deficiency (in IBD and psoriasis) are likely to influence disease development. Therefore, there have been focused efforts to discover the therapeutic potential of recombinant IL-10 (rIL-10) and anti-IL-10 antibodies against these diseases. The therapeutic effects of rIL-10 were tested in clinical trials in psoriasis patients. It has been shown that rIL-10 treatment decreases pro-inflammatory cytokines in these patients, but there was only temporary clinical improvement after 6 weeks (Asadullah et al., 2003). In SLE, high levels of autoantibodies generate immune complexes, causing tissue damage. The levels of IL-10 in SLE patients are significantly higher compared to healthy volunteers (Park et al., 1998). Depletion of IL-10 by anti-IL-10 antibody treatment of SLE patients significantly decreased autoantibody production (Asadullah et al., 2003). In this context IL-10 is pathologic as it induces auto-antibody

production. The potential of IL-10 as a therapeutic agent has been limited by variations in treatment. So far the only treatment that has proven successful is allogeneic hematopoietic stem cell transplantation (HSCT), which was performed in patients with mutations in the IL-10 signalling pathway (IL-10 deficiency or IL-10R deficiency) (Engelhardt et al., 2013). Transplanted patients were cured of colitis, wound healing improved and they showed no signs of active inflammation. However HSCT and the other IL-10 related diseases are very demanding on the patients and therefore a better understanding of the molecular mechanism of IL-10-mediated effects may lead to the discovery of new drug targets. Identifying the molecular mechanisms that regulate IL-10 expression are critical to develop therapeutic strategies directed against pathology-associated impaired IL-10 production.

1.3.4 IL-10 signalling

After TLR activation IL-10 can be secreted. Following this, IL-10 binds as a homodimer to a heterodimeric receptor complex, consisting of IL-10R1 and IL-10R2 subunits located on the cell surface (Kotenko et al., 1997). These IL-10R1/2 subunits activate the kinase function of Janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2). Activation of JAK1 initiates phosphorylation of the IL-10R1 α chain and two tyrosine residues. These then dock to the SH2 domains of the inactive signal transducer and activator of transcription (STAT) resulting in its phosphorylation by JAK1 (Darnell, 1997). STAT phosphorylation leads to homodimerisation and translocation to the nucleus to promote the expression of IL-10 responsive genes (Finbloom and Larner, 1995). STAT is a transcription factor that can transcribe genes like Suppressor of cytokine signalling (SOCS), whose production can inhibit inflammatory signalling (Figure 1.5) (Murray, 2006, Cassatella et al., 1999). However the precise details of how STAT inhibits production of the cytokines TNF-α, IL-6 and IL-12 are not fully

understood. It has been shown that IL-10 signalling inhibits NF-κB activity through a dual mechanism whereby IL-10 blocks NF-κB nuclear translocation by inhibiting IKK activity. IL-10 signalling is also able to block NF-κB from binding to DNA, this occurs when NF-κB is already present in the nucleus. This possibly occurs by blocking p65/p50 heterodimer translocation to the nucleus (Clarke et al., 1998, Driessler et al., 2004, Asadullah et al., 2003).

1.3.5 Regulation of IL-10 expression

IL-10 and can also act to modulate a number of miRNAs. For example, it up regulates miR-187 (Rossato et al., 2012) and miR-146b (Curtale et al., 2013) to increase the anti-inflammatory response and down regulates the miRNA that are highly pro-inflammatory such as miR-155 (Figure 1.5). IL-10 was shown to potently down regulate the induction of miR-155 by LPS (McCoy et al., 2010). IL-10 targeting of miR-155 enabled it to control the miR-155 target gene SHIP1. Blocking miR-155 activity resulted in increased SHIP1 expression, which is an important negative regulator of TLR signalling. Therefore, IL-10 was able to further modulate the anti-inflammatory response (McCoy et al., 2010).

Different steps in the response to a pathogen can control the regulation of IL-10. Its gene expression can be regulated by different transcription factors, but IL-10 can also be regulated post-transcriptionally.

1.3.5.1 Transcriptional regulation of IL-10

There are different transcription factors that regulate IL-10 expression. The IL-10 promoter structures in human and mouse have high levels of homology, particularly around binding sites for transcription factors. The IL-10 promoter contains a TATA box and a CCAAT box (CCAGT in mice) that can be recognised by transcription

factors. However, the presence of a conserved binding site in a gene promoter does not guarantee transcription factor binding (Gabrysova et al., 2014). The transcription factors known to regulate IL-10 in macrophages are activating transcription factor 1 (ATF1), C/EBPβ, c-Maf, CREB, NF-κB through p50 homodimers and the p65 subunit, pre-B-cell leukemia transcription factor 1 (PBX1), E26 transformation-specific 1 (ETS1), specific protein 1 (SP1) and PBX-regulating protein (PREP1) (Saraiva and O'Garra, 2010). The expression of *IL10* or indirect expression of *IL10* depends on transcription factor binding, however the transcription factor that binds on the IL-10 promoter is dependent on the cell type and on the stimulus used (Saraiva and O'Garra, 2010). For example, it has been shown in THP1 cells that LPS stimulation versus cyclic AMP stimulation results in different transcription factors such as CREB and NF-κB being active on the IL-10 promoter (Ma et al., 2001, Brenner et al., 2003). The choice of transcription factor in different situations reflects the complexity that the expression of IL-10 demands.

1.3.5.2 Post-transcriptional regulation of IL-10

Modulation of inflammatory responses involves a coordinated system that resolves inflammation. This includes the mechanism of transcriptional regulation through transcription factors but also requires post-transcriptional mechanisms that regulate transcript stability. Like many transcripts, IL-10 mRNA is subject to rapid degradation after synthesis as IL-10 mRNA can be detected within a few hours after LPS stimulation in macrophages. IL-10 mRNA contains clusters of adenosine- and uridine-rich elements (ARE) located on the 3' untranslated region (UTR). AREs are one of the most common determinants of RNA stability in mammalian cells. The ARE binding protein tristetrapolin (TTP) can degrade IL-10 mRNA by the mRNA decay machinery, which includes mRNA de-capping proteins, exosome endonuclease activity and RNA-

induced slicing complex (RISC) members (Brooks and Blackshear, 2013). TTP knockout mice have reduced IL-10 mRNA degradation in primary macrophages (Stoecklin et al., 2008). TTP-mediated mRNA degradation progresses through p38-MK2 (Chrestensen et al., 2004, Stoecklin et al., 2004). Moreover, activation of p38 has been reported to stabilize IL-10 mRNA by inhibiting the action of TTP (Tudor et al., 2009), suggesting that the MAPKs are involved in both IL-10 transcription as well as post-transcriptional regulation to promote IL-10 production.

IL-10 mRNA stability can also be positively and negatively regulated through small non-coding RNA sequences called microRNA (miRNA). IL-10 can be directly post-transcriptionally regulated by several miRNA, including miR-106a, miR-4661, miR-98, miR-27, let7 and miR-1423p/5p (Sharma et al., 2009, Liu et al., 2011, Ma et al., 2010, Jiang et al., 2012, Ding et al., 2012).

1.3.6 PDCD4 as an IL-10 controller

In 2006 mice deficient in programmed cell death protein 4 (PDCD4) were characterized by Hilliard et al. (2006). They found that these PDCD4-deficient mice developed spontaneous lymphoma after 80 weeks and had reduced autoimmune diseases. It was demonstrated that these PDCD4-deficient mice were resistant to models of inflammatory disease, such as experimental autoimmune encephalomyelitis (EAE) and streptozin toxin induced type II diabetes. This was shown to be due to the role of PDCD4 as a translational inhibitor of complex mRNAs, specifically those for IL-10, IL-4 and IFN-γ (Hilliard et al., 2006). PDCD4 was shown to be a pro-inflammatory protein that promotes activation of NF-κB and suppresses IL-10. It was further shown that miR-21 can regulate PDCD4 expression after LPS stimulation (Sheedy et al., 2010) (Figure 1.5).

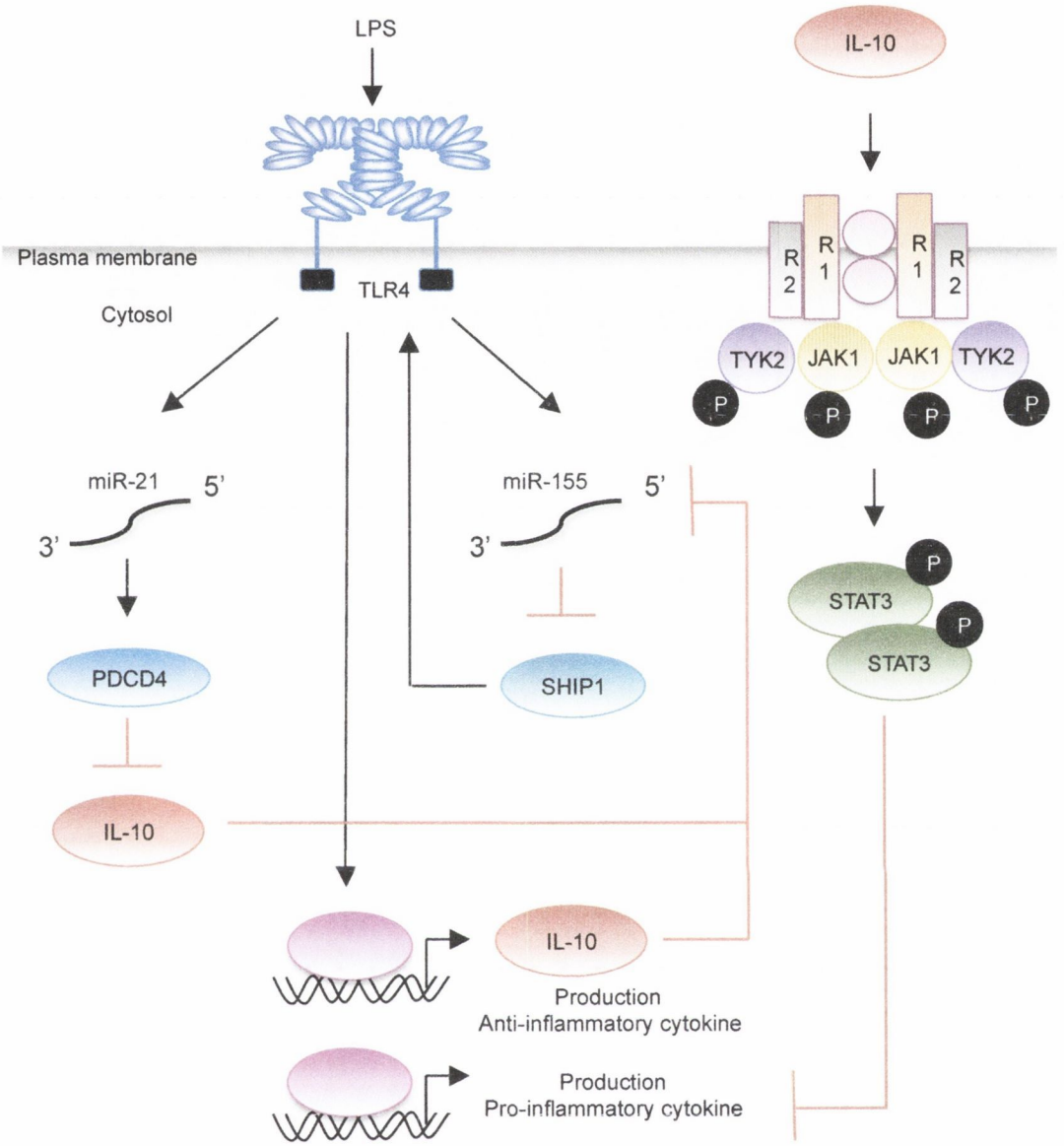


Figure 1.5 – IL-10 signalling after LPS response

After LPS stimulation the production of pro-inflammatory cytokines occurs as well as the anti-inflammatory cytokines such as IL-10 are induced. IL-10 is able to bind to its receptor and therefore activates the dimerization of STAT3. STAT3 can in its turn inhibit the pro-inflammatory cytokine production. LPS stimulation can also induce the miRNAs miR-21 and miR-155. miR-155 targets SHIP1. The function of SHIP1 is to inhibit TLR4 signalling however when miR-155 inhibits SHIP1 TLR4 signalling will increase. miR-21 also targets PDCD4. When miR-21 inhibits PDCD4 the IL-10 production will increase. The increasing levels of IL-10 have a negative feedback on miR-155; restoring the SHIP1 levels and inhibiting TLR4 but also promotes the inhibition of pro-inflammatory cytokine production through the IL-10 receptor.

1.3.7 PDCD4

PDCD4 was originally described as having a role in apoptosis, which plays an essential role during biological processes. During investigation of apoptosis *pdcd4* was first identified as an upregulated gene when cells were treated with tumor promoter 12-O-tetradecanoylphorbol-13-acetate (PMA), ionomycin, or dexamethasone (Shibahara et al., 1995). However, other inducers of apoptosis including UV irradiation or topoisomerase inhibitors had no such effect (Onishi and Kizaki, 1996, Onishi et al., 1998). PDCD4 mRNA levels are also induced by cytokines such as IL-12 but downregulated by IL-2 and IL-15 (Azzoni et al., 1998). These studies suggest that PDCD4 protein levels depend on the stimulus used. The fact that PDCD4 had been known as a tumor suppresser protein therefore made it a potential target for anticancer therapies for several years. Today there is increasing evidence that PDCD4 influences transcription and translation in different signalling transduction pathways, besides acting as a tumor suppressor (Lankat-Buttgereit and Goke, 2009).

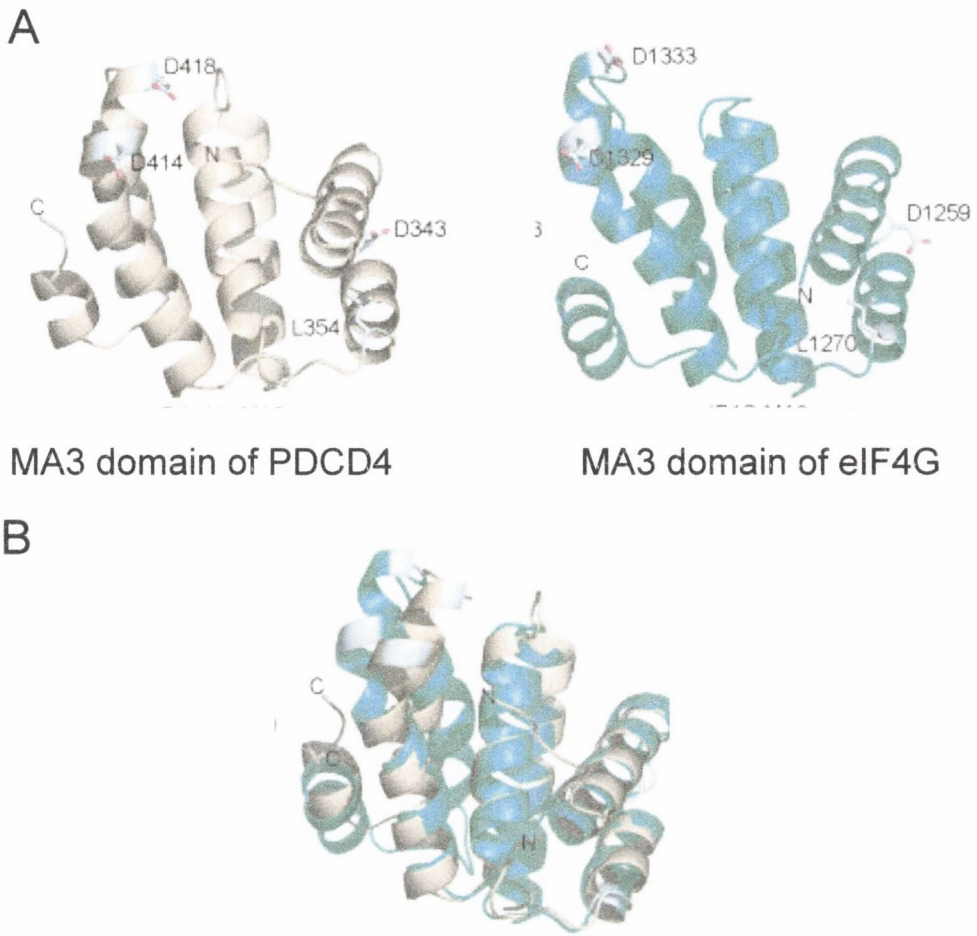
PDCD4 contains 469 amino acids, with two basic domains at the N-terminus and C-terminus and two conserved α-helical MA-3 domains (Lankat-Buttgereit and Goke, 2009). The MA-3 domains are involved in the protein-protein interaction between PDCD4 and eukaryotic translation initiation factors eIF4A and eIF4G. Co-immunofluorescence microscopic studies suggested that PDCD4 interaction disrupts the function of eIF4A and eIF4G (Yang et al., 2003a, (Goke et al., 2002). Mutations in the MA-3 domains of PDCD4 led to a complete loss of eIF4 binding (Yang et al., 2004a). Following studies with crystal and solution structures of the C-terminal MA-3 domain of PDCD4 it was found that PDCD4 interacts with eIF4A and that this was sufficient to inhibit translation initiation (Figure 1.6) (LaRonde-LeBlanc et al., 2007, Waters et al., 2007, Suzuki et al., 2008). The translation initiation factors, including eF4A, are required for the cap-dependent translation of mRNAs, including IL-10 or

IL-4 mRNA (Yang et al., 2003, Loh et al., 2009, Hilliard et al., 2006). It has been shown that the induction of IL-10 by LPS requires degradation of PDCD4, allowing translation to occur (Sheedy et al., 2010). All these studies proposed a role for PDCD4 in translational regulation.

There are different phosphorylation sites found in the amino acid sequence of PDCD4 and phosphorylation of PDCD4 was first demonstrated in HeLa cells (Lankat-Buttgereit and Goke, 2009). It has been shown that Akt specifically phosphorylates PDCD4 at Ser67 and Ser457, resulting in nuclear translocation of PDCD4 and the loss of its function to inhibit the AP-1 transcription factor (Palamarchuk et al., 2005). In addition, phosphorylation of PDCD4 on Ser67 initially and then Ser71 and Ser76 by the S6K1 results in degradation by the ubiquitin ligases $SCF^{\beta\text{-}TRCP}$ (Dorrello et al., 2006) A study showing a decrease in PDCD4 protein levels after PMA treatment in keratinocytes and HEK293 cells confirmed the phosphorylation sites of PDCD4 (Schmid et al., 2008). However, Schmid et al (2008) found that the decrease of PDCD4 is due to increased proteasomal degradation of PDCD4. This degradation is mediated by activation of Akt and S6K1, as well as MEK-ERK signalling (Schmid et al., 2008). Recently it has also been found that PDCD4 proteasomal degradation requires PI3K-mTOR signalling in tumor cells in an inflammatory tumor microenvironment (Schmid et al., 2011). The degradation of PDCD4 might allow protein synthesis and cell growth by releasing translational initiation factors as well as transcription factors. Therefore, PDCD4 targeting by mTOR relate to our understanding of mTOR in the immunity.

Besides eIF4A, another protein that is known to interact with PDCD4 is the transcription factor Twist1 (Figure 1.7). This interaction also occurs through the MA3 domain of PDCD4 and has been shown to down-regulate Y-Box binding protein-1 (YB-1) expression (Shiota et al., 2009). YB-1 has been reported to have a crucial role

in various biological activities of cancer cells, such as cell proliferation and drug resistance (Shiota et al., 2009, Kohno et al., 2003). The Twist family will be discussed in more detail below.



Alignment of the MA3 domains of PDCD4 and eIF4G

Figure 1.6 – The interaction of the MA3 domains of PDCd4 and eIF4G

The MA3 domains of Pdcd4 and eIF4G have similar structures. (A) Ribbon representation of the MA3 domains of Pdcd4 (brown) and eIF4G (cyan) showing the locations of residues that influence interaction with eIF4A. (B) Alignment of the ribbon representation of the MA3 domains of Pdcd4 and eIF4G (LaRonde-LeBlanc et al., 2007).

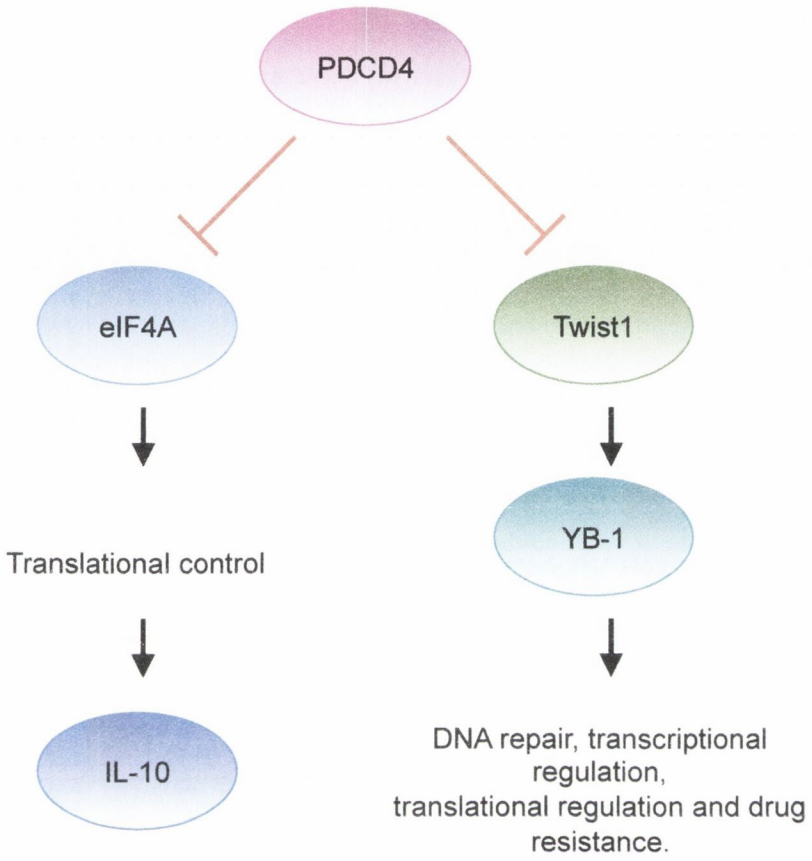


Figure 1.7 – PDCD4 inhibits eIF4 and Twist1 actions through interaction with the MA3 domains.

PDCD4 can interact with eIF4 through the MA3 domains and inhibits the eIF4 translational activity in the cap-dependent translation of mRNA, which includes IL-10 mRNA. However, PDCD4 is also able to bind to Twist1 and therefore downregulate YB-1 expression. YB-1 has been shown to play a role in biological activities of cancer cells and drug resistance.

1.4 mTOR pathway

mTOR is a serine/threonine protein kinase which belongs to the phosphatidylinositol 3-kinase-related kinase protein family. mTOR regulates cell growth, cell proliferation, cell motility, cell survival, transcription and protein synthesis. mTOR has been described with different names such as mechanistic target of rapamycin, mammalian target of rapamycin (mTOR) or FK506-binding protein 12-rapamycin-associated protein 1 (FRAP1). The mTOR signalling pathway is conserved from yeast to human and can be activated by a variety of stimuli including growth factors, such as insulin via the insulin-like growth factor 1 (IGF-1) receptor, epidermal growth factors (EGF) and nutrients, for example amino acids and ATP (Yang and Guan, 2007) (Figure 1.8). The mTOR complex acts as a critical PDCD4 controller and is a major focus of this thesis. Therefore, the regulation of the mTOR signalling network will next be described.

1.4.1 Regulation of the mTOR signalling network

Firstly, mTOR is a key signalling kinase that affects cellular functions including growth, survival, metabolism, aging and memory. The mTOR system contains two different complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2). mTORC1 includes mTOR in a complex with mammalian lethal with SEC13 protein 8 (mLST8), regulatory-associated protein of mTOR (RAPTOR) in an active state (Figure 1.9). But mTORC1 is also known to contain proline-rich AKT substrate of 40 kDa (PRAS40) and DEP domain-containing mTOR-interacting protein (DEPTOR) in an inactive state. mTORC1 is sensitive to inhibition by rapamycin. mTORC2 comprises mTOR, rapamycin-insensitive companion of mTOR (RICTOR), Sin1 and

mLST8 in an active state and DEPTOR in an inactive state. mTORC2 is considered to be rapamycin-insensitive (Benjamin et al., 2011).

1.4.1.1 Insulin/IGF-1-mediated mTORC1 signalling

Insulin and IGF-1 through the insulin/IGF1 receptor mediate the activation of class I phosphoinositide 3–kinase (PI3K) (Fruman, 2004). PI3K phosphorylates phosphatidylinositol 4,5–biphosphate (PIP2) to generate phosphatidylinositol 3,4,5 – trisphosphate (PIP3). This leads to the recruitment of the serine-threonine kinase Akt or Protein Kinase B (PKB) to the cell membrane. Akt can be phosphorylated at threonine 308 through phosphoinositide-dependent kinase (PDK1) or by mTORC2 complex at serine 473 (Weichhart and Saemann, 2009). The direct mechanism by which Akt regulates cell growth was for a long time unknown. However, in 2002 a study was published showing that Akt directly phosphorylates tuberous sclerosis complex (TSC) protein 2 (TSC2) at the conserved residues serine 924 and threonine 1518. It was shown that mutation of these sites reduces TSC2 insensitivity to Akt signalling (Potter et al., 2002). TSC2 forms a heterodimeric complex with TSC1. When Akt activates TSC2 it relieves the inhibitory activity of the TSC1-TSC2 complex on mTORC1 by activation of the GTPase Ras homolog enriched in brain (Rheb) (Stocker et al., 2003). Rheb in turn associates with mTORC1 by an unknown mechanism and promotes mTORC1 signalling to downstream substrates of mTORC1 (Figure 1.9).

1.4.1.2 Ras-MAPK pathway-mediated mTORC1 signalling

There are different signals that can regulate TSC1-TSC2 complex. One of them is the direct phosphorylation of Akt to block TSC2 function, the upstream inhibitor of

mTORC1. Another signal to inhibit TSC1-TSC2 function is through the activation of the Ras-MAPK pathway. The EGF-mediated activation of Ras follows through the growth factor receptor-bound protein-2 (GRB2). GRB2 links the EGF receptor to the guanine nucleotide-exchange factor son of sevenless (SOS). SOS catalyses GDP release and GTP binding to Ras. Ras binds to Raf kinase, which brings the complex to the plasma membrane where they are activated. Activated Raf kinase phosphorylates and activates both MAPKs MEK1/2, which then phosphorylate ERK1/2. ERK1/2 activates RSK. RSK is also activated by PDK1 (Anjum and Blenis, 2008). RSK is known to phosphorylate TSC2 at Serine1798 and inactivates its tumour-suppressor function, thereby promoting mTOR signalling and translation (Roux et al., 2004). RSK has also been shown to phosphorylate RAPTOR, providing another link between RSK and the mTOR pathway (Carriere et al., 2008).

1.4.1.3 Energy, nutrients and stress-mediated mTORC1 signalling pathways

When the cellular AMP/ATP ratio is increased, AMP-activated protein kinase is phosphorylated and activated. Liver kinase B1 (LKB1) is an upstream kinase of AMP-activated protein kinase (AMPK). AMPK is known as a regulator of cellular energy metabolism but can also phosphorylate TSC2 on Ser1345 to enhance TSC function and suppress mTORC1 signalling (Inoki et al., 2003). Thus, TSC2 phosphorylation either promotes or inhibits TSC function dependent upon the sites of phosphorylation, and therefore also promotes or inhibits mTORC1 activity. AMPK is also known to inhibit mTORC1 by direct phosphorylation of Raptor (Foster and Fingar, 2010). Various forms of cell stress including hypoxia and DNA damage can reduce mTORC1 signalling by increasing the inhibitory function of TSC. A lack of amino acids, particularly leucine and isoleucine, is known to inhibit mTORC1 signalling directly through mTORC1, however, the mechanism remains unknown.

1.4.1.4 mTOR substrates

Activation of mTORC1 results in the activation of the best-known downstream substrates S6 kinase (S6K1) and eukaryotic initiation factor 4E-binding protein (4E-BP1). The phosphorylation of S6K1 promotes mRNA translation and cell growth through programmed cell death protein 4 (PDCD4). The phosphorylation of 4E-BP1 prevents it from inhibiting eIF4 factors and therefore stimulates protein synthesis (Luo et al., 1996, von Manteuffel et al., 1997, Weichhart and Saemann, 2009) However more mTOR substrates were discovered by using the inhibitor rapamycin and TSC2 knock-out such as apoptotic chromatin condensation inducer 1(ACIN1), inhibitor of Bruton Agammaglobulinemia tyrosine kinase 1 (IBTK1) and lysosomal-associated membrane protein 1 (LAMP1) (Hsu et al., 2011). ACIN1 is known to be involved in in chromatin condensation and nuclear fragmentation (Sahara et al., 1999). IBKT1 down regulates Bruton tyrosine kinase-mediated calcium mobilization and is also known to negativily regulate the activation of NF-κB transcription (Liu et al., 2001). LAMP1 is a family member of glycoproteins and provides selectins with carbohydrate ligands (Chen et al., 1988).

1.4.1.5 Feedback inhibition of the mTORC1 pathway

There are different feedback loops to complicate mTORC regulation. Firstly PTEN, a dual protein-lipid phosphatase, can dephosphorylate PIP3 produced by PI3K and interrupts the downstream activation of Akt (Rosivatz, 2007, Maehama and Dixon, 1999, Georgescu, 2010). Also, WNT pathway is known to influence mTORC1 by inhibiting glycogen synthase kinase 3 (GSK3), which in the absence of WNT proteins negatively regulates the mTORC1 pathway. This is because GSK3 can phosphorylate TSC2 to enhance its inhibitory function (Inoki et al., 2006). Activated S6K1 can also feedback negatively to PI3K/Akt signalling by facilitating the degradation of insulin

receptor substrate 1 (IRS1), which is the molecular intermediate between the insulin receptor and PI3K (Foster and Fingar, 2010) So far it has not been shown whether S6K1 can negatively regulate other receptors active in PI3K-mTOR signalling. It has been shown that under certain conditions S6K1 can positively regulate mTOR activity by negatively regulating GSK3 (Thomson et al., 2009).

1.4.1.6 mTORC2 regulation

The knowledge of mTORC2 is more limited compared to mTORC1. This is because there are fewer specific mTORC2 inhibitors available. However, studies targeting RICTOR through small interfering RNA (siRNA) and studies with RICTOR deficient mice have shown the importance of mTORC2 in mammalian development and cellular processes (Sarbassov et al., 2004, Jacinto et al., 2004). Knockdown of RICTOR does not decrease S6K1 activation, indicating that mTORC2 does not activate mTORC1 signalling (Guertin et al., 2006). IGF-1 activity through PI3K promotes mTORC2-mediated Akt Ser473 phosphorylation (Guertin et al., 2006).

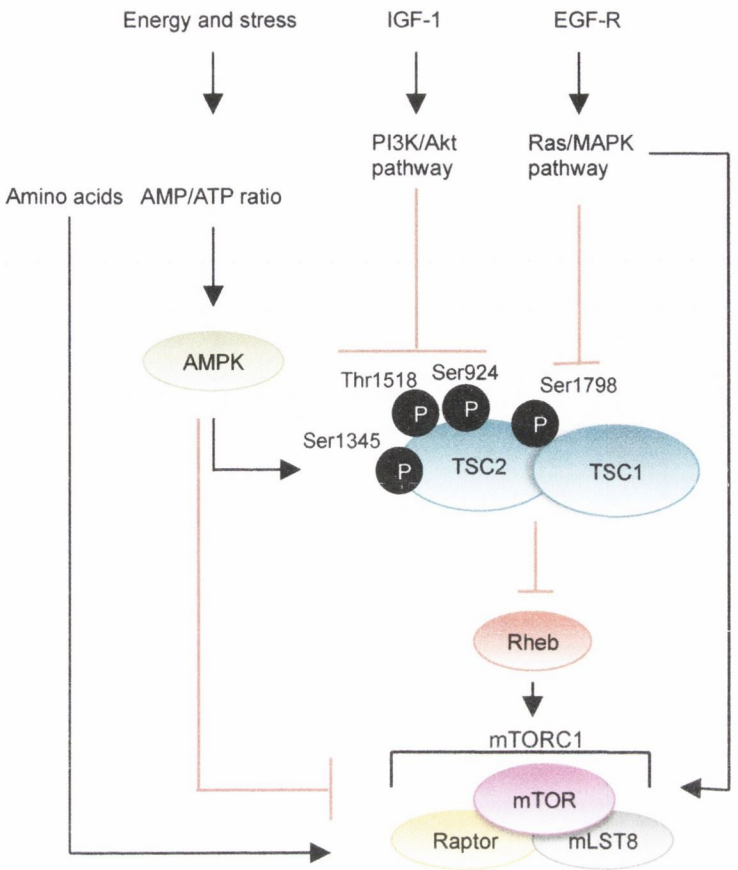


Figure 1.8 – Regulation of mTORC signalling networks.

Growth factors, insulin and nutrients including amino acids promote mTORC1 signalling via phosphorylation of TSC2 and mTORC1. Insulin signals via IGF-1 receptor to activate the PI3K/Akt/TSC/Rheb pathway, which can block the inhibitory function of TSC2 on mTORC1 by phosphorylation of TSC2 on Ser1798. EGF signals via EGF-R to activate the Ras/Raf/Mek/MAPK/RSK pathway, which can also block TSC2 function by phosphorylation of TSC2 on Thr1518 and Ser924. RSK can phosphorylate raptor directly. Amino acids are also known to activate mTORC1 directly. Energy sufficiency suppresses AMPK, which is known to activatethe TSC2/TSC1 complex by phosphorylation of Ser1345, and can also inhibit mTORC1 directly.

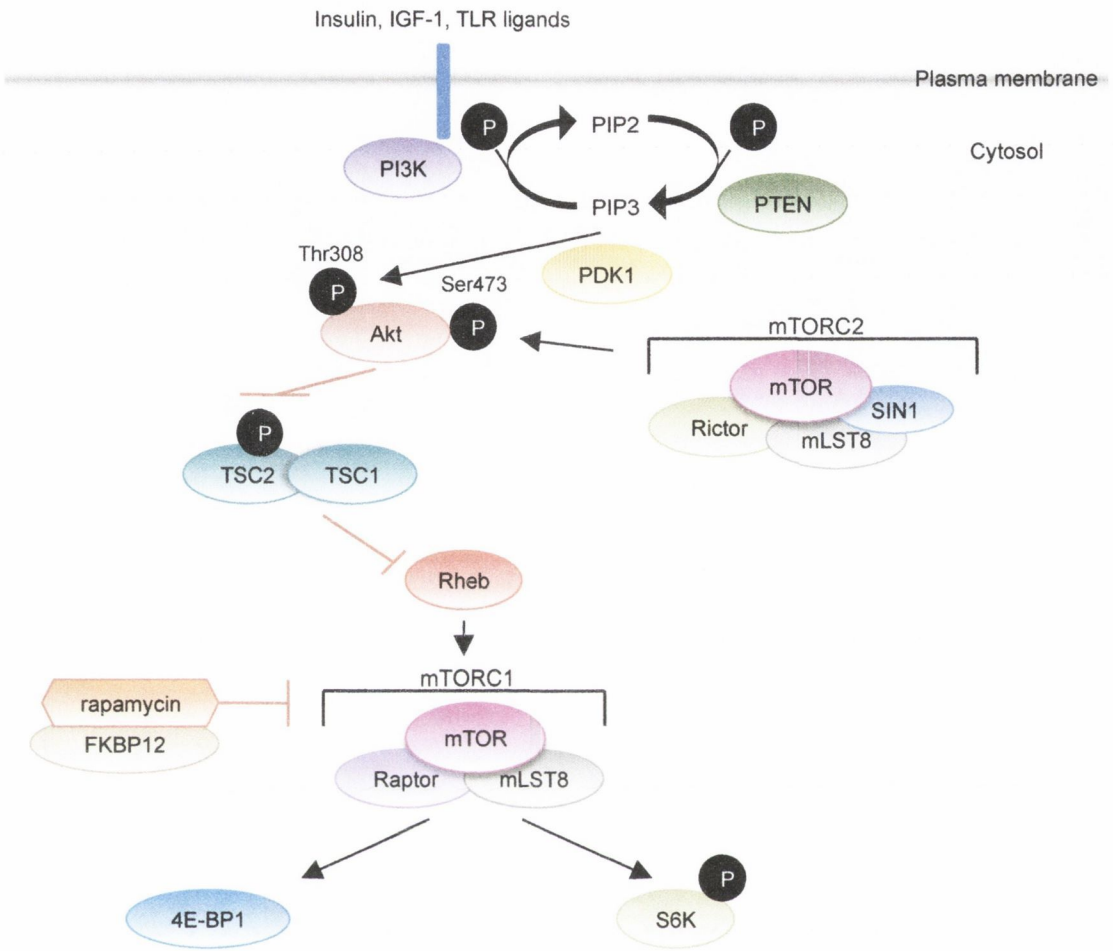


Figure 1.9 – Activation of the mTOR pathway.

mTOR is found in two distinct protein complexes mTORC1 and mTORC2. mTORC1 (composed of mTOR, Raptor, and mLST8) is rapamycin-sensitive and is regulated by growth factors, TLR ligands, and nutrients. In contrast, mTORC2 (composed of mTOR, Rictor, Sin1, and mLST8) is not directly inhibited by rapamycin. Growth factors (such as insulin or IGF-1) and TLR ligands trigger mTORC1 activity via PI3K. PI3K stimulates PIP3 production, which recruits Akt to the cell membrane, resulting in its phosphorylation via PDK1 and by the mTORC2 complex. Active Akt phosphorylates TSC2 and thereby relieves the inhibitory activity of the TSC1–TSC2 complex on mTORC1. AMPK in turn inhibits mTORC1 by directly phosphorylating TSC2, leading to enhanced suppressive activity of TSC1–TSC2 towards mTORC1. Moreover, AMPK directly phosphorylates Raptor and decreases mTORC1 kinase activity. Amino acids induce binding of the Rag GTPases to mTORC1 to enhance its activity. Active mTORC1 phosphorylates its downstream substrates 4E-BP1 and p70S6K, thus stimulating protein synthesis and cell growth. Rapamycin binds FKBP12 and, in the form of this complex, inhibits mTORC1 (Weichhart and Saemann, 2009).

1.4.2 mTOR complex and rapamycin

The name TOR (target of rapamycin) is derived from its inhibitor rapamycin Rapamycin was initially isolated from soil bacterium on Rapa Nui (Easter Island) and developed as an antifungal agent to inhibit the growth of yeast, but was found to decrease proliferation of T lymphocytes (Martel et al., 1977). Rapamycin is also known as sirolimus and belongs to the macrolide class drugs. It inhibits the enzymatic activity of mTORC1 (Martel et al., 1977). Rapamycin interacts with the cellular protein FK506-binding protein 12 (FKBP12), and this complex directly binds to the TOR FKBP12-Rapamycin-Binding (FRB) domain, located outside the ATP-binding pocket of free mTOR, i.e. not in complex with associated its regulatory proteins. Rapamycin treatment inhibits mammalian TORC1 (mTORC1) but not mTORC2 (Foster and Fingar, 2010). However, it has been shown that high-dose (0.2 – 20 μM) rapamycin and long treatment times inhibit mTORC2 signalling in certain cell types (Sarbassov et al., 2006, Chen et al., 2005). The efficiency of rapamycin has been shown to be dependent on phosphatidic acid (PA), which is required for the assembly of both mTORC1 and mTORC2. Phospholipase D (PLD) can generate PA from phosphatidylcholine (PC) (Foster and Toschi, 2009). Rapamycin interacts with mTOR in a competitive manner with PA (Figure 1.10). For example, high levels of PA can be found in cancer cells and therefore there is an increase in rapamycin concentration needed to suppress mTOR activity (Chen et al., 2005,(Foster et al., 2014). Suppression of PA production as well as using rapamycin inhibits the association between mTOR and Raptor and the association of mTOR and Rictor (Foster and Toschi, 2009, Foster et al., 2014). Because mTORC2 is more stabile than mTORC1, this possibly explains the resistance of mTORC2 to rapamycin.

1.4.2.1 Rapamycin and its therapeutic implications

To date, rapamycin has been approved by the FDA for immunosuppression for organ transplantation, prevention of restenosis, chemotherapy, angioplasty and soft tissue and bone sarcomas (Yang and Guan, 2007). It has been shown that complete mTOR knockout mice are embryonic lethal (Gangloff et al., 2004, Murakami et al., 2004). However tissue-specific knockout of mTOR complex components in selected organs indicates the importance of mTOR signalling in growth and metabolism as it has been linked to cancer (Gu et al., 2011). Mice treated with rapamycin have an increased lifespan compared to untreated control group (Harrison et al., 2009). These studies indicate that rapamycin might have major clinical benefits for the fight against cancer (Blagosklonny, 2013).

It has been shown that LY294002 and rapamycin co-operate to inhibit T-cell proliferation (Breslin et al., 2005). T-cell proliferation is critical for mounting an effective adaptive immune response. The proliferation is regulated by signals through co-stimulation and IL-2, which activates PI3K. The first cell cycle proteins cyclin D2 and cyclin D3 are regulated by PI3K and induced in T-cell proliferation and transcription. LY294002 and rapamycin prevents the induction of the cyclin D proteins. Rapamycin was also noted to control a critical cell cycle checkpoint protein E2F through S6K in T- cells (Brennan et al., 1999). Therefore combination of these inhibitors can be useful as alternative immunosuppressive therapies to limit T-cell proliferation (Breslin et al., 2005).

Rapamycin has also been shown to reverse the decrease in TLR4-mediated TNF-α release from human immunodeficiency virus (HIV) infected macrophages through the prolongation of the MAPK/mTOR signalling pathway (Li et al., 2011). Furthermore, HIV inhibits the autophagy function of dendritic cells by mTOR activation and blocks

therefore early innate and adaptive immune responses (Blanchet et al., 2010). Therefore inhibition of mTORC1 by rapamycin might be a potential therapeutic target to up-regulate macrophage and dendritic cells responses in HIV-positive patients (Katholnig et al., 2013b).

Because of the inhibitory actions of rapamycin on DCs and effector T cells but not Treg cells, rapamycin is a useful therapeutic agent for the prevention of transplantation rejection and might be a treatment for autoimmune disease (Thomson et al., 2009). For instance, rapamycin combined with IL-10 blocks type I diabetes and induces long-term tolerance without chronic immunosuppression in non-obese diabetic mice (Thomson et al., 2009). This is due to the rapamycin-mediated increase in suppressive forkhead box P3 (FOXP3) Treg cells in the pancreas (Battaglia et al., 2006). Rapamycin is also effective for the treatment of autoimmune hepatitis following human liver transplantation (Montano Loza and Czaja, 2007) and has recently been used to treat Crohn's disease (Massey et al., 2008). Currently much research is still ongoing about the effect of rapamycin on cytokine secretion after TLR responses.

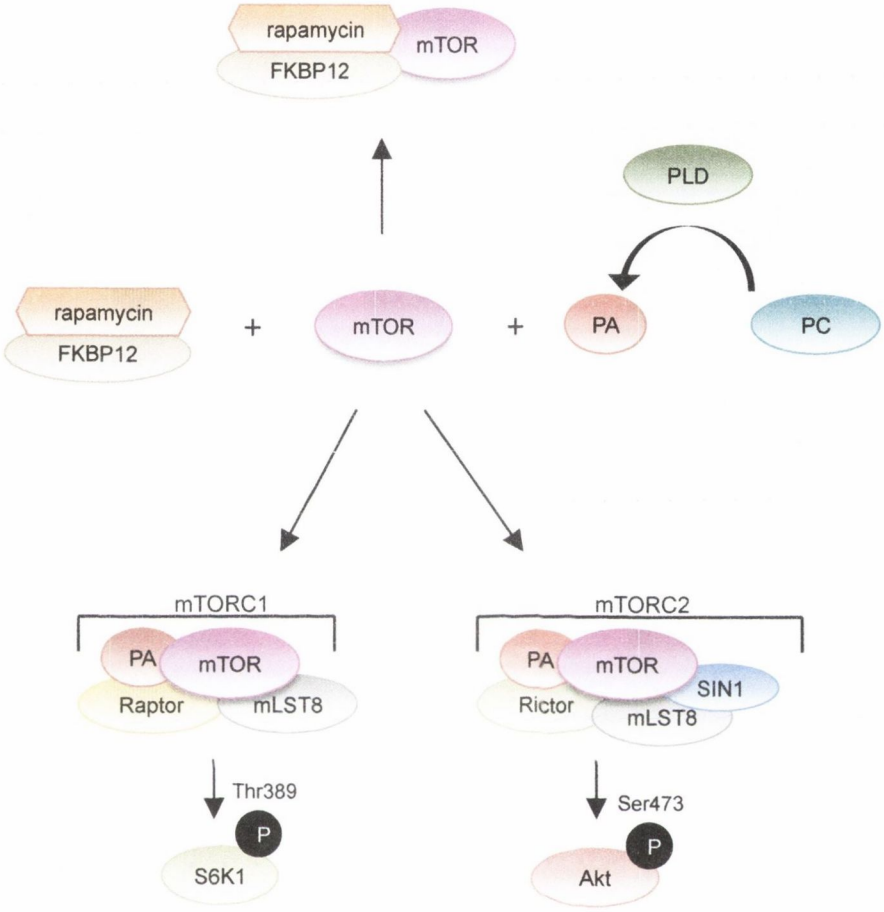


Figure 1.10 – Simplified model of the differential effect of rapamycin on mTOR.

When mTOR is not in complex with its regulatory-associated proteins Raptor or Rictor it can bind to the FKBP12-Rapamycin complex or PA. The choice of mTOR regarding whether to bind to PA or the FKBP12-Rapamycin complex is competition based as well as rapamycin dose dependent. PA can be generated by PLD from PC. PA is required for the assembly of both mTORC1 and mTORC2. The stability of mTORC2 is stronger than mTORC1, so there are fewer dissociations of mTORC2 to PA and a higher dose of rapamycin needed.

1.4.3 mTOR and the immune system

There are different studies showing that the mTOR pathway is linked to the immune system. mTOR plays a role in regulating diverse immune cells, including neutrophils, mast cells, natural killer cells, B cells and T cells as well as APCs; macrophages, dendritic cells. See Figure 1.11 for the effect of mTOR in various cell types. In macrophages and dendritic cells LPS and other TLR ligands have been shown to induce mTORC1 and mTORC2 activation (Weichhart et al., 2008, Schmitz et al., 2008, Weintz et al., 2010).

The mTOR pathway can be activated by TLRs via PI3K, as pharmacological inhibitors of PI3K have been shown to regulate cytokine production. Suppression of PI3K activity increases IL-12 but reduces IL-10 production (Weichhart et al., 2008, Martin et al., 2003, Williams et al., 2004). It has also been demonstrated that TLR directly activates mTOR via PI3K but that mTOR is then able to associate physically with the adaptor molecule MyD88. This activation leads to the activation of transcription factors IRF5 and IRF7, which are responsible for the induction of cytokines and IFN-α (Schmitz et al., 2008).

Recently it has been suggested that the MAPK p38α activates the mTOR patwhay in TLR-stimulated macrophages and monocytes (Figure 1.12) (Katholnig et al., 2013a). p38α is a well establised signalling partner in TLR4 signalling. It has also been shown to phosphorylate MK2, which then phosphorylates TSC2 at Ser1210 (Katholnig et al., 2013a). In LPS-treated BMDM lacking the p38α gene and mouse embryonic fibroblasts (MEF) deficient difficient in the PI3K subunit p85, it was shown that p38α and PI3K independently regulate mTORC1. Furthermore, rapamycin did not modulate the phosphorylation of p38 or its downstream kinase MK2 after stimulation of human monocytes with LPS, but blocked the phosphorylation of the mTOR substrates

p70S6K and 4EBP1 (Katholnig et al., 2013a). Another upstream regulator named tpl2 kinase has been shown to activate mTORC1. Tpl2 is responsible for the activation of the MKK1/ERK1/2 pathway after LPS stimulation, as it has been shown to reduce mTORC1 and increase IL-12p40 in LPS-stimulated tpl2-deficient BMDM (Lopez-Pelaez et al., 2011). Furthermore, tpl2 has been shown to regulate cap-dependent mRNA translation via mTOR/4E-BP1 in TLR-activated macrophages (Lopez-Pelaez et al., 2012).

There are other interactions between the mTOR pathway and TLR signalling. mTORC1 inhibition by rapamycin decreases the production of nitric oxide in LPS-induced macrophages. This decrease of nitric oxide is an effect of reduced IFN-β secretion, as IFN-β is a cofactor for nitric oxide (Weinstein et al., 2000). Inhibition of mTORC1 with rapamycin during TLR stimulation also increases the expression of IL-12 via NF-κB and TNF-α, whereas the release of IL-10 is reduced via inhibited STAT3 activation (Weichhart et al., 2008, Baker et al., 2009). However conversely, knockdown of TSC2 in human monocytes results in activation of mTORC1 and enhances IL-10 but inhibits IL-12 (Weichhart et al., 2008, Chen et al., 2012). Furthermore, T cells stimulated with cytokines have been shown to induce IL-10 production in macrophages in a PI3K-dependent and S6K1-dependent manner (Figure 1.12) (Foey et al., 2002). Foey et al (2002) showed this in BMDMs co-cultured with cytokine activated T-cells using the inhibitors PI3K inhibitors wortmannin and LY294002 and mTOR inhibitor rapamycin. They also suggested that this reaction might have implications for rheumatoid arthritis patients, as spontaneous IL-10 production by rheumatoid arthrits synovial-membrane mononuclear cells and co-culture of rheumatoid arthrits T cells and macrophages was observed (Foey et al., 2002).

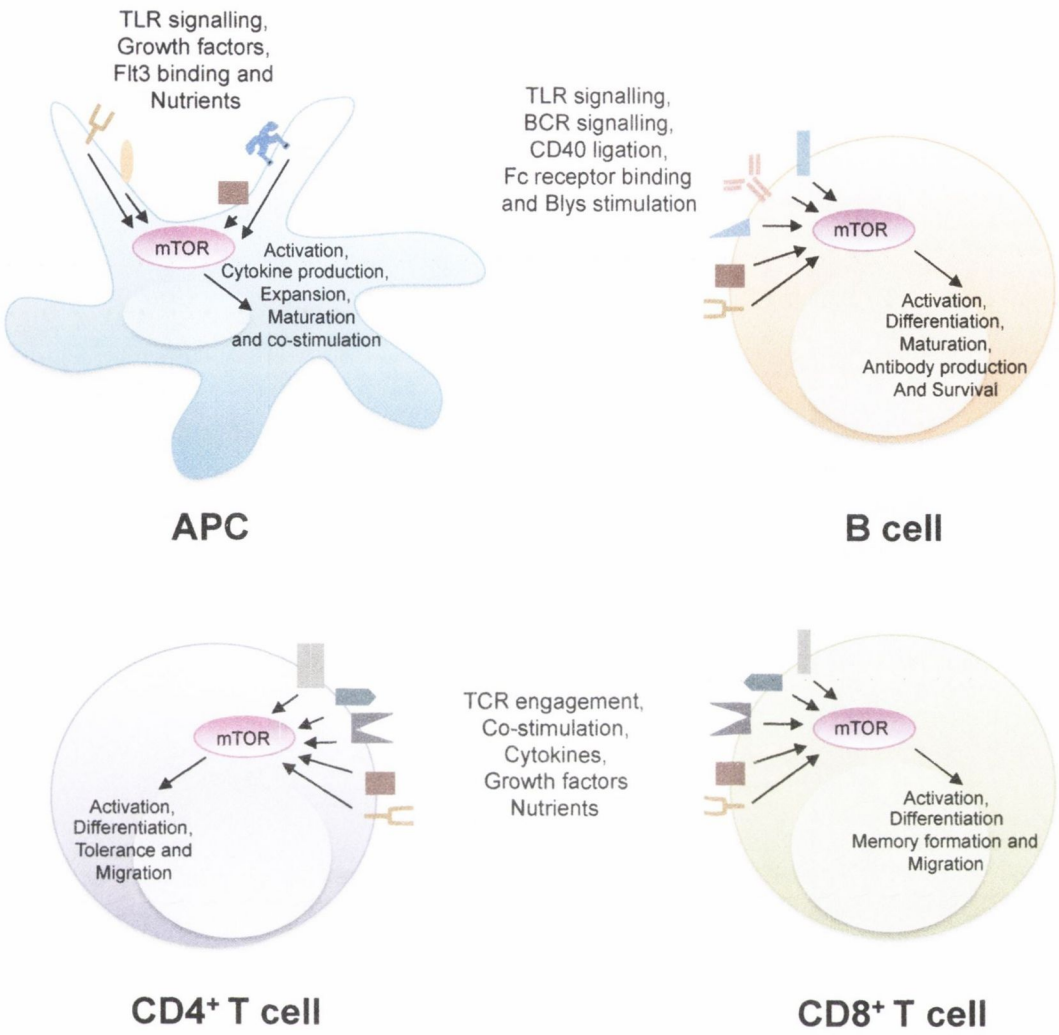


Figure 1.11 – mTOR and its role in regulating diverse immune cells.

mTOR is a central regulator of immune responses in antigen presenting cells, T cells and B-cells. Activation of the mTOR signalling by different stimuli results in the activation of each of these cell types, which have a specific response including APC cells will be activated upon mTOR activation by producing cytokines. Activation of mTOR in B-cells will lead to differentiation and maturation of these cells as well as start the production of antibodies.

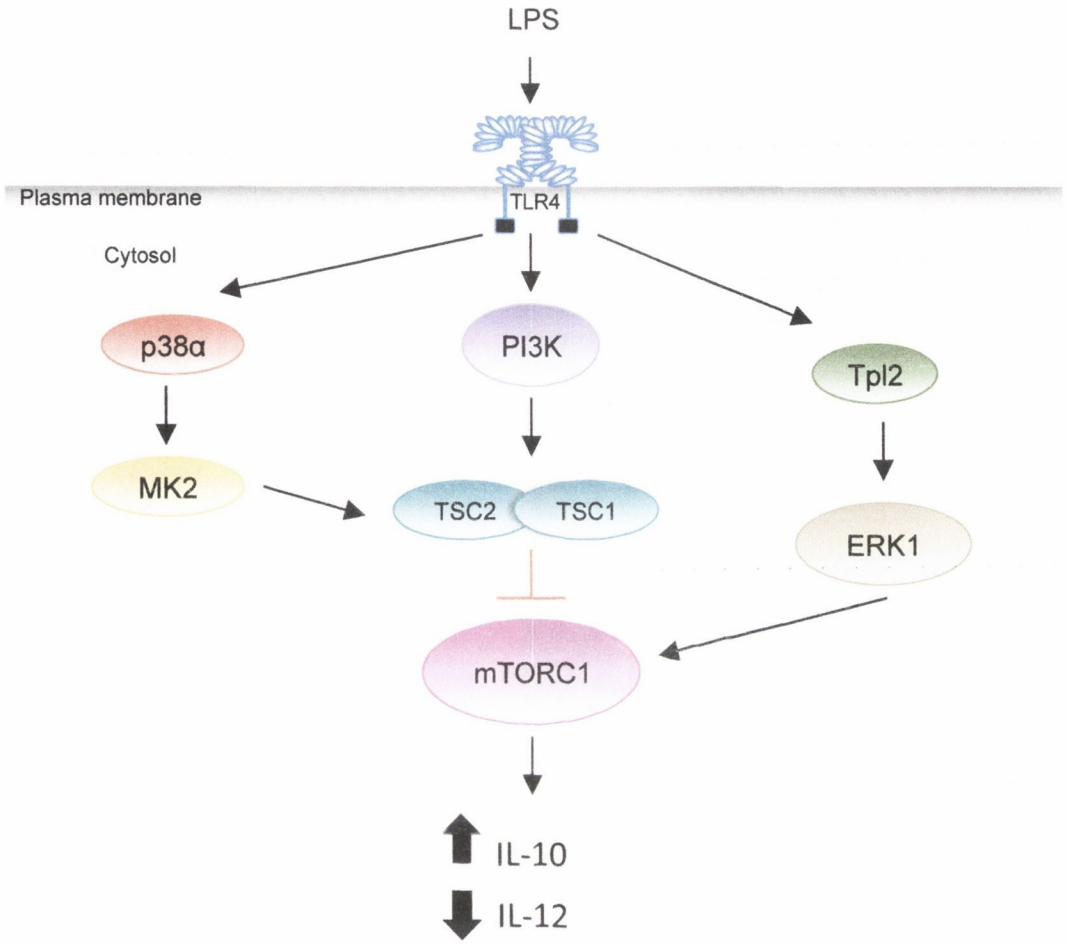


Figure 1.12 – TLR4 signalling activates the mTOR-signalling pathway in macrophages.

There are different signalling pathways involved in activation of the mTOR pathway. The activation of PI3K in response to LPS is the most studied one resulting in the activation of mTORC1. However the activation of p38α by LPS results in the activation of MK2, which can phosphorylate TSC2. Another kinase that regulates mTORC1 is Tpl2. It can activate mTORC1 via ERK1.

1.5 Twist transcription factors

As described above, the mTOR pathway acts as a regulator of PDCD4. mTOR will give rise to PDCD4 degradation, releasing translational factors such as eIF4A and the transcription factor Twist1 (Shiota et al., 2009). In mammals, there are two Twist family members, Twist1 and Twist2 (Wolf et al., 1991, Li et al., 1995). This section will describe why Twist2 might be a candidate for regulating IL-10 expression through PDCD4.

1.5.1 Twist transcription factor family

Both of the Twist proteins are between 30-45% conserved members of the Twist subfamily of Class B basic helix-loop-helix (bHLH) transcription factors (determined using basic local alignment search tool from NCBI) (Thisse et al., 1987). Other members are Paraxis, Scleraxis, Hand1 and Hand2. However, Twist1 and Twist2 are 66% identical. There are two other bHLH transcription factor classes; the ubiquitous Class A and the tissue restricted Class B bHLH transcription factors lacking the basic region to contact DNA. In the Twist subfamily, Twist1 and Twist2 exhibit a high degree of sequence similarity suggesting that their functions might be redundant. However studies have shown that their functions as gene regulators are unique (Franco et al., 2011). bHLH transcription factors like Twist1 and Twist2 act as homo or heterodimers through their HLH motifs to form a DNA-binding domain. The binding domain is able to recognise 5'CANNTG-3', a specific DNA sequence named an Enhancer box (E-box) formation on the promoters of target genes (Murre et al., 1989).

The first Twist protein described was the *Drosophila Twist* (DTwist), as one of the zygotic genes required for dorso-ventral patterning during embryogenesis (Simpson, 1983). However, it was later named Twist1. Twist2 was first identified from a yeast–two-hybrid screen and named Dermo-1 for its expression pattern in the dermis of mouse early embryo, although Dermo-1 was temporally expressed after Twist1 (Li et al., 1995). Later Dermo-1 was renamed Twist2 based on its high homology and overlapping expression pattern with Twist1. Twist2 is 66% identical to Twist1 and identity increases to 95% in the bHLH region and 100% in the C-terminal Twist Box (Figure 1.13). The major differences between the two proteins are found in the N-terminal region where Twist1 has two-glycine rich regions. However, these are absent in Twist2, making Twist1 202 amino acids bigger than Twist2, which has 160 amino acids. At gene level, *Twist1* and *Twist2* share common introns and exons that likely reflect evolutionary gene duplication (Wolf et al., 1991, Tukel et al., 2010). There are several pathways that control their expression, which include NF-κB- and STAT3-mediated cytokine signalling (Sosic and Olson, 2003, Cheng et al., 2008), the WNT/β-catenin (Corbin et al., 2009) and TGFβ pathways (Yu et al., 2009) and induction through hypoxia-inducible factor 1α (HIF1α) (Sun et al., 2009). However, specifics of how these pathways activate *Twist1* and *Twist2* gene regulation is not well understood.

The Twist proteins' classic roles are to inhibit differentiation of mesenchymal cells in muscle and bone. Muscle differentiation is activated by the myogenic bHLH transcription factor subfamily, with members such as MyoD. Twist1 and Twist2 are able to interact with E-protein E12 and MyoD and inhibit their specific function to activate myogenesis (formation of muscular tissue, particularly during embryonic development), by inhibiting specific genes. (Spicer et al., 1996). Id proteins also compete for binding with MyoD, Twist1 and Twist2 add an additional level of

regulation for dimer choice formation. This provided more evidence for how bHLH protein levels can influence partner choice and therefore affect gene expression.

MyoD-mediated activation of muscle differentiation requires the binding of myocyte enhancer factor-2 (MEF2) and involves the recruitment of histone acetyltransferase (HAT) to the promoter site of myogenetic genes (Spicer et al., 1996). It has been shown that direct binding of Twist1 or Twist2 to MEF2 acts as a repressor of muscle differentiation by promoting deacetylation, either by blocking HAT activity (Twist1 only) or by encouraging the recruitment of histone deacetylases (HDAC), which are known to block transcription (Spicer et al., 1996)

Twist1 and Twist2 are also key regulators of osteogenesis. A homodimer of Twist1 can activate fibroblast growth factor receptor-2 (Fgfr2) expression but when Twist1 is in an interaction with E12 it blocks Fgfr2 expression. Fgfr2 has been related to the expression of Runt-related transcription factor 2 (Runx2). Runx2 is considered to be a master regulator of osteogenesis due to its indispensable role in the regulation of most genes involved in maturing osteoblasts (Bialek et al., 2004). Twist1 and Twist2 can both bind to the Runx2 promoter, but binding was not correlated to changes in Runx2 expression (Zhang et al., 2008). Both Twist proteins are also known to interact at the protein level with Runx2 thereby inhibiting its binding to DNA and thus blocking the expression of osteogenic-specific genes (Bialek et al., 2004).

Despite the high similarity between Twist1 and Twist2, there is evidence that their functions as gene regulators are unique (Table 1.1) (Sosic and Olson, 2003). However, there are several factors that influence the functions of Twist1 and Twist2, including dimer choice, phosphoregulation and protein-protein interaction. The dimer choice is mainly influenced by the availability of other bHLH transcription factors in the cell but can also be regulated by the phosphorylation state of specific threonine

and serine residues that are conserved in the first α-helix of the HLH domain of Twist proteins (Figure 1.13). Mutations of these residues not only alter partner choice but also E-box binding (Firulli et al., 2007).

Table 1.1 comparing Twist1 and Twist2 functions

	Twist1 functions	Twist2 functions
Role in immune system development and response	Twist1 and Twist2 inhibit NF-κB mediated expression of pro-inflammatory cytokines (Pan et al., 2009).	
	Twist1 and Twist2 bind both direct E-boxes in promoters of cytokines such as TNF-α and IL-1β (Sosic et al., 2003).	
		Twist2 inhibits the production of pro-inflammatory cytokines as IL-12 and IFNγ (Sharabi et al., 2008).
		Twist2 promotes IL-10 cytokine production through c-Maf (Sharabi et al., 2008).
	Twist1 and Twist2 are both critical regulators of energy homeostasis involving adipose tissue (Pan et al., 2009, Pettersson et al., 2010).	
		Twist2 is an inhibitor of transcription factors ADD1/SREBP1c, which are involved in adipocyte differentiation (Lee et al., 2003, Kahn et al., 2006).
		Twist2 is a regulator of development of immune cells (Sharabi et al., 2008, Koh et al., 2009).
		Twist2 is a negative regulator of myeloid lineage development by inhibiting proliferation and differentiation of macrophage progenitors (Runx1) (Sharabi et al., 2008).
	Twist1 is able to protect B-cells from apoptosis through mediating IL-17 (Doreau et al., 2009).	
		Twist2 was found to be overexpressed in SLE patients (Doreau et al., 2009).
Role in Cancer	Twist1 plays a role in migration of neural crest cells during embryogenesis (O'Rourke et al., 2002).	
	Twist1 and Twist2 function both as a proto-oncogenes by protecting cells from Myc and p53-dependent apoptosis (Maestro et al., 1999).	
	Twist1 is an upregulator of proto-oncogenes YB-1 and AKT2 (Shiota et al., 2008, Cheng et al., 2007).	
	Twist1 and Twist2 regulate cyclin-dependent kinase inhibitor p21, which is involved in growth (Funato et al., 2001, Murakami et al., 2008).	
	Twist1 binds to miRNA promoters such as miR-199/214 and miR-10b, which results in repression of target genes implicated in development and cancer (Lee et al., 2009, Ma et al., 2007).	
	Twist1 and Twist2 both inhibits apoptosis and lose of cell adhesion, which are important for wound healing and correlated with high Twist1 and Twist2 expression in cancer patients (Barnes et al., 2009).	
Role in muscle and bone differentiation	Twist1 and Twist2 interact with MyoD, MEF2 and HDAC proteins, which are required for myogenesis. (Spicer et al., 1996, Hamamori et al., 1997).	
	Twist1 and Twist2 interact with Runx2 protein resulting in inhibition of osteogenic specific genes which are required for maturing osteoblasts (Bialek et al., 2004, Komaki et al., 2007).	

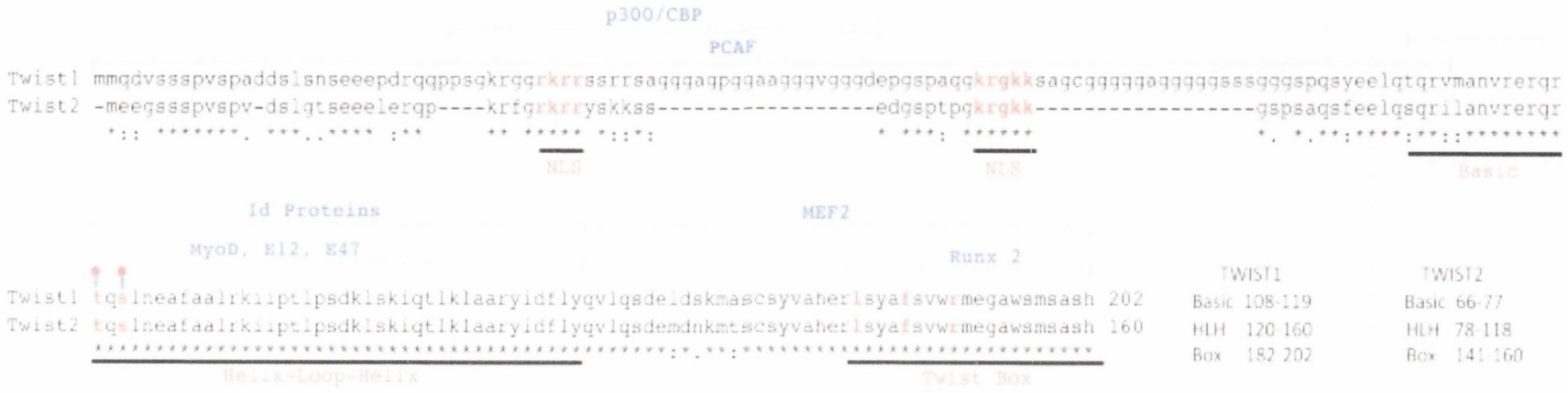


Figure 1.13 Amino acids sequence alignment between Twist1 and Twist2.

The functional motifs are delineated with black bars including the two nuclear localization sequence (NLS), the basic Helix-Loop-Helix and the Twist box. Conserved threonine and serine phosphor-regulated residues involved in dimer choice and DNA-binding site selection are indicated, and protein-protein interaction domains are located above the alignment. It is interesting to note that the Twist box has been characterized as both an activation domain with the amino-acid motif LX3FX3R (indicated in red) and as a repressor domain (Franco et al., 2011).

1.5.2 Twist2 and its role in the immune system

Twist2 and Twist1 are key regulators of the development of immune cells. Twist2 can be found in B cells from patients with systemic lupus erythematous (SLE) (Doreau et al., 2009). In chronic inflammatory diseases, the proliferation and differentiation of B cells into immunoglobulin-secreting cells, are influenced by IL-17 and B cell activating factor (BAFF) (Doreau et al., 2009). Twist1 was shown to play a central role as a mediator of IL-17 and BAFF. The result of this is to protect B-cells from apoptosis through the induction of anti-apoptotic genes such as Twist2. After Twist2 induction by Twist1, Twist1 levels decrease while Twist2 levels are maintained (Doreau et al., 2009). This suggests that Twist2 is the primary anti-apoptotic effector in the B-cell response. Twist2 has also been shown to be a key negative regulator of myeloid lineage development as it inhibits proliferation and differentiation of macrophages (Sharabi et al., 2008). The repression by Twist2 of macrophage proliferation is mediated by the direct interaction with and inhibition of the transcription factor Runx1, a family member of Runx2 and CCAAT/enhancer binding protein (C/EBP) (Sharabi et al., 2008). Runx1 is known to regulate haematopoiesis which is the proliferation of haematopoietic stem cells in the bone marrow to different mature blood cells and tissue.

It has also been shown that Twist2 can interact with transcription factor NF-κB via its C-terminal domain (Sosic et al., 2003). Sosic et al. (2003) have proposed a negative feedback loop in which inflammatory cytokines activate NF-κB and NF-κB activates the expression of cytokines via both Twist1 and Twist2 (Sosic et al., 2003, Franco et al., 2011). In turn, it has been demonstrated that Twist2 modulates the expression of pro-inflammatory cytokines through inhibition of NF-κB activation by interacting with the p65 subunit (Sosic et al., 2003). Twist2 and Twist1 are also known to bind to E-boxes in the promoters of cytokines regulated by NF-κB as shown in Figure 1.14 (Sosic et al., 2003). Studies with Twist2-deficient mice show a significant perinatal death rate and high levels of pro-inflammatory cytokines. Further studies have shown that Twist2 regulates cytokine production by inhibiting pro-inflammatory cytokines such as IL-12 and IFN-γ. Twist2 also promotes production of the anti-inflammatory cytokine IL-10 by regulating expression of c-maf, a transcription factor responsible for IL-10 transcription (Sharabi et al., 2008). Twist2 plays a more critical role in the direct regulation of inflammatory cytokines than Twist1. This may account for the immune dysfunction observed specifically in Twist2-deficient mice (Sosic et al., 2003). However, the subsequent induction and maintenance of Twist2 levels after Twist1 disappearance in immune system proliferation and responses suggest that Twist2 plays a more critical role in the direct regulation of inflammatory cytokines than Twist1.

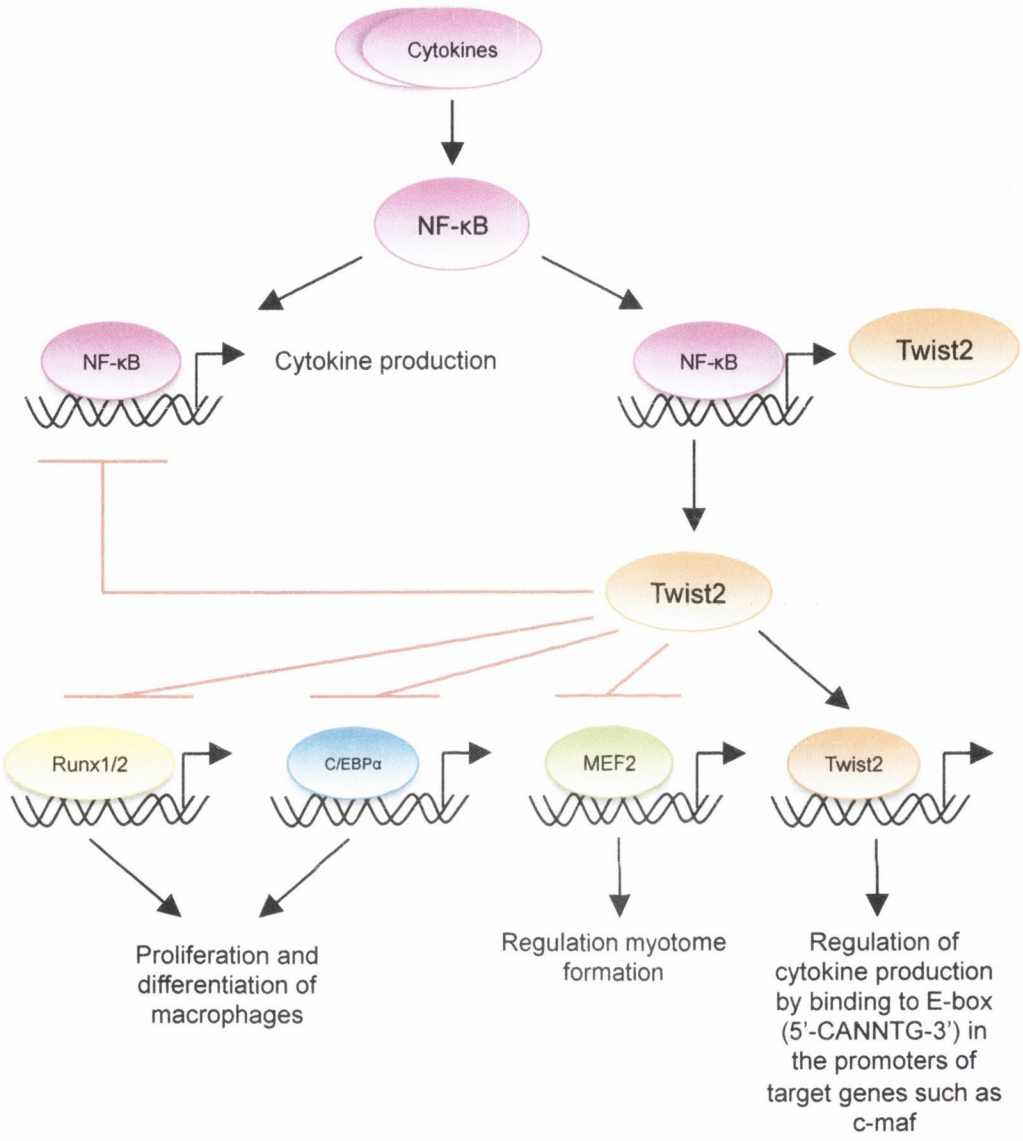


Figure 1.14 – Effects of Twist2 on transcription.

Cytokines can activate the transcription factor NF-κB resulting in the transcription of cytokines but NF-κB can also influence Twist2 transcription as the Twist2 promoter contains a NF-κB binding site. Twist2 is a transcription factor, which can modulate the expression of pro-inflammatory cytokines through inhibition of NF-κB activation, as Twist2 is able to inhibit NF-κB p65 subunit through protein interaction. But Twist2 can also recognise E-box formation (5'-CANNTG-3') in the promoter regions of target genes regulated by NF-κB, by its DNA binding domain. Besides this, Twist2 can interact with RUNX1/2 and C/EBPα resulting in inhibition of the proliferation and differentiation of macrophages. Twist2 is also known to interact with the transcription factor MEF2, which is known to regulate myotome formation in muscles.

1.6 c-Maf transcription factor

It has been shown by Sharabi et al. (2008) that Twist2 binds to the c-Maf promoter. Furthermore, IL-10 production by T helper 17 cells has been shown to be directly regulated by c-Maf, since c-Maf was able to bind to the Maf recognition element (MARE) in the IL-10 promoter (Xu et al., 2009). It also has been shown that IL-10 gene expression in macrophages lacking c-Maf was impaired, although c-Maf alone is not sufficient to induce *Il10* expression (Cao et al., 2005).

c-Maf is the cellular counterpart of oncogenic v-Maf, which was originally discovered in an oncogenic retrovirus (AS42) and induces musculaoponeurotic fibrosarcoma (Maf) *in vivo* (Nishizawa et al., 1989). c-Maf belongs to the family of basic region leucine zipper domain transcription factors, as they all share the same b-Zip motif which mediates DNA binding and dimer formation. There are two forms of human c-Maf mRNA, c-Maf-long and c-Maf-short, as the result of differential splicing (Valanciute et al., 2004, Chesi et al., 1998). c-Maf is known to recognise MARE in DNA and can heterodimerize with the transcription factor AP-1, which can be activated by TLR signalling (Kataoka et al., 1994, Motohashi et al., 1997). The cell lineage-specific targets of c-Maf have been identified. For example, it has been shown that c-Maf is the key mediator of the TGF-β-dependent suppression of IL-22 in T helper 17 cells (Rutz et al., 2011), suggesting that c-Maf is one of the master regulators of Th2 differentiation. It has also been shown to target the crystalline genes in lens fiber cells (Kawauchi et al., 1999, Kim et al., 1999), the insulin gene in islet β cells (Matsuoka et al., 2003), p53 (regulates cell-death)(Hale et al., 2000), and L7 (neuron specific gene)(Kurschner and Morgan, 1995). The first direct in vivo demonstration of a physiological role of c-Maf was provided by studies in c-Maf-null mice (Kawauchi et al., 1999, Kim et al., 1999, Ring et al., 2000). Disruption of the c-Maf gene affected both intrauterine and postnatal survival (Kim et al., 1999). Recently

c-Maf has been shown to be involved in autoimmune diseases as increased c-Maf expression was involved in enhanced IL-21 production by regulatory T cells (Iwamoto et al., 2014). Therefore it appears that c-Maf is both a developmentally and immunologically important gene.

1.7 Project Aims and Objectives

IL-10 is a critical immunomodulatory cytokine. The role of IL-10 production in response to LPS is not fully understood. This project builds on previous observations implicating PDCD4 as a negative regulator of IL-10 production and a role for the central immune kinase mTOR in PDCD4 regulation. It further builds on observations regarding Twist1 as a key PDCD4-interacting protein and the possibility of Twist2 being involved in the regulation of IL-10 transcription via c-Maf. The degradation of PDCD4 and the increase of IL-10 after LPS stimulation form the basis for this research project. The overall aim is to investigate the mTOR/PDCD4 axis in the control of IL-10 in LPS-activated macrophages.

The project has two broad aims:

1. To investigate if mTOR signalling is required for LPS-induced PDCD4 degradation and subsequent induction of IL-10.

2. To investigate whether PDCD4 is controlling Twist2 to limit c-Maf induction and subsequent IL-10 production. .

The overall goal is therefore to explore whether induction of IL-10 by LPS involves the targeting of PDCD4 by the mTOR pathway, leading to Twist2 release with subsequent induction of c-Maf, resulting in IL-10 production. The study could therefore provide exciting new insights into the mechanism of induction of the critically important immunomodulatory cytokine IL-10.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 General Laboratory Chemicals

All general laboratory chemicals were purchased from Sigma (Poole, Dorset, UK).

2.1.2 Drugs

LPS from E. Coli (L4391 10mg, dissolved in sterile water and aliquotted in concentration of 2mg/ml), Wortmannin (W1628 1mg, dissolved in sterile DMSO with a concentration of 1mg/ml), MG132 (M7449 1mg, ready made solution), PF-4708671 (PF0143 5mg, dissolved in sterile DMSO with a concentration of 1mg/ml) and LY294002 (W9908 1mg, dissolved in sterile DMSO with a concentration of 1mg/ml), were all purchased from Sigma (Poole, Dorset, UK). Rapamycin (R-5000 >99% 250mg) was purchased from LC Laboratories (Boston, USA). 10mg rapamycin powder was for every needed day measured on a scale as sterile as possible. The 10mg was dissolved in sterile DMSO to a final concentration of 10mg/ml. The concentrated rapamycin stock was used for lower concentrations if needed.

2.1.3 Cell culture media

DMEM was purchased from Gibco Biosciences (Dun Laoghaire, Ireland). Foetal calf serum was purchased from Biosera (East Sussex, UK). Penicillin, streptomycin and trypsin were purchased from Sigma (Poole, Dorset, UK).

2.1.4 Cell Lines

RAW264.7 cells were obtained from ATCC (Manassas, USA). HEK293TLR4-MD2-CD14 cell lines were obtained from Invivogen. C57/BL6 PDCD4 knockout bone marrow-derived macrophages (BMDMs) were a kind gift from Derek Johnson, University of Pennsylvania, USA.

2.1.5 Animals

Animals were maintained under specific pathogen-free conditions in line with Irish and European Union regulations. Experiments were approved by local ethical review and were carried out under the authority of Ireland's project license. Wild-type C57/BL6 mice were obtained from the Bioresources unit at Trinity College Dublin.

2.1.6 ELISA Reagents

Tween-20 and Bovine Serum Albumin (BSA) were purchased from Sigma (Poole, Dorset, UK). Mouse IL-6 and mouse IL-10 ELISA Duoset kits were purchased from R&D Biosystems (Abingdon, UK). TMB substrate solution was purchased from R&D Biosystems (Abingdon, UK).

2.1.7 PCR Reagents

RNase Zap, RNase-free H_20 and RNeasy Plus Mini Kit were purchased from Qiagen. PCR plates, PCR fast plates, 10x RT buffer, dNTPs, RNase Inhibitor, Reverse Transcriptase, 2x Fast PCR buffer were purchased from Applied Biosystems (Huntington, UK). SYBR green PCR master mix was purchased from Kappa PCR Biosystems (Dun Laoghaire, Ireland).

2.1.8 DNA Transfection Reagents

GeneJuice® was purchased from Novagen. Lipofectamine 2000 Transfection reagent was purchased from Invitrogen.

2.1.9 Western Blotting Reagents

APS, N, N, N, N Tetra Methylethylenediamine (TEMED), 10% SDS, Tris-HCL, Acrylamide: Bisacrylamide were all purchased from Sigma (Poole, Dorset, UK), ECL LumiGlow from Cell Signalling, Spectra™ multicolor marker was purchased from Thermo scientific X-Ray film was purchased from Kodak.

2.1.10 Antibodies

The mouse β-Actin, mouse FLAG, mouse HA, rabbit IgG antibodies were all purchased from Sigma (Poole, Dorset, UK). The rabbit PDCD4 antibody (#9535), Akt thr 308 (#13038), Akt Ser473 (#4060), S6K thr389 (#9234), 4EBP1 (#2855) and pS6 Ser235/236 (#4858) was purchased from Cell Signalling and the rabbit Twist2 (ab66031) antibody and c-Maf (ab72584) was purchased from AbCam (Cambridge, UK). The rabbit Twist antibody (H-81) (sc-15393) was purchased form Santa Cruz Biotechnology (Heidelberg, Germany).

2.1.11 Plasmids

The human HA-tagged PDCD4 expression vector was kindly provided by Dr. Michele Pagano and subcloned into pc.DNA3.1. The human Myc-DDK-tagged (FLAG-tag) ORF clone of Twist2, cloned into pCMV6, was purchased from OriGene Technologies (Rockville, USA).

2.1.12 Primers

The Taqman primers used for Real Time PCR were purchased from Applied Biosystems (Huntington, UK). The primers for SYBR Green used in the ChIP assay and Mutagenesis of Twist2 and the biotin-tagged primers used in the oligonucleotide pull down were designed and purchased from Eurofins MWG Operon (Ebersberg, Germany). The primers used are detailed below in Table 2.1

2.1.13 Bacteria

For transformation, aliquots of *DH5α E.coli* competent cells from Invivogen were used.

Table 2.1 – Oligonucleotides for TaqMan Real Time PCR, SYBR green and Oligonucleotide pull down.

	Oligo nucleotide	Sense primer sequence	Antisense primer sequence
TaqMan PCR probe	mGAPDH Mm99999915	Not available on website	Not available on website
	mIL-10 Mm00439614	Not available on website	Not available on website
	mIL-6 Mm00446190	Not available on website	Not available on website
	mTwist2 Mm00492147	Not available on website	Not available on website
	mPDCD4 Mm01266062	Not available on website	Not available on website
SYBR green primers (ChIP)	c-Maf	TGACGTCAGGCTCAAATGGGAA	AGTCAGCTGATTGGGCTGTGAT
	c-Maf neg	TTCCCAGTTCACATTCAGCCCT	ACGCATCATCATTCCTGGCT
	b-actin	TGGTGTCCGTTCTGAGTGATCC	TCGCTCTCTCGTGGCTAGTACC
	IL-10 primer set 1	TGGGAGTGCGTGAATGGAAT	TGTATTTCCTGAGGCAGACAGC
	IL-10 primer set 2	ATTATGACCTGGGAGTGCGTG	GAGGCAGACAGCTGTTCTATG
	IL-10 neg	CAGTCAGGAGAGAGGGCAGTG	TTTCCAACAGCAGAAGCAAC
Oligonucleotide pull down probe	c-Maf	BIO-CAGGCTCAAATGGGAAATTG	CAATTTCCCATTTGAGCCTG
Mutagenesis of Twist2	Twist2 Q118-Stop	CATAGACTTCCTCTACTAGGTCCTG CAGAGCGA	TCGCTCTGCAGGACCTAGTAGAGGAAG TCTATG
	Twist2 Q66-Stop	GGAGCTGCAGAGCTAGCGCATCCTG GC	GCCAGGATGCGCTAGCTCTGCAGCTCC
	Twist2-FLAG T78A/S81A	AGCGCCAGCGCGCCCAGGCGCTCAA CGAG	CTCGTTGAGCGCCTGGGCGCGCTGGCG CT
	Twist2 I92-Stop	GCGCTGCGCAAGATCTAGCCCACGC TGCCCTCT	AGAGGGCAGCGTGGGCTAGATCTTGCG CAGCGC
	Twist2-FLAG I92T/I93S/Y113C/I114S	CGCGGCGCTGCGCAAGACCAGCCCC ACGCT	AGCGTGGGGCTGGTCTTGCGCAGCGCC GCG
	Twist-FLAG (Y113C/I114S)	AAGCTGGCCGCCAGGTGCAGAGACT TCCTCTACC	GGTAGAGGAAGTCTCTGCACCTGGCGG CCAGCTT

2.1.13 Buffers and Media

The buffers and solutions used in the laboratory are detailed in Table 2.2 below. The tissue culture media used for cell culture are detailed in Table 2.3 below.

Table 2.2 – Buffers and solutions.

Buffer solutions	Compositions	
1% electrophoresis running buffer	25nM Tris, 1.92 Glycine and 1% (w/v) SDS	
Phosphate Buffered Saline (PBS)	37nM NaCl, 2.7mM KCl, 4.3 mM Na2HPO4 and 1.4 mM KH2PO4	
Sample Lysis buffer (5x)	10% (v/v) Glycerol, 1% (v/v) SDS, 80% (v/v) Triton Lysis buffer and a few grains of Bromophenol Blue, 1mM DTT	
Transfer Buffer	48mM Tris-Acetate and 1mM EDTA	
Tris Buffered Saline/ TWEEN (TBST)	50mM Tris pH 7.5, 150mM NaCl and 0.2% (v/v) TWEEN-20	
Co-IP lysis buffer	50mM Hepes pH7.5, 100mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40, 1mM PMSF, 1µg/mL Leupeptin, 11.5µg/mL Aprotinin, H_2O	
ChIP buffer	ChIP lysis buffer	100mM NaCl, 50 mM Tris pH 8.1, 5mM EDTA pH 8.0, 0.02% NaN_3, 0.5% SDS, H_2O
	Triton dilution buffer	100mM Tris pH 8.6, 100mM NaCl, 5 mM EDTA, 0.02% NaN_3, 5% Triton-X-100, H_2O
	Mixed Micelle Wash Buffer, 1mL	150mM NaCl, 200mM Tris pH8.1 5mM EDTA, 65% w/v sucrose, 1%w/v Triton-X-100, 0.2% SDS 0.02% NaN_3, H_2O
	Buffer 500	1% w/v deoxycholic acid, 1mM EDTA, 50mM HEPES pH7.5, 1%w/v Triton-X-100, 0.02% NaN_3, H_2O
	Li/Cl detergent solution	0.5% w/v deoxycholic acid, 1mM EDTA, 250mM LiCl, 0.5% w/v NP-40, 10mM Tric pH 8.0, 0.02% NaN_3, H_2O
Oligonucleotide pull down buffers	2x Stock	50mM Tris pH7.9, 100mM EDTA pH8.0, 10% Glycerol, 10mM NaF pH8, H_2O
	Buffer A	1x 2x stock, 150mM NaCl, 1% NP40, 1µg/mL Leupeptin, 1µg/mL Aprotinin, 1mM DTT, 1mM Na3VO4, 1mM PMSF, H_2O
	Buffer B	1x 2x stock, 1% NP40, 1µg/mL Leupeptin, 1µg/mL Aprotinin, 1mM DTT, 1mM Na3VO4, 1mM PMSF, H_2O

Table 2.3 – Media solutions and compositions used for cell culture.

Media Solution		Composition
Supplemented DMEM medium (High glucose Dulbecco's Modified Eagle Medium)	RAW264.7 cells	DMEM containing 10%(v/v) heat in activated foetal calf serum (FBS), 1%(v/v) penstrep (comprising the antibiotics 100µg/mL Peniclillin, 100µg/mL streptomycin)
	HEK293TLR4-MD2-CD14	DMEM containing 10%(v/v) heat in activated foetal calf serum (FBS), 50mg/mL Noromycin, 100µg/mL Blsaticidin, 100µg/mL Hydrogold
	BMDM (BMDM medium)	DMEM containing 10% (v/v) heat inactivated foetal bovine serum, 1%(v/v) penicillin/streptomycin (comprising the antibiotics 100µg/mL Peniclillin, 100µg/mL streptomycin) and 20% L929 cell media

2.2 Methods

2.2.1 Primary cell culture

2.2.1.1 Generation and plating of bone marrow-derived macrophages

Bone marrow was flushed from both femurs of mice with sterile Phosphate Buffer Saline (PBS). The PBS/bone marrow suspension was passed through a sterile cell strainer (100 μM), collected and centrifuged at 300x g for 5 minutes. The supernatant was then aspirated and the remaining pellet was resuspended in 20 mL of BMDM medium. This was then plated onto two 10 cm bacterial dishes. Cells were cultured for 7 days at 37°C and 5% CO_2.

After 7 days of growth, BMDMs were passaged using a sterile RNAse free cell scraper, into a 12- or 24-well plate in preparation for stimulation. Cells were prestarved with 1% FCS and 1% L929 medium. These were incubated overnight at 37°C and 5% CO_2 in preparation for stimulation the following day.

2.2.1.2 Stimulation of bone marrow derived macrophages

BMDMs were cultured for 7 days and split into 12- or 24-well plates with 1% FCS and 1% L929 medium. The following day the BMDMs were pretreated with 500nM rapamycin and stimulated with 100 ng/mL lipopolysaccharide (LPS), for 1, 2, 4, 6, 8 or 20 hours. Unstimulated cells were used as a control. After stimulation the medium was removed from the cells and stored at -80° C for subsequent measurement of cytokine concentrations, as described in section 2.2.4.7 Cytokine measurements. The cells were lysed for RNA isolation as described in section 2.2.5 Analysis of cellular nucleic acids, or for analysis of cellular protein (section 2.2.4).

2.2.2 Mammalian cell lines

2.2.2.1 RAW264.7

RAW264.7 cells were cultured in T175 flasks in RAW264.7 medium. Once cells were 80-90% confluent they were passaged by washing with 10 mL PBS and scraping the cells with a cell scraper in supplemented DMEM medium. The cell suspension was plated at a ratio of 1:10 in T175 flasks for starting an experiment, the cell suspension was counted with Trypan Blue, which is excluded by live cells and taken up by non-viable cells, using a haemocytometer and bright light microscope and passaged at a ratio of $5x10^5$ cells per mL on a 12-well plate or 10 or 15 cm dish (for ChIP or oligonucleotide pull down), or plated at a ratio of $1x10^5$ cells per mL on a 12- or 24-well plate (for transfections), and placed at 37°C and 5% CO_2.

2.2.2.2 HEK293TLR4-MD2-CD14 cells

HEK293TLR4-MD2-CD14 cells were cultured in T175 flasks in supplemented DMEM medium containing 10% FCS as well as the antibiotics 50mg/ml Noromycin, 100µg/ml Hydrogold and 100µg/ml Blastinomycin. Once 80-90% confluent, cells were passaged by washing with 10 mL PBS and incubated for 5-10 minutes with 3 mL Trypsin. After incubation, 7 mL of medium was slowly added and the lifted cells were centrifuged for 5 minutes at 300g. The pellet was replated at a ratio of 1:10 in T175 flasks. For starting an experiment, the cell suspension was counted using Trypan Blue, which is excluded by live cells and taken up by non-viable cells, using a haemocytometer and bright light microscope, and passaged at a ratio of $5x10^5$ cells per mL on a 10 or 15 cm dish and placed at 37°C and 5% CO_2.

2.2.3 Site-directed mutagenesis of Twist2

The QuickChange site-directed mutagenesis kit (Stratagene) was use to mutate or delete certain basis in the Twist2 gene, which were obtained from the pCMV6 plasmid containing the Myc-DDK-tagged (FLAG-tag) ORF clone of Twist2. The manufacturer's instructions were followed using primers incorporating the desired mutations and deletions. The plasmid containing Twist2 was being mutated to find a mechanistic function for Twist2. Therefore, the amino acids located on 79 and 81 in the DNA binding domain were mutated in Thr78Ala and Ser81Ala. Previously it has been shown that PDCD4 interacts with Twist1 (Shiota et al., 2009) and eIF4A (Yang et al., 2003a). It was found that the amino-terminal region of eIF4A possesses sequence homology with the bHLH of Twist1 (Shiota et al., 2009) and Twist2 (data not shown), which is evolutionarily conserved. Therefore amino acids located on 92, 93, 113 and 114 in the protein binding domain of Twist2 where mutated in the protein binding domain in Ile92Thr, Ile93Ser, Tyr113Cys and Ile114Ser. The identity of all Twist2 mutations was confirmed by sequencing the plasmid (see DNA sequencing 2.2.5).

2.2.4 Determination of DNA concentration

To determine the concentration of a DNA solution, the plasmids were diluted in sterile MilliQ water and quantified with the Nanodrop 2000. For a DNA solution, a 260:280 ratio of 1.6 or greater is indicative of a highly purified sample. As a blank sterile MilliQ water was used. The DNA samples were used for transformation and DNA sequencing or stored at -20°C.

2.2.5 DNA sequencing

For DNA sequencing, triplicates of each sample were sent for sequencing. DNA

sequencing was performed by TrinSeq from Trinity Genome Sequencing Lab located in the Institute of Molecular Medicine, Trinity College Dublin. Sequencing samples were prepared from 150-500 ng DNA. The primers were selected in the TrinSeq program and the primers frequently used for sequencing are indicated here: CMV profor: 5'-GGCGGTAGGCGTGTA-3' were used for pCMV6 plasmids containing FLAG-tagged Twist2 sequence. T7: 5'-GTAATACGACTCACTATAGGGC-3' were used for pc.DNA3.1 plasmids containing HA-tagged PDCD4. Sequencing results were viewed and DNA alignments using ApE Universal software.

2.2.6 Transformation of competent E. coli

For transformation, an aliquot of *DH5α E.coli* competent cells was thawed on ice. Plasmid DNA (100 ng – 1µg) was added, mixed gently and incubated on ice for 5 minutes. The cells were heat shocked in a water bath at 42°C for exactly 90 seconds, and returned to ice for a further 2 minutes. Then, 500µL LB medium was added and incubated at 37°C for 30 - 60 minutes prior to plating on three selective LB agar plates containing 100µg/ml ampicillin (pc.DNA3.1 HA-tagged PDCD4 plasmid) or 50µg/ml kanamycin (pCMV6 FLAG-tagged Twist2 plasmids). Colonies were grown at 37 °C overnight.

2.2.7 Purification of plasmid DNA

To prepare micrograms quantities of plasmid DNA, an individual colony of plasmid-transformed bacteria was added into 4 ml (or 500 ml) of LB broth containing 100µg/ml ampicillin (pc.DNA3.1 HA-tagged PDCD4 plasmid) or 50µg/ml kanamycin (pCMV6 FLAG-tagged Twist2 plasmids) and incubated overnight at 37°C in a shaking incubator. Bacterial cells were harvested by centrifugation at 3000 rpm for 15 min at 4°C in a centrifuge. Microgram quantities of plasmid DNA were purified from the

bacterial pellet using the QIAprep maxi system (Qiagen) or QIAprep mini system (Qiagen), according to the manufacturer's instructions. The QIAprep mini system was mainly used when sequencing plasmid DNA were required.

2.2.8 Transfection of cells

2.2.8.1 siRNA transfection of RAW264.7 cells

RAW264.7 cells were plated at a ratio of $1x10^5$ cells/mL in a 24 well plate in antibiotic-free and 1% FCS DMEM medium. 2µL Lipofectamine 2000 with 100µL Optimem or DMEM was added per well and siRNA specific for PDCD4 or S6K1 or a negative control (6.25, 25nM) was added to each well. Complexes were incubated for 5 minutes before being combined and incubated again for a further 20 minutes. 200µL of the mixture was added per well and cells were incubated at 37°C and 5% CO_2 for 24 hours.

2.2.8.2 Plasmid transfection of RAW264.7 and HEK293TLR4-MD2-CD14 cells

RAW264.7 cells and HEK293TLR4-MD2-CD14 were plated at a ratio of $5x10^5$ cells/mL in a 10 or 15 cm dish in antibiotic-free and 1% FCS DMEM medium except for HEK293-TLR4-MD2-CD14 for which 50mg/ml Noromycin, 100µg/ml Hydrogold and 100µg/ml Blastinomycin is needed. 15-18µL GeneJuice with 235µl DMEM was added per well and 2µg DNA plasmid (pc.DNA3.1 HA- tagged PDCD4, pCMV6 FLAG-tagged Twist2 or empty vector) and 250µl DMEM medium was added to each plate. Complexes were incubated for 5 minutes before being combined and incubated again for a further 20 minutes. The mixture of 500µL was added per plate and cells were incubated at 37°C and 5% CO_2 for 24 hours.

2.2.9 Analysis of cellular proteins

2.2.9.1 SDS lysis for total denatured protein

To isolate total cellular protein for immune blotting, the medium was removed from the tissue culture plates. Cells were lysed using SDS Lysis Buffer in the required volume depending on well size. The plates were scraped using a pipette tip or cell scraper and transferred to a 1.5 mL microcentrifuge tube. The samples were heated to 95°C for 5-15 minutes and passed through a 25 gauge needle to break the aggregated chromatin. The protein concentration was not measured due to the presence of SDS and bromophenol blue in the lysis buffer. The protein samples were stored at -20°C until required.

2.2.9.2 Protein quantification

Protein concentration of samples lysed in lysis buffer without SDS were measured using the Bradford Assay. In a spectrophotometer cuvette, 1 or 2 µL of the protein sample was added to 1 mL of Coomassie Bradford Reagent. The cuvettes were mixed using a vortex. A control of Coomassie Bradford Reagent alone was used to normalize the spectrophotometer at 595 nm. Duplicates or triplicates of each absorbance were averaged and the protein concentration was calculated by reference to a standard BSA solution of known concentration.

2.2.9.3 SDS polyacrylamide gel electrophoresis

The protein samples and a marker (Spectra™ multicolor) were separated on a 12% separating gel and stacking gel using 1% electrophoresis buffer. The separating gel

includes 10% Acrylamide, 1.5 M Tris-HCl pH 8.6, 0.01% SDS, 0.08% TEMED, and 0.03% APS and the stacking gel includes 3.8% Acrylamide, 2 M TRIS-HCl pH 6.8, 0.01% SDS, 0.1% TEMED, and 0.08% APS. Gels were run at a constant 25mA per gel for approx. 1.5 hours, until the dye front had run to the bottom of the gel.

2.2.9.4 Electrophoretic transfer of proteins

After electrophoresis, the protein samples in the gel were transferred onto an Immobilon polyvinylidene difluoride (PVDF) membrane using semi-dry transfer apparatus. Four pieces of filter paper were cut into rectangles and the first piece of filter paper was soaked in buffer 1 (0.3M Tris pH10.4 and 20% MeOH) and placed at the bottom of the semi-dry transfer plate. The second piece was soaked in Buffer 2 (0.025M Tris pH10.4 and 20% MeOH) and placed on top of the first paper. Following this the PVDF membrane was soaked in 100% MeOH and placed on top of the papers. The gel used in the SDS polyacrylamide gel electrophoresis was placed into the PVDF membrane followed by two filter papers soaked in Buffer 3 (0.025M Tris pH 9.7, 0.04M Caproic Acid and 20% MeOH). A small roller was used to remove the existing air bubbles. An Avantec power supply was used to provide the current for transfer and was set at 15V, 800mA and 300W for 35 minutes. Following this the membranes were quick-stained with Ponceau S to check that the proteins were present on the membranes.

2.2.9.5 Antibody incubation and visualization

After transfer, primary antibodies (section 2.1.10 Antibodies) were used to detect specific proteins. First the PVDF membranes were blocked by incubation with 5% (w/v) skimmed milk powder in 10% Tris buffered Saline (TBS) and 0.1% Tween

(1%TBST) for one hour at room temperature. They were then incubated overnight in primary antibody in 5% (w/v) BSA or 5% (w/v) skimmed milk powder in 1% TBST at 4°C overnight. The following day the membranes were washed three times for at least 10 minutes per wash in 10% TBST. After the wash step, the membranes were incubated for one hour in 5% (w/v) skimmed milk powder in 1% TBST containing horseradish peroxidase (HRP)-conjugated secondary antibody specific to the primary antibody. After incubation, the membranes were washed three times for at least 10 minutes per wash with TBST and bands on the membrane were visualized with ECL Western Blotting Detection reagents. Bands were exposed on a medical X-Ray film, which were developed in an automatic film developer and the BioRad ChemiDoc.

Important note: Protein samples were run on multiple gels and transferred onto membranes. The membranes were sometimes cut to allow protein detection using multiple antibodies. This may led to different patterns of bands for the same samples. The figures containing multiple gels for the same samples and/or cut are; Figure 3.5, Figure 3.6, Figure 3.7C, Figure 3.8, Figure 3.9, Figure 3.10, Figure 4.1, Figure 4.2, Figure 4.3, Figure 4.4 and Figure 4.5.

2.2.9.6 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 1% TBST and then incubated for 10 minutes in Restore™ PLUS western blot stripping buffer. The membrane was then washed and blocked by incubation with 5% (w/v) skimmed milk powder for 30 minutes. The membrane was then probed and developed as described in section 2.2.4.5.

2.2.9.7 Cytokine measurements

Cytokine concentrations in cell supernatants were detected using ELISA Duoset kits for mouse IL-6 and mouse IL-10 (R&D systems) according to the manufacture's instructions. The optical density values were measured at 450 nm and cytokine concentrations were determined using a standard curve.

2.2.10 Analysis of cellular nucleic acids

2.2.10.1 Genomic DNA PCR

Genomic DNA, specific primers as listed in table 2.1, and KAPA 2G Fast mix were assembled in 0.2mL 8-strip tubes, which are capped and incubated in a 96-well Thermal Cycler (Mastercycler gradient, Eppendorf) for 2 minutes at 95°C, 31 cycles of 15 seconds at 95°C, 15 seconds at 60°C, 15 seconds at 72°C, and then held at 4°C. The resulting cDNA was stored at -20° C until needed.

2.2.10.2. DNA Agarose gel

After PCR a 1% DNA agarose gel was generated by using 1g agarose in 100 mL 1% TBS (descripted in table 2.2). This mixture was boiled and 5 µL Nancy DNA stain (non toxic replacement for ethidium bromide) was added. After cooling, the mixture was placed in a gel holder until it was set. Gels were run in 1% TBS for 15 minutes at 80V.

2.2.10.3 RNA extraction

Total RNA was isolated using an RNeasy® Micro kit (Qiagen). Cells were washed with 3 mL PBS and lysed in RLT buffer. The lysate for each sample was placed into a QIAshredder spin column and centrifuged for 2 minutes at 8000x g in preparation for RNA purification. The flow-through (with RNA) was stored at -80° C until purification was carried out using the RNeasy® Micro kit (Qiagen), following the manufacturer's protocol for purification of total RNA from animal and human cells. The quantification of RNA was determined with a Nanodrop 2000. The isolated RNA was used for the reverse transcriptase reactions and quantitative PCR analysis, or stored at -80° C.

2.2.10.4 Reverse transcriptase reactions

For analysis of mRNA transcripts, 0.2–1 µg total RNA was reverse transcribed using an Applied Biosystems Taqman Reverse Transcription Kit. Mixes were assembled in 0.2mL 8-strip tubes, capped and incubated in a 96-well Thermal Cycler (Mastercycler gradient, Eppendorf) for 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and then held at 4°C. The resulting cDNA was stored at -20°C until needed. 1:10 diluted cDNA samples were quantified and used to approximate mRNA expression in these samples.

2.2.10.5 Quantitative PCR Analysis of mRNA induction

Real time PCR was carried out using Taqman gene-specific probes or Sybr green-based PCR to quantify RNA. These reactions were set up as following the manufacturer's protocol. The primers used are listed in Table 2.2. Plates were loaded onto the Applied Biosystems Real time PCR machine (AB7500FAST) and cycling was

performed as followed; 40 cycles of 5 seconds at 95°C, 5 seconds at 60°C. Relative quantification was performed and the ΔΔCt method was used to calculate fold changes in mRNA expression relative to mRNA from unstimulated samples. For expression analysis, 18S or GAPDH was used as a normalization control for each reverse transcription reaction.

2.2.11 Binding Assays

2.2.11.1 Co-Immunoprecipitation

Transfected HEK293TLR4-MD2-CD14 cells were lysed in 800µl of low stringency lysis buffer (as shown in table 2.3) and incubated on ice for 15 minutes. Lysates were transferred to 1.5 mL microcentrifuge tubes and centrifuged at 1610xg for 10 minutes at 4°C. Lysates were pre-cleared for 30 minutes with 20µL Protein A/G Plus agarose beads, rotating at 4°C. Prior to incubation with lysate 2µg of relevant antibodies were incubated with 30µL Protein A/G Plus agarose beads overnight, rotating at 4°C. The lysates were then incubated with the pre-coupled antibodies/beads for 2 hours, rotating at 4°C. The lysates and beads were centrifuged at 80x g for 2 minutes at 4°C, the supernatant was removed and the beads were washed 3 times in 1mL low stringency lysis buffer. The immune complexes were eluted by adding 50µL sample buffer and analysed by SDS-PAGE and electrophoretic transfer as described in section 2.2.4 Analysis of cellular proteins

2.2.11.2 Chromatin Immunoprecipitation

Transfected RAW264.7 cells were plated in 15cm dishes at $5x10^5$ cells/mL, (3 dishes per condition) and were left untreated or were pre-treated with 500nM rapamycin for 30 minutes followed by 6 hours 100ng/mL LPS stimulation or no stimulation. At the time of lysis paraformaldehyde was added at 2%, to cross link the DNA, for 10 minutes maximum. Glycine was then added to stop the reaction at 1/20 v/v for 5 minutes. The medium was removed and cells were washed with PBS ensuring they were not allowed to dry out in between washes. 6mL ChIP Lysis buffer (as described in table 2.2) was then added to the cells, 3 dishes were combined and cells were scraped into 15mL falcon tubes, which were immediately snap frozen in liquid nitrogen. Lysates were thawed on ice, centrifuged at 100 x g at 4°C for 15 minutes, and resuspended in 4mL ice cold I.P. Buffer (SDS Buffer: Triton Dilution Buffer 1:0.5). Samples were sonicated using the Branson sonicator machine at 22% intensity, 10 x 30 sec per sample, being placed on ice in between pulses. Efficiency of sonication was checked by running samples of purified DNA on 1% agarose gels. Protein A/G Plus agarose beads were washed 3 times in ChIP Lysis buffer and blocking was carried out at 4°C for 20 minutes using TE containing 0.2mg/mL sonicated herring sperm DNA and 0.5mg/mL lipid-free BSA. 25µL/mL blocked Protein A/G PLUS agarose beads were added to lysates and incubated for 10 minutes at 4°C. Lysates were then centrifuged for 30 minutes at full speed to preclear the lysates. 10µL of this crude lysate was removed and stored at 4°C overnight as the 1/100 input. Primary antibodies were added at 1-10µg/mL and incubated at 4°C overnight, rotating at 4°C. Samples were then centrifuged at 4°C for 30min at full speed and supernatants transferred to fresh tubes. 40µl blocked protein A/G PLUS agarose beads were added to the samples, which were incubated on a rotating wheel for 1 hour at 4°C and then centrifuged at 300 x g for 2 minutes. Beads were washed by twice adding 1mL Mixed Micelle Wash Buffer and centrifuging for 1min at 4°C, followed by 1mL Buffer 500, 1mL Li/Cl detergent solution twice each respectively and finally 1mL TE.

DNA/antibody/Protein A/G PLUS agarose beads were then resuspended in 250µL 1% SDS, 0.1M NaHCO and incubated for 60 min at 65°C on a horizontal shaker to keep the beads in suspension. Samples were briefly centrifuged and transferred to fresh 1mL tubes. 1/100 input crude lysate samples stored overnight were recovered and 240µL 1% SDS, 0.1M NaHCO was added. All samples were then incubated overnight at 65°C to reverse crosslinks. 250µl TE was added to each sample the next morning. Samples were RNase treated at 1/100 at 37°C for 1 hour, then Proteinase K treated at 0.2µg/µL at 55°C for 2 hours. Samples were prepared for PCR using the Promega SV Wizard PCR clean up kit and eluted in 50µl H_20. Samples could then be run on Real Time PCR using specific primers (see table 2.1) and binding calculated as percentage input, or samples could be amplified by PCR and analysed with a 1% agarose gel.

2.2.11.3 Oligonucleotide pull down

Oligonucleotides were designed based on the binding site of the transcription factor of interest, with a Biotin tag on the 5' end of the forward oligonucleotide. Oligonucleotides were annealed at 90-95°C for 3-5 minutes and then heat block allowed to cool to room temperature. Primers used are described in table 2.1. Raw 264.7 cells were plated at $5x10^5$ cells/mL in 10cm dishes, one dish per condition and left overnight. Cells were then and pre-treated with or in absence of 500nM rapamycin for 30 minutes followed by 6 hours 100ng/mL LPS stimulation or left unstimulated. Fresh Oligonucleotide binding lysis buffers were made up (+/- NaCl) according to table 2.2. Streptavidin-Agarose beads were prepared by taking 50µL beads per condition (using a cut pipette tip), centrifuging for 2 minutes at 300 x g rpm to eliminate original buffer and washing three times in PBS. Cells were washed and scraped in 1mL PBS, placed in 1.5 micro centrifuge tubes and centrifuged for 3

minutes at 1300rpm. 100μl Buffer A was added to the cell pellet to lyse the cells. Lysates were snap frozen in liquid nitrogen and stored at -80°C. Lysates were thawed on ice and 900μl Buffer B was added per tube (1mL final volume), centrifuged for 5 minutes at 11000 x g at 4°C, to eliminate cell debris. Supernatant was transferred to a fresh tube and 10μl was removed, to which 40μl Sample Loading Buffer was added and stored at -20°C. Samples were then pre-cleared with 20μl pre-washed streptavidin-agarose beads, rotating at 4°C for 15 minutes and centrifuged at 350 x g for 5 minutes at 4°C. Supernatants were removed to a fresh tube with 30μl pre-washed streptavidin-agarose beads and 30μg of 5'Biotinylated-Oligonucleotide. Binding was performed for 2 hours at 4°C, rotating. Samples were centrifuged for 5 minutes at 4°C, and supernatant removed. 1mL of Buffer B was added, tubes inverted and centrifuged at 2500rpm, 4°C to wash the beads, repeating three times. 50μl Sample Loading Buffer was then added to the beads, and samples boiled for 10 minutes at 95°C. Samples were loaded on a 12% Gel and Twist2 binding detected by SDS-PAGE and Electrophoretic transfer as previously described in section 2.2.9 Analysis of cellular proteins.

2.2.12 Statistical analysis

2.2.12.1 Calculation of Errors

When using quantitative methods of data collection from biological samples there is an intrinsic element of variability that results from differences between the original samples. This variation is accounted for in the presented results by including error bars representing the standard error of the mean (SEM) of at least 3 replicates. The SEM is calculated as detailed below in Excel:

$$SEM = \frac{1}{\sqrt{n}} \sqrt{\frac{\sum (x - \bar{x})^2}{n-1}}$$

Where n is the number of replicates, and x is the mean of the measured values x1 through xn.

2.2.12.2 Student's T-test

Student's T-test is used to determine the significance of differences between mean values derived from small datasets. When considering factors which fall into a normal distribution it is likely that chance will cause the mean, and thus standard deviation, calculated from a small number of samples to deviate from that which would result from a larger dataset. Student's T-test takes this into account, delivering a p-value representing the probability that the difference between two groups would occur due to random chance. The form of the T-test used in Excel is shown below:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s_{(\bar{x}_1 - \bar{x}_2)}}$$

Where xn is the mean of a set, and s(x1−x2) is determined from the size and variation within the two sets according to the equation below, where sn is the standard deviation of a data set.

$$s_{(\bar{x}_1 - \bar{x}_2)} = \sqrt{\frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{n_1 + n_2 - 2} \cdot (1/n_1 + 1/n_2)}$$

CHAPTER 3

mTOR SIGNALLING IS REQUIRED FOR LPS-INDUCED PDCD4 DEGRADATION.

3.1 Introduction

The mTOR pathway is a well-established signalling pathway, involved in biological processes such as cell growth, cell proliferation, cell motility, cell survival, transcription and protein synthesis. Furthermore, over the last number of years there have been an increasing number of studies linking mTOR signalling to inflammatory responses through TLR signalling. Most of the studies have been carried following activation of the LPS sensor TLR4. As described in chapter 1, TLRs are very important for detecting pathogens and initiating an adaptive inflammatory response through cytokine production. However TLRs must be tightly regulated by negative control mechanisms to avoid endotoxic shock or the development of chronic inflammation. The mTOR pathway has been described to regulate the induction of IL-10 through the activation of STAT3 (Weichhart et al., 2008).

It has previously been shown that LPS can activate the mTOR pathway. The activation of the mTOR pathway leads to phosphorylation of the mTORC1 complex. mTORC1 can in its turn activate S6K1. S6K1 has multiple substrates including PDCD4. PDCD4 is a well-known tumor suppressor initially discovered during experiments on apoptosis and later implicated in tumor suppression (Jansen et al., 2005). PDCD4 plays a role in apoptosis by stopping protein translation. PDCD4 deficient animals have been generated and work on these mice demonstrated that whilst they develop spontaneous lymphoma, they were resistant to models of inflammatory disease tested such as autoimmune encephalomyelitis (EAE) and streptozin toxin induced type II diabetes. This was shown to be due to the role of PDCD4 as a translational inhibitor of complex mRNAs, specifically those for IL-10, IL-4 and IFN-γ (Hilliard et al., 2006). As it has been shown that PDCD4 is able to inhibit

eIF4A and eIF4G translation function by protein-protein interaction through its α-helical MA3 domain (Yang et al., 2003a, Goke et al., 2002).

It has recently been discovered that PDCD4 has an important role in inflammation. For instance, PDCD4 deficient mice are protected from lethality of LPS (Sheedy et al., 2010). These mice have also been shown to display increased IL-10 production. Therefore, it seemed likely that PDCD4 regulated the translation of the anti-inflammatory cytokine IL-10, as it had been proposed to occur through eIF4 factors, which are required for the cap-dependent translation of mRNA, including IL-10 mRNA (Yang et al., 2003, Loh et al., 2009, Hilliard et al., 2006). The increased production of IL-10 might therefore protect the host against inflammatory disease. However, at the same time, the immune system of these mice became compromised allowing the spread of tumors and the development of lymphoma. These PDCD4-deficient animals also showed higher prevalence of B cell derived lymphomas, multi-organ cysts and decreased susceptibility to experimentally induced autoimmune inflammation to the brain and spinal cord (encephalomyelitis) (Wang et al., 2013, Schmid et al., 2008, Hilliard et al., 2006).

In this chapter several inhibitors are used to investigate the role of PDCD4 in TLR4 regulated signalling through mTOR (Figure 3.1). At the start of the project, the inhibitor rapamycin was used to provide more evidence that PDCD4 was involved in the mTOR pathway. However, to examine the upstream effect of the mTOR pathway, the PI3K inhibitors wortmannin and LY294002 were used. Wortmannin is a steroid metabolite of the fungus *Penicilllium (Talaromyces) wortmannii* and is a specific covalent inhibitor of PI3K (Ui et al., 1995). It has been shown *in vitro* to be a more potent inhibitor than LY294002. At higher concentrations, these inhibitors were shown to inhibit other PI3K related enzymes such as mTOR (Ui et al., 1995, Sarbassov et al., 2005). To examine whether PDCD4 degradation is involved the proteasome

inhibitor MG132 was used. Also a specific S6K inhibitor PF-470861 (Pearce et al., 2010) was used to examine whether S6K is involved in the degradation of PDCD4. In this chapter, I examine whether the mTOR pathway and proteasome degradation are involved in PDCD4 regulation during LPS signalling to IL-10.

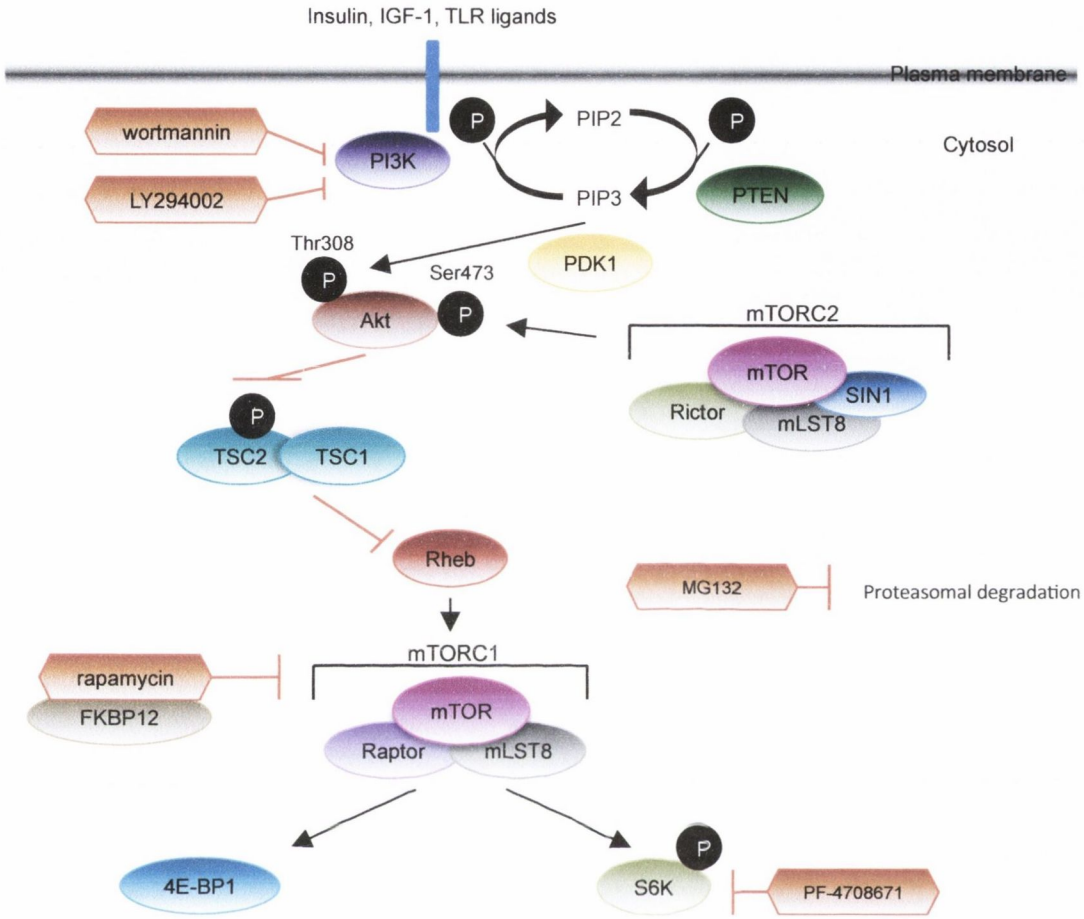


Figure 3.1 – mTOR pathway and the different inhibitors blocking mTOR signalling.

Growth factors (such as insulin or IGF-1) and TLR ligands trigger mTORC1 activity via PI3K. PI3K stimulates PIP3 production, which recruits Akt to the cell membrane, resulting in its phosphorylation via PDK1 and by the mTORC2 complex, which is considered rapamycin insensitive. Active Akt phosphorylates TSC2 and thereby relieves the inhibitory activity of the TSC1–TSC2 complex on mTORC1. Active mTORC1 phosphorylates its downstream substrates 4E-BP1 and p70S6K, thus stimulating protein synthesis and cell growth (Weichhart and Saemann, 2009). There are different inhibitors which are able to block mTOR signalling. The first described inhibitor is rapamycin, which binds FKBP12 and, in the form of this complex, inhibits mTORC1. Other inhibitors are the PI3K inhibitors wortmannin and LY294002. MG132 is a compound known to block proteasomal degradation by reducing the degradation of ubiquitin-conjugated proteins. PF-4708671 has been used as a specific S6K inhibitor.

3.2 Results

3.2.1 PDCD4 protein levels are regulated by LPS in RAW264.7 macrophages and primary BMDMs.

First, the expression of PDCD4 was examined in the RAW264.7 macrophage cell line in response to serum. After serum starvation, RAW264.7 macrophages were stimulated with 10% FCS. PDCD4 protein decreased at 6 and 20 hours after stimulation with FCS, as shown in Figure 3.2 lanes 3 and 4. This result suggests that PDCD4 protein degradation is serum-dependent, as shown by others (Dorrello et al., 2006), and therefore further experiments were conducted in serum-starved cells. Next, the modulation of PDCD4 by LPS was examined in RAW264.7 cells. Figure 3.3A shows a time course of PDCD4 protein levels in RAW264.7 cells following LPS stimulation. LPS stimulation results in higher expression of PDCD4 protein between 0 - 4 hours (Figure 3.3A lane 1-4) and following this, PDCD4 protein levels decrease from 4 hours onward with virtually no detectable PDCD4 protein 20 hours (Figure 3.3A lane 6) post LPS stimulation. The effect of LPS on PDCD4 protein levels was analysed further using a dose response of LPS concentrations at the 6 hour time point as shown in Figure 3.3B. The LPS-induced decrease of PDCD4 can be seen at concentrations as low 1ng/mL LPS (Figure 3.3B lane 8 compare to lane 8) following 6 hours stimulation. The decrease in PDCD4 protein was also evident in primary BMDMs stimulated with LPS, as shown in Figure 3.3C. 1 – 2 hours (Figure 3.3C lane 14 and lane 15). Stimulation with LPS resulted in an increase in PDCD4 protein, followed by a decrease, with no detectable PDCD4 protein at 20 hours (Figure 3.3C lane 18). The decrease in PDCD4 protein at 6 hours (Figure 3.3C lane 17) was less dramatic than that seen in the RAW264.7 cell line (Figure 3.3A lane 5).

RAW264.7

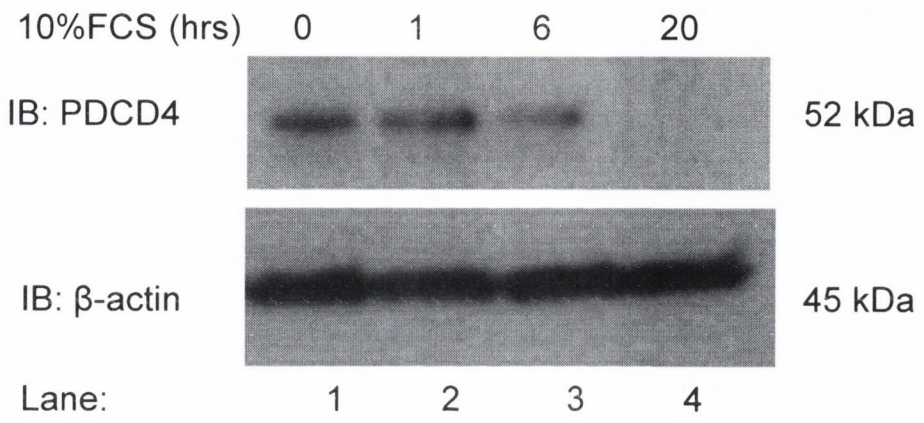


Figure 3.2 – PDCD4 protein levels are regulated by FCS over time in RAW264.7 cells.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 12-well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. The following day, cells were left unstimulated or were stimulated with 10% FCS for the indicated time. Protein lysates were prepared and analyzed via Western blotting followed by immuno-blotting (IB) for PDCD4. The expression of β-actin is presented as a loading control. The experiment shown is representative of at least three independent experiments.

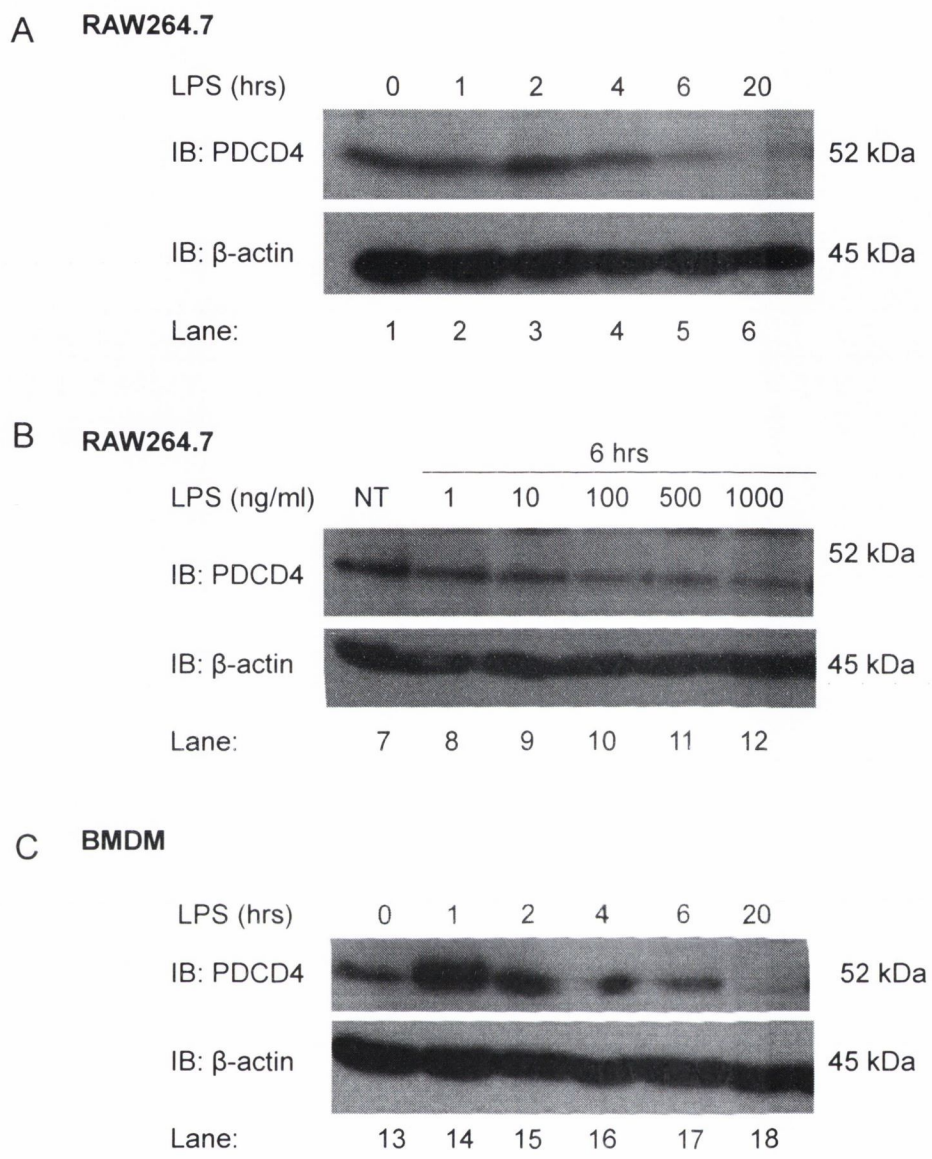


Figure 3.3 – PDCD4 protein expression is regulated by LPS in RAW264.7 and BMDMs.

Cells were plated at $5x10^5$ cells/mL in 12-well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. (A) The following day, RAW264.7 cells were left unstimulated or were stimulated with 100ng/ml LPS for the indicated time. (B) RAW264.7 cells were stimulated for 6 hours with various LPS concentrations as indicated above or were left untreated. (C) Wild-type BMDM were left unstimulated or were stimulated with 100ng/mL LPS for the indicated time. After stimulation protein lysates were prepared and analyzed via Western blot followed by immuno-blotting (IB) for PDCD4. The expression of β-actin is presented as a loading control. The experiments shown are representative of at least three independent experiments.

3.2.2 LPS signalling through PDCD4 is mTOR dependent

3.2.2.1 Rapamycin stabilizes PDCD4

To investigate whether the effect of LPS on PDCD4 was mTOR dependent, RAW264.7 cells were pre-treated with rapamycin followed by a time course of LPS stimulation. As can be seen in Figure 3.4A, PDCD4 protein levels decreased from 4 hours (Figure 3.4A compare lanes 4-6 to lane 1), with virtually no detectable PDCD4 protein at 20 hours (Figure 3.4A lane 6) post LPS stimulation. However RAW264.7 macrophages pre-treated with the mTOR inhibitor rapamycin showed that inhibition of mTOR by rapamycin inhibited LPS-induced PDCD4 protein degradation after 4 – 20 hours, as shown in Figure 3.4A comparing lanes 10-12 to lanes 4-6. The inhibitory effect was also evident in BMDMs. LPS-induced degradation of PDCD4 after 6 and 20 hours stimulation (Figure 3.4B lanes 17 and 18), and pre-treatment with rapamycin inhibited this effect, as shown in Figure 3.4C lanes 23-24 compared to lanes 17-18. Furthermore, as shown in Figure 3.5, 6 hours of LPS stimulation decreased PDCD4 protein levels (Figure 3.5 compare lane 2 to lane 1), whereas pre-treatment with rapamycin followed by 6 hours of LPS stimulation prevented the degradation of PDCD4 protein in a dose-dependent manner (Figure 3.5 compare lane 2 to 3-7). This inhibition could be seen at a concentration as low as 1nM rapamycin (Figure 3.5 comparing lane 3 to lane 2). To establish whether the dose of 500nM rapamycin could be providing erroneous results for off target effects such as inhibition of mTORC2 and Akt signalling, the phosphorylation of Akt was determined (Figure 3.5). There was virtually no difference seen in Akt phosphorylation after pre-treatment with rapamycin in a dose-dependent manner (Figure 3.5 lanes 3-7). Akt phosphorylation on Ser473 would relate to inhibition of mTORC2. Inhibition of the phosphorylation of S6K1 on Thr389 could be seen at a concentration as low as 1nM rapamycin (Figure 3.5 lane 3). There was also inhibition visible of 4E BP1 activity on phosphorylation

site Thr36/37 as low as 1nM rapamycin (Figure 3.5 lane 3). To determine if the doses rapamycin used would have influence on MAPK signalling the phosphorylation of p38 was examined and no effect was observed (Figure 3.5 lane 3-7).

3.2.2.2 PDCD4 half-life is extended upon rapamycin treatment

To verify that the increase in PDCD4 protein in response to rapamycin was a result of increased protein stability, I next determined mTOR-inhibition by rapamycin using cycloheximide to block translation. RAW264.7 cells were pre-treated with rapamycin followed by LPS stimulation. After 20 hours of LPS stimulation cells were post-treated with a time course of cycloheximide. As can be seen in Figure 3.6 the RAW264.7 cells in absence of rapamycin but stimulated with LPS followed by treatment with cycloheximide, showed a decrease in PDCD4 protein levels (Figure 3.6 lanes 6-8). However, PDCD4 protein levels remained high in cells pre-treated with rapamycin (Figure 3.6, lanes 9-11). This indicates that the increase of PDCD4 after rapamycin pre-treatment was a result of protein stability.

3.2.2.3 The upstream pathway of mTOR signalling and proteosomal degradation are involved in LPS signalling

To show that phosphatidylinositol-3-kinase (PI3K), known to signal upstream of mTOR, was involved in LPS signalling, RAW264.7 cells were pre-treated with the PI3K inhibitors LY294002 and Wortmannin, followed by LPS stimulation. As shown in Figure 3.7A, LPS-induced a decrease in PDCD4 protein at 6 and 20 hours (Figure 3.7A lanes 3 and 4). Pre-treatment with LY294002 inhibited the LPS-induced response (Figure 3.7A lanes 7 and 8 compare to lanes 3-4). Also, pre-treatment with Wortmannin inhibited the LPS-induced response compared to cells stimulated with

LPS alone (Figure 3.7B lanes 15 -16 compare to lanes 11-12). In order to establish the role for proteosomal degradation of PDCD4, the effect of the 26S proteasome inhibitor MG132 was tested. As shown in Figure 3.7C, MG132 pre-treatment inhibited the induction of PDCD4 degradation in response to LPS (Figure 3.7C compare lane 22 to lane 19). To establish the effect of rapamycin on protein further upstream and downstream of mTOR itself in the mTOR pathway, LY294002, wortmannin and MG132 were tested (Figure 3.8). Stimulation of RAW264.7 cells with LPS resulted in the degradation of PDCD4 (Figure 3.8 PDCD4 panel, lane 2). However, pre-treatment with rapamycin, wortmannin or LY294002 in the presence or absence of 6 hours LPS stimulation inhibited PDCD4 degradation (Figure 3.8 PDCD4 panel, with LPS lanes 3-6, without LPS lanes 7-10). The inhibitors alone caused an increase in PDCD4 protein compared to the non-stimulated cells. (Figure 3.8 PDCD4 panel, compare lanes 7-10 to lane 1).

LPS stimulation can increase the phosphorylation of Akt as well as S6K1 (Figure 3.8 Ser473 and ℗S6K1 Thr389 panels lane 2). Both the inhibitors, wortmannin and LY294002, showed an inhibition of LPS-induced Akt phosphorylation as well as S6K phosphorylation (Figure 3.8 ℗Akt Thr308, Ser473 and ℗S6K1 Thr389 panels, lanes 4-5). The same result can be seen in the absence of LPS stimulation (Figure 3.8 ℗Akt Thr308, Ser473 and ℗S6K1 Thr389 panels, lanes 8-9). Pre-treatment with rapamycin followed by LPS stimulation showed no difference in Akt phosphorylation on Thr308 compared to LPS alone (Figure 3.8 ℗Akt Thr308 panel compare lane 3 to lane 2). However the Akt phosphorylation on Ser473 after pre-treatment with rapamycin was unexpectedly slightly decreased compared to LPS alone (Figure 3.8 ℗Akt Ser473 panel, compare lane 3 to lane 2).

Pre-treatment with MG132 followed by LPS resulted in inhibition of PDCD4 degradation. However this was not as great in magnitude as the effect seen using not rapamycin or LY294002 (Figure 3.8 PDCD4 panel lane 6). Akt phosphorylation for Thr308 and Ser473, as well as S6K1 phosphorylation, are notably reduced after pre-treatment with MG132 (Figure 3.8 ℗Akt Thr308, Ser473 and ℗S6K1 Thr389 panels, lane 6). These effects might be due to the decease seen in total Akt protein (Figure 3.8 total AKT panel, lane 6), as it has shown before that MG132 does not block Akt or S6K phosphorylation.

After LPS treatment there was an increase observed in the phosphorylation of 4EBP1 on Thr37/46 compared to unstimulated RAW264.cells (Figure 3.8 ℗4EBP1 Thr37/46 panel, compare lane 2 to lane 1). However, pre-treatment with the different inhibitors showed an inhibition of the phosphorylation of 4EBP1 on Thr37/46 compared to LPS treated cells (Figure 3.8 ℗4EBP1 Thr37/46 panel compare lanes 3-6 to lane 2). p38 phosphorylation on Tyr182 is increased after LPS stimulation (Figure 3.8 ℗p38 Tyr182 lane 2). The inhibitors were shown not to influence the MAPK pathway in the presence of LPS stimulation as p38 phosphorylation on Tyr182 was not affected (Figure 3.8 ℗p38 Tyr182 lanes 3-6).

3.2.2.4 S6K1 is involved in the degradation of PDCD4 in the LPS signalling pathway

It has previously been shown that phosphorylation by S6K1 is critical for the proteasomal degradation of PDCD4 (Dorrello et al., 2006, Schmid et al., 2008). I next tested the effect of knocking down S6K1 with siRNA on the LPS-induced degradation of PDCD4. A significant decrease in endogenous S6K1 expression as well as

phosphorylation of S6K on Thr389 was observed (Figure 3.9 lanes 5, 6 for 6.25 nM, 8, 9 for 25 nM siRNA) except for 6.25nM S6K1 where there was still an increase seen in S6K phosphorylation (Figure 3.9 lanes 5 and 6). The decrease in S6K1 prevented the induction of PDCD4 degradation in response to LPS (Figure 3.9, comparing lane 3 to lane 6). These results show that S6K1 is critical for PDCD4 degradation.

3.2.2.5 PF-4708671 prevents LPS-induced PDCD4 degradation

Another method to implicate S6K1 in this response was to pre-treat RAW264.7 cells with the specific S6K1 inhibitor PF-4708671. The cells pre-treated with PF-478671 followed by 6 hours LPS stimulation also led to an inhibition of LPS-induced PDCD4 protein degradation (Figure 3.10 compare lane 3-4 with 7-8). As a control S6 was used and shows that PF-470861 indeed blocked S6 phosphorylation on Ser235/236 site but did not influence the total levels of S6. This has been shown before by Pearce et al (2010) Another read out was to probe for phosphor 4E-BP1 as the PF-470861 should not influence phosphor 4E-BP1 as can be seen in Figure 3.10 lanes 5-8. PF-4670861 did not influence p38, as phosphorylation on Tyr182 was not affected. This result suggests a role for mTOR and its downstream kinase S6K1 in the degradation of PDCD4 in response to LPS.

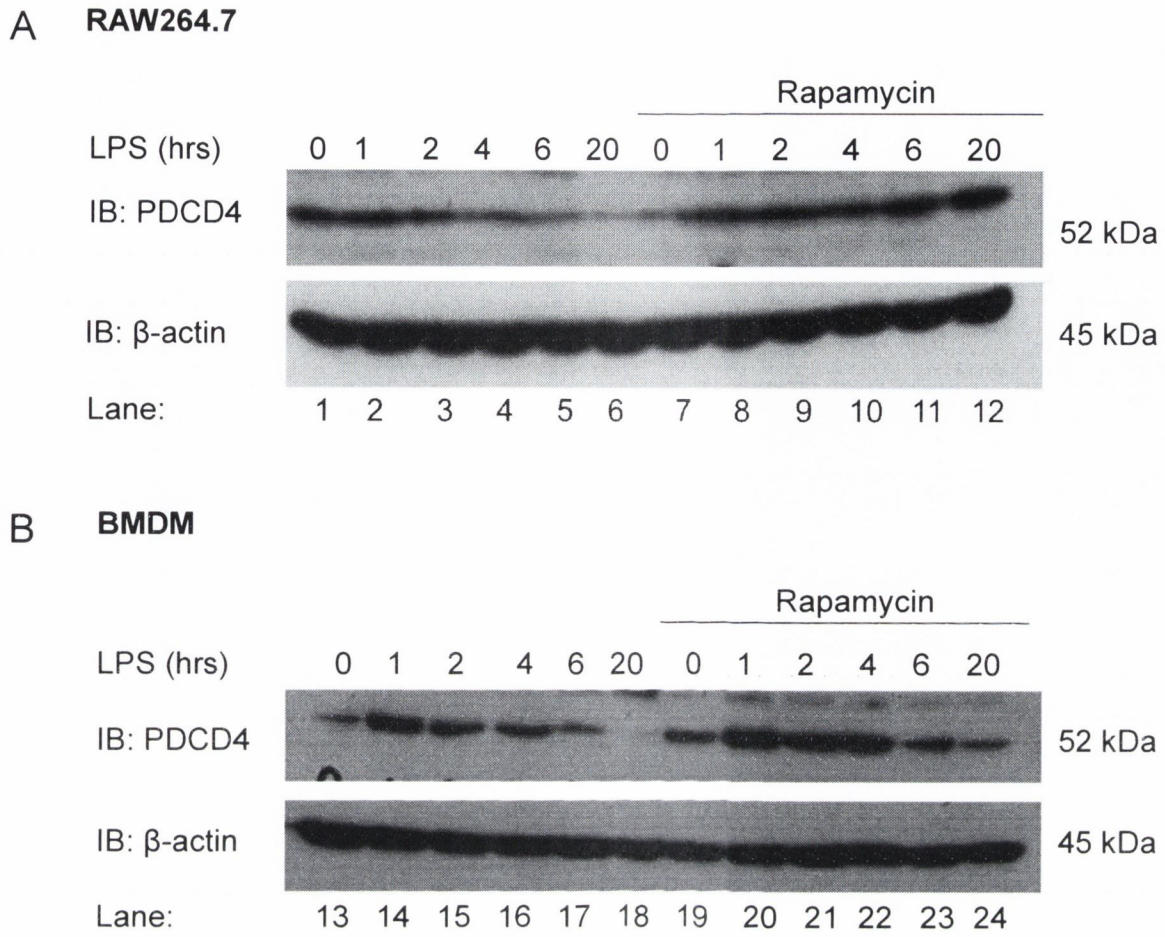


Figure 3.4 – Rapamycin inhibits the LPS-induced decrease in PDCD4 protein levels.

Cells were plated at $5x10^5$ cells/mL in 12-well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. (A) RAW264.7 cells were pretreated with DMSO or 500nM rapamycin for 30 minutes followed by 100ng/ml LPS for the indicated time. (B) Wild-type BMDM were pretreated with DMSO or 500nM rapamycin for 30 minutes followed by 100ng/ml LPS for the indicated time. After stimulation protein lysates were prepared and analyzed via Western blot followed by immuno-blotting (IB) for PDCD4. The expression of β-actin is presented as a loading control. The experiments shown are representative of at least three independent experiments.

RAW264.7

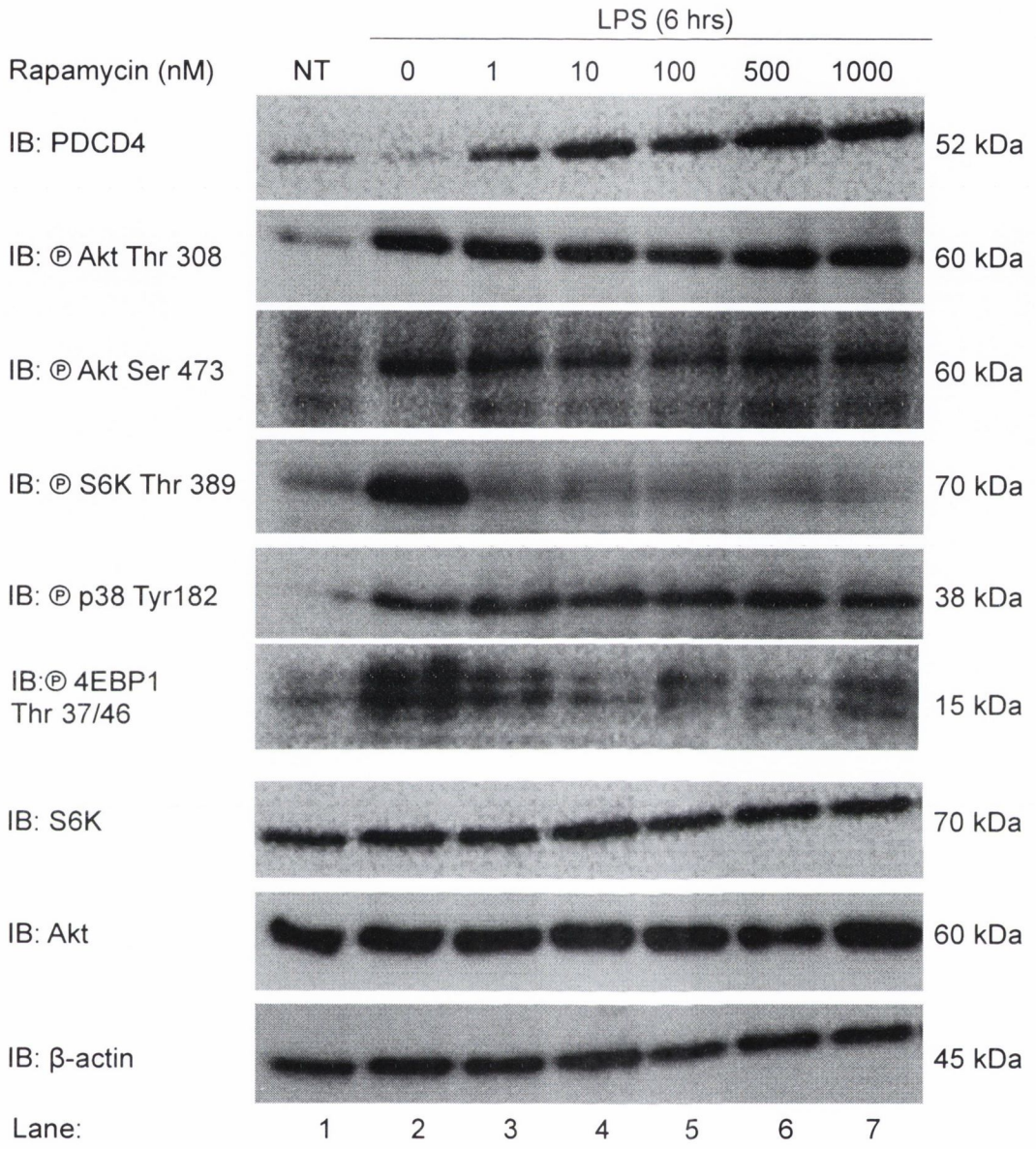


Figure 3.5 – Rapamycin in dose-dependent manner inhibits LPS-induced PDCD4 degradation.

Cells were plated at $5x10^5$ cells/mL in 12-well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. RAW264.7 cells were left untreated (NT) or were pretreated for 30 minutes with DMSO control or with various rapamycin concentrations as indicated above each lane followed by 6 hours LPS stimulation. After stimulation protein lysates were prepared and analyzed via Western blot followed by immuno-blotting (IB) for PDCD4, phosphorylated Akt Thr308, Akt Ser473, S6K Thr 389 total S6K or total akt. The expression of β-actin is presented as a loading control. The experiments shown are representative of at least three independent experiments.

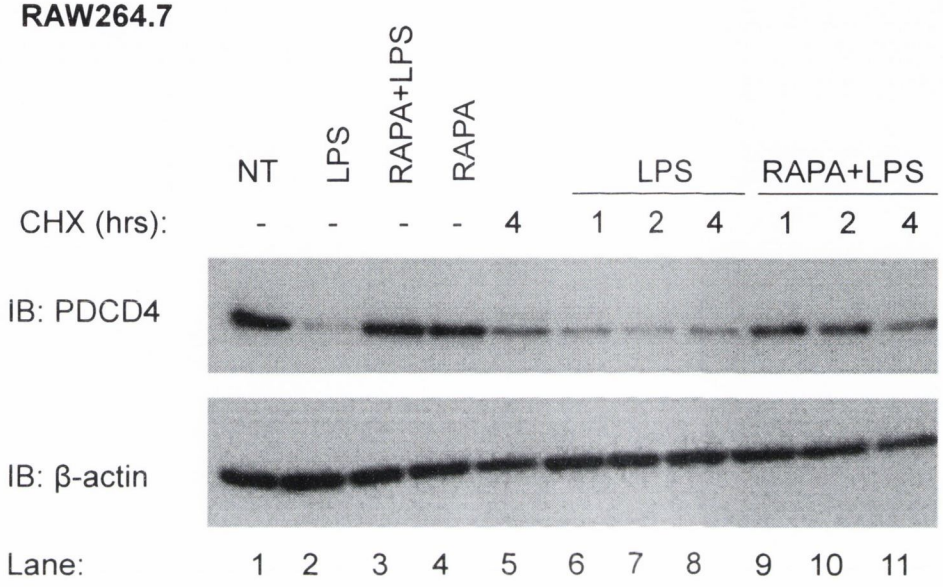


Figure 3.6 – PDCD4 half-life is extended upon rapamycin treatment.

Cells were plated at $5x10^5$ cells/mL in 24-well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. RAW264.7 cells were left unstimulated (NT) or were pretreated with DMSO (LPS) or 500nM rapamycin for 30 minutes followed by 100ng/ml LPS (RAPA + LPS). After 20 hours cells were treated with 10μM cycloheximide (CHX) for the indicated time. After stimulation protein lysates were prepared and analyzed via Western blot followed by immuno-blotting (IB) for PDCD4. The expression of β-actin is presented as a loading control. This experiment shown is representative of at least three independent experiments

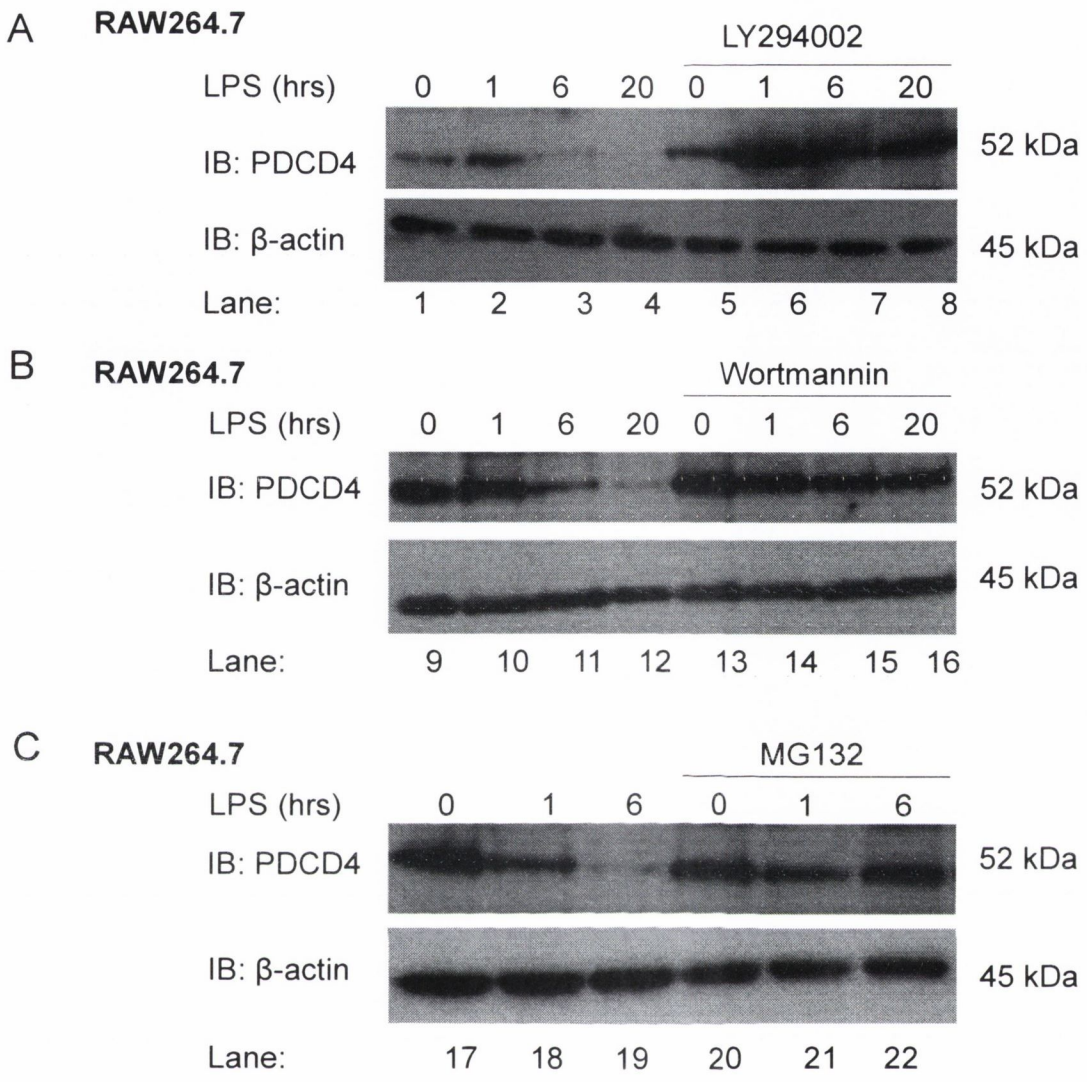


Figure 3.7 – Treatment with the PI3K inhibitors LY294002, Wortmannin or the proteasome inhibitor MG132 before LPS stimulation restores PDCD4 protein expression in RAW264.7 cells.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 12-well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. (A) The following day, cells were pretreated with 20μM LY294002 or DMSO for 30 minutes followed by 100ng/ml LPS for the indicated time. (B) Cells were pretreated with 5μM Wortmannin or DMSO for 30 minutes followed by 100ng/ml LPS for the indicated time. (C) Cells were stimulated with 5μM MG132 or DMSO for 30 minutes followed by 100ng/ml LPS for the indicated time. After stimulation protein lysates were prepared and analyzed via Western blotting followed by immuno-blotting (IB) for PDCD4. The expression of β-actin is presented as a loading control. The experiment shown is representative of at least three independent experiments.

RAW264.7

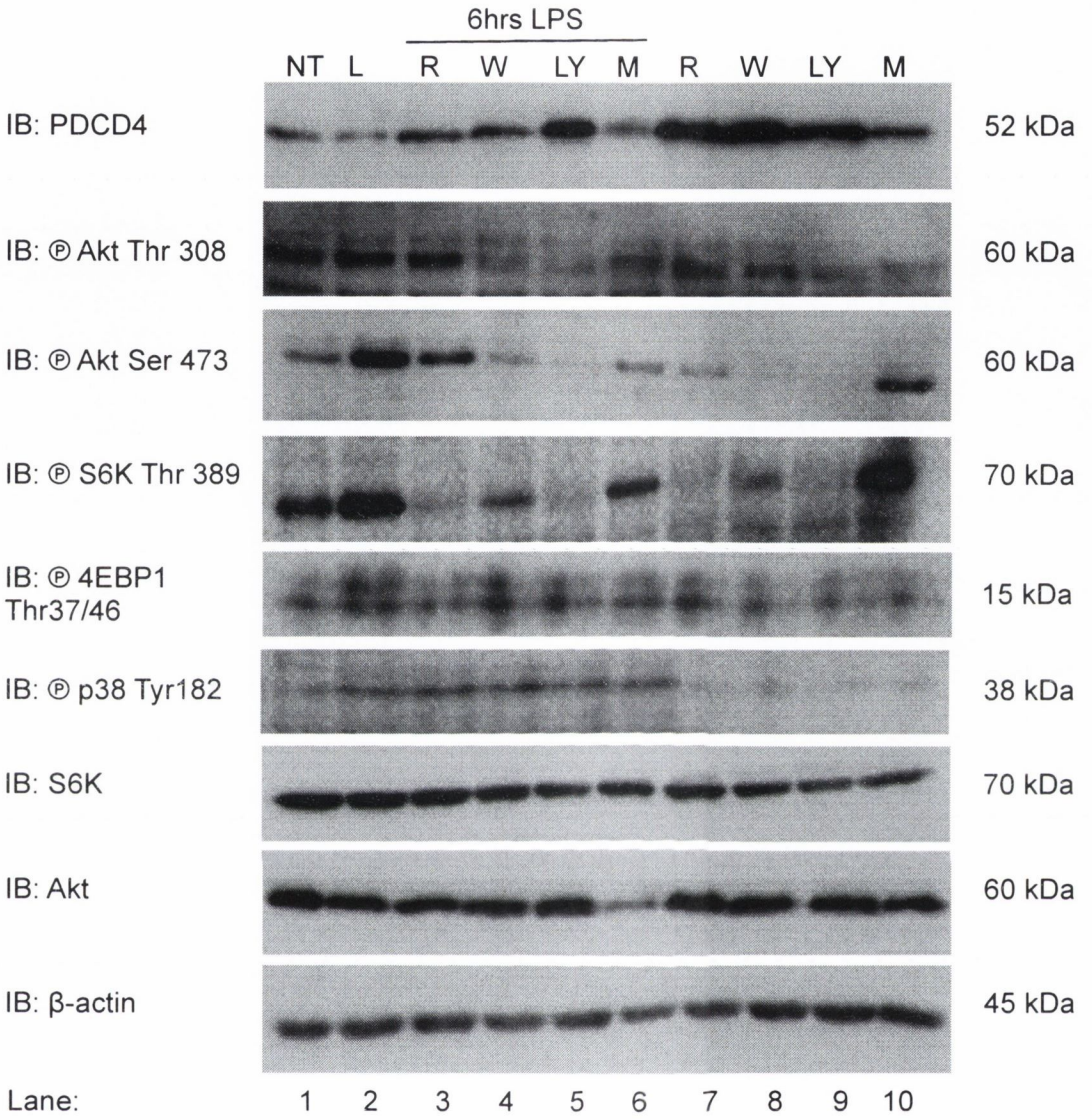


Figure 3.8 – Treatment with the PI3K inhibitors LY294002, Wortmannin or the proteasome inhibitor MG132 before LPS stimulation restores PDCD4 protein expression in RAW264.7 cells after 6 hours LPS stimulation.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 12-well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. The following day, cells were left untreated (NT) or were pretreated with DMSO (L) or 500nM rapamycin (R), 20μM LY294002 (LY), 5μM Wortmannin (W), 5μM MG132 (M) for 30 minutes followed by 100ng/ml LPS for 6 hours. After stimulation protein lysates were prepared and analyzed via Western blot followed by immuno-blotting (IB) for PDCD4, phosphorylated Akt Thr308, Akt Ser473, S6K Thr 389 total S6K or total akt. The expression of β-actin is presented as a loading control. The experiments shown are representative of at least three independent experiments.

RAW264.7

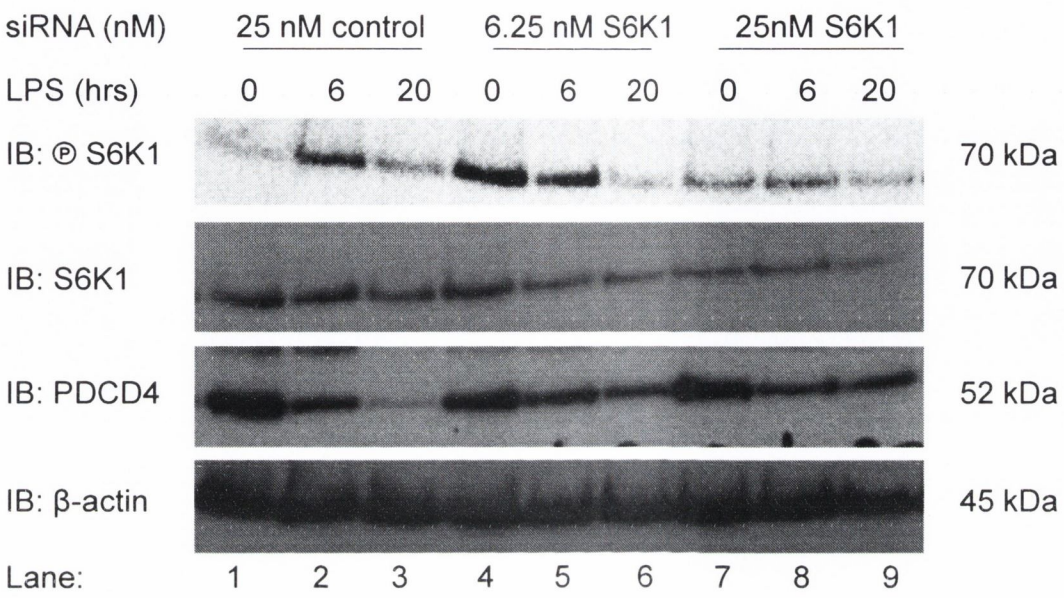


Figure 3.9 – S6K1 siRNA decreases endogenous S6K1 and prevents LPS-induced PDCD4 degradation.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 24 well plates and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, penicillin and streptomycin (100µg/mL). The following day, cells were transfected with 6.25 nM or 25 nM siRNA targeting S6K1 or control siRNA oligonucleotide (Control) and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. Cells were stimulated with 100ng/L LPS for the indicated time. The protein lysates were prepared and analyzed via Western blotting followed by immuno-blotting (IB) for PDCD4 and S6K1. The expression of β-actin is presented as a loading control. Results are representative of three independent experiments.

RAW264.7

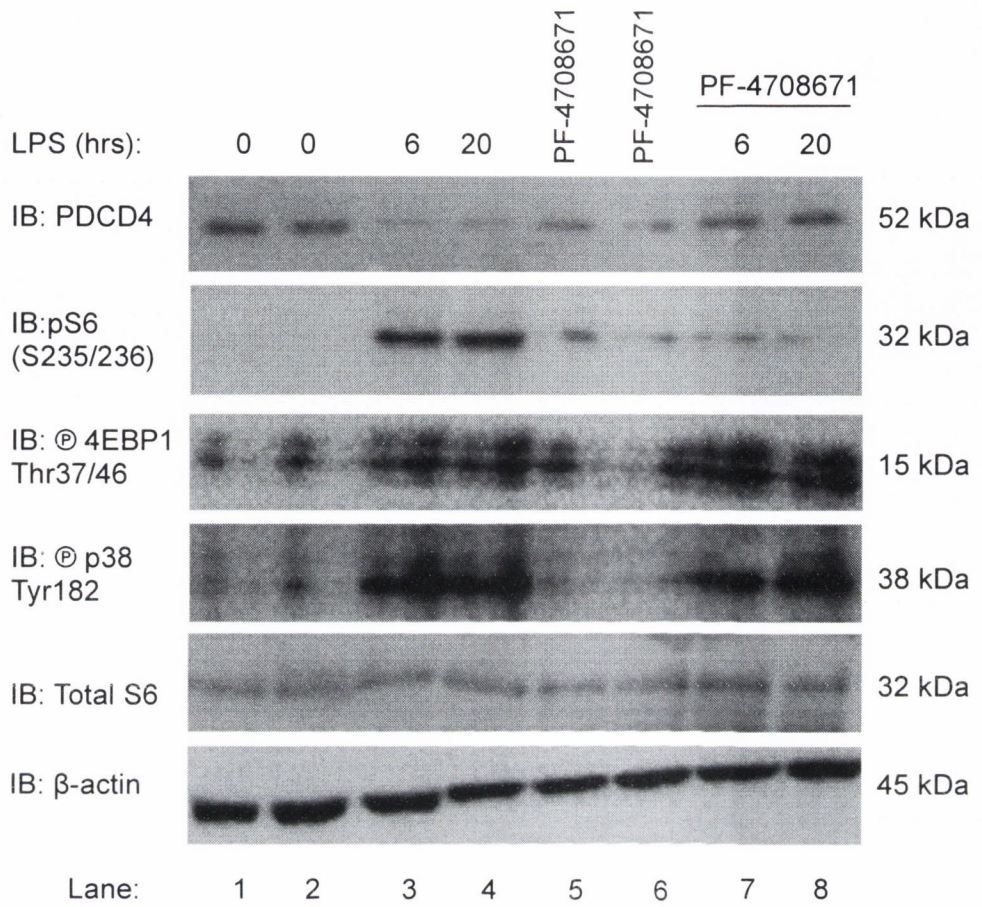


Figure 3.10 – The S6K inhibitor PF-4708671 prevents LPS-induced PDCD4 degradation.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 24 well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS, penicillin and streptomycin (100µg/mL). The following day, cells were pretreated with 10µM PF-47086871 or DMSO for 30 minutes followed by 100ng/ml LPS for the indicated time. The protein lysates were prepared and analyzed via Western blotting followed by immuno-blotting (IB) for PDCD4, pS6 (S235/236), pE4BP1 and total S6. The expression of β-actin is presented as a loading control. A similar result was obtained in another independent experiment.

3.2.3 Rapamycin inhibits LPS-induced IL-10 expression through stabilization of PDCD4.

3.2.3.1 The effect of rapamycin on IL-10 expression

As shown before, one of the consequences of inhibiting PDCD4 degradation is to decrease IL-10 production in response to LPS (Sheedy et al., 2010). I next examined the effect of LPS on IL-10 production in RAW264.7 cells. As shown in Figure 3.11A, LPS-induced an increase in IL-10 production at a concentration as low as 1ng/mL LPS following 6 hours of stimulation. The effect of rapamycin on this response was also examined in RAW264.7 macrophages. As shown in Figure 3.11B, a significant decrease in IL-10 production can be seen at a concentration as low as 10 nM rapamycin. This effect was evident over a time course of LPS treatment as shown in Figure 3.11C. The same effect can be seen on IL-10 mRNA expression as shown in Figure 3.11D. The increase in IL-10 mRNA expression peaks at 4 hours of LPS stimulation and decreases slightly after 6 and 20 hours LPS stimulation. However, cells pre-treated with rapamycin before LPS stimulation display a decrease in IL-10 mRNA expression. The effect on IL-10 was somewhat specific since rapamycin had no effect on IL-6 induction by LPS as shown in Figure 3.12A for IL-6 protein and 3.12B for IL-6 mRNA.

3.2.3.2 The effect of PI3K inhibitors and a proteasome inhibitor on IL-10 expression

Next the effect of the PI3K inhibitors LY294002 and Wortmannin and the proteasome inhibitor MG132 on IL-10 production were examined. Figure 3.13A shows increased

IL-10 production after 6 hours LPS stimulation. However, when macrophages were pre-treated with LY294002, Wortmannin, MG132 or rapamycin before 6 hours of LPS stimulation, there was a significant reduction in IL-10 production in all cases. The same result can be determined in the IL-10 mRNA samples Figure 3.13B. The inhibitors had no effect on IL-6 induction by LPS as shown in Figure 3.14A for IL-6 protein and Figure 3.14B for IL-6 mRNA.

3.2.3.3 S6K1 is required for IL-10 induction

Furthermore, when S6K1 was knocked down in RAW264.7 macrophages there was a decrease in IL-10 production after 6 and 20 hours of LPS stimulation, as shown in Figure 3.15A. This effect was only evident using 25 nM siRNA. When using the specific S6K inhibitor PF-4708671 there was also a decrease in IL-10 production after 6 and 20 hours of LPS stimulation (Figure 3.15B). These results provide more evidence that S6K is required for LPS-induced IL-10 production.

3.2.3.4 The effect of PDCD4 knockdown on IL-10

Sheedy et al. (2010) had previously demonstrated elevated IL-10 production in macrophages lacking PDCD4 (Sheedy et al., 2010). Since rapamycin prevented the LPS-induced degradation of PDCD4, I next tested was whether rapamycin could inhibit IL-10 in PDCD4 deficient cells. First it was tested whether PDCD4 was knocked down in RAW264.7 macrophages using siRNA against PDCD4, as shown in Figure 3.16A lanes 5 and 6. Next, RAW264.7 macrophages were transfected with 25 nM control siRNA or 25nM PDCD4 siRNA and challenged with LPS for 6 or 20 hours following pre-treatment with rapamycin. As shown in Figure 3.16B, LPS-induced degradation of PDCD4 after 6 and 20 hours stimulation in cells transfected with the

control siRNA (Figure 3.16B lanes 8 and 9) Pre-treating with rapamycin prevented this degradation (Figure 3.16B lanes 14 and 15). However, in cells transfected with siRNA specific for PDCD4, no detectable PDCD4 protein expression can be seen (Figure 3.16B lanes 10- 12 and 16-18). Rapamycin pre-treatment prevented the degradation of PDCD4 induced by LPS in the control-transfected cells (Figure 3.16B lanes 14 and 15). In Figure 3.17A a 3 fold increase in IL-10 production in PDCD4 knockdown cells can be seen after 6 hours LPS stimulation in comparison to cells transfected with control siRNA (Figure 3.17A, 6 hours LPS treated samples, comparing grey bar to white bar). Furthermore, rapamycin had no effect on the induction of IL-10 by LPS in the cells in which PDCD4 was knocked down (Figure 3.17A, 6 hours LPS treated samples compare grey hatched bar to grey bar). Rapamycin inhibited the IL-10 response in cells transfected with control siRNA (Figure 3.17A, 6 hours treated samples compare black to white bar). Similar results were observed in IL-10 mRNA (Figure 3.17B). However when the IL-6 protein (3.18A) and IL-6 mRNA (3.18B) were measured there was unexpectedly a slight decrease in the samples in the presence and absence of rapamycin followed by 6 hours LPS (black, grey and grey hatched bars) except in the samples transfected with control siRNA stimulated with LPS (white bar). These data indicate that inhibition of IL-10 by rapamycin involves the targeting of PDCD4.

3.2.3.5 The effect of PDCD4 deficiency on IL-10 expression

I next examined the effect of rapamycin on IL-10 production in PDCD4 deficient cells. As shown in Figure 3.19A, LPS-induced degradation of PDCD4 in the wild-type matched BMDMs (Figure 3.19A lanes 4-5). Rapamycin blocked this effect, as expected (Figure 3.19A lanes 14 and 15). PDCD4 was absent in the PDCD4-deficient cells (Figure 3.19A lanes 6-10 and 16-20). Figure 3.18B shows a significant increase

in IL-10 cytokine production in the PDCD4-deficient BMDMs compared to the wild-type BMDMs after 6 hours LPS stimulation (Figure 3.19B, 6 hours LPS treated samples, compare grey bar to white bar). When the cells were pre-treated with rapamycin before LPS stimulation, the wild-type BMDMs show a significant decrease in IL-10 after 6 hours (Figure 3.19B 6 hours LPS treated samples, compare black bar with white bar). However this effect of rapamycin is absent in the PDCD4-deficient BMDMs (Figure 3.19B, 6 hours LPS treated samples, compare grey hatched bar with grey bar). Figure 3.19C shows an increase in IL-6 cytokine production in the PDCD4-deficient BMDMs compared to the wild-type BMDMs (Figure 3.19C, 6 hours LPS treated samples compare grey bar with white bar). Cells pre-treated with rapamycin before LPS stimulation showed no difference compared to the cells without rapamycin pre-treatment (Figure 3.19C, 6 hours LPS treated samples compare grey hatched bar with grey bar). These results confirm the observation made using siRNA to PDCD4.

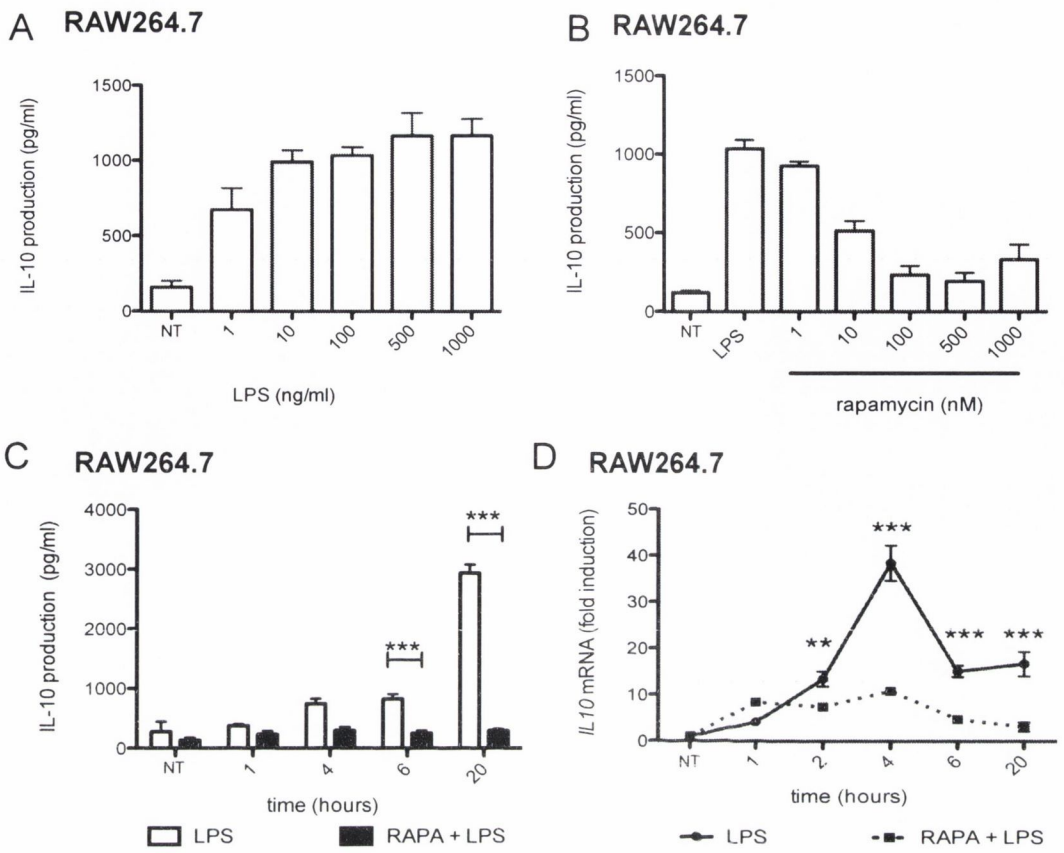


Figure 3.11 – Rapamycin pretreatment results in inhibition of LPS-induced IL-10.

RAW264.7 cells were plated at $5x10^5$ cells/mL in a 12-well plate and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. (A) The following day, cells were left untreated (NT) or were stimulated with various LPS concentrations, as indicated, for 6 hours. (B) Cells were pretreated with various concentrations of rapamycin for 30 minutes followed by 6 hours of stimulation with 100 ng/ml LPS. (C-D) Cells were pretreated with DMSO (LPS) or 500nM rapamycin (RAPA+LPS) for 30 minutes followed by 100ng/ml LPS for indicated time. (A-C) After the indicated treatments IL-10 was measured in supernatants by ELISA. (D) mRNA was extracted and analyzed using TaqMan RT-PCR probes specific for murine IL-10 and using ΔΔCT method. Data were normalized to GAPDH mRNA expression and fold induction was calculated relative to untreated cells at 0 hours. The data shown represents the mean of three independent experiments, each carried out in triplicate ± S.E.M. ** ; P<0.01 ***; P<0.001

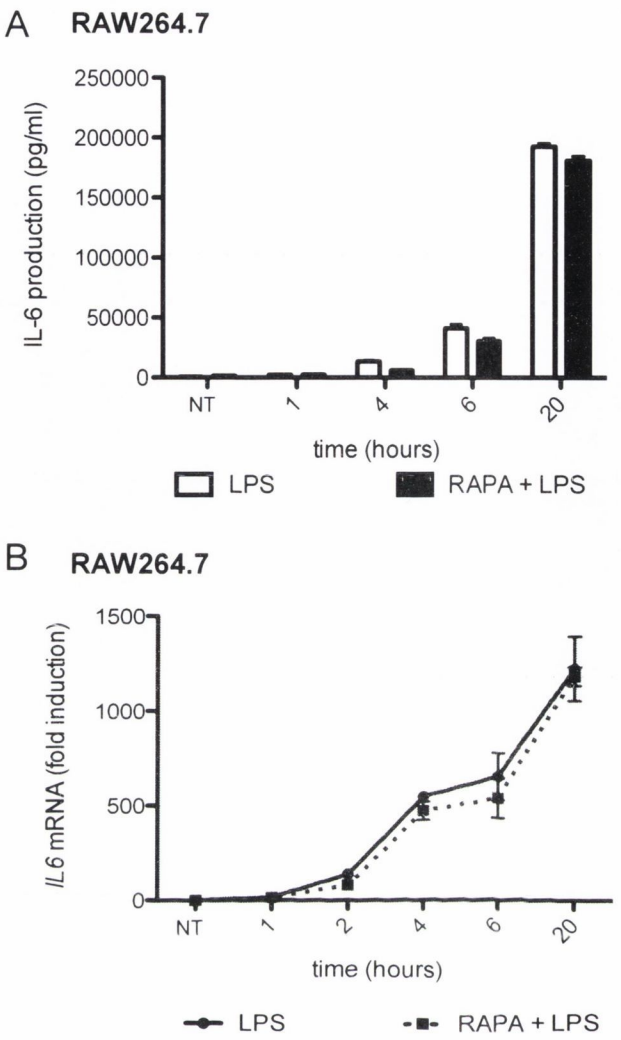


Figure 3.12 – Rapamycin pre-treatment has no effect on IL-6.

RAW264.7 cells were plated at $5x10^5$ cells/mL in a 12-well plate were incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. The following day, cells were left untreated (NT) or were pretreated with DMSO (LPS) or 500 nM rapamycin (RAPA+LPS) for 30 minutes followed by 100 ng/ml LPS stimulation for the indicated time. (A) IL-6 in cell supernatants was measured by ELISA. (B) mRNA was extracted and analyzed using TaqMan RT-PCR probes specific for murine IL-6 and using ΔΔCT method. Data were normalized to GAPDH mRNA expression and fold induction was calculated relative to untreated cells at 0 hours. The data shown represents the mean of three independent experiments, each carried out in triplicate ± S.E.M.

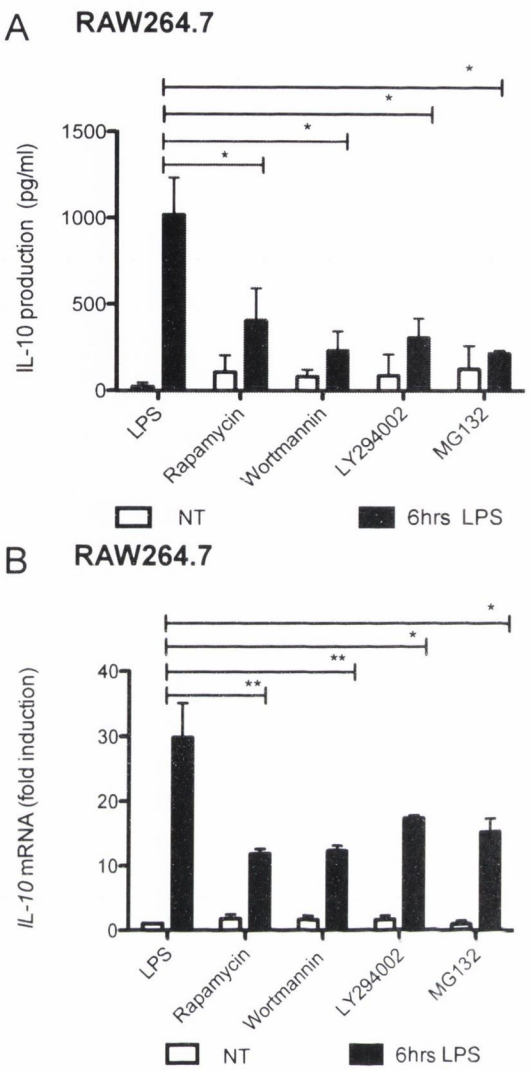


Figure 3.13 – Pre-treatment with rapamycin or PI3K inhibitors results in inhibition of LPS-induced IL-10.

RAW264.7 cells were plated at $5x10^5$ cells/mL in a 12-well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. The following day, cells were left untreated (NT) or were pretreated with 20μM LY294002, 5μM Wortmannin, 5μM MG132 or 500nM rapamycin for 30 minutes followed by 100ng/ml LPS for 6 hours. (A) IL-10 in cell supernatants was measured by ELISA. (B) mRNA was extracted and analyzed using TaqMan RT-PCR probes specific for murine IL-10 and using ΔΔCT method. Data were normalized to GAPDH mRNA expression and fold induction was calculated relative to untreated cells at 0 hours. The data shown represents the mean of three independent experiments, each carried out in triplicate ± S.E.M.

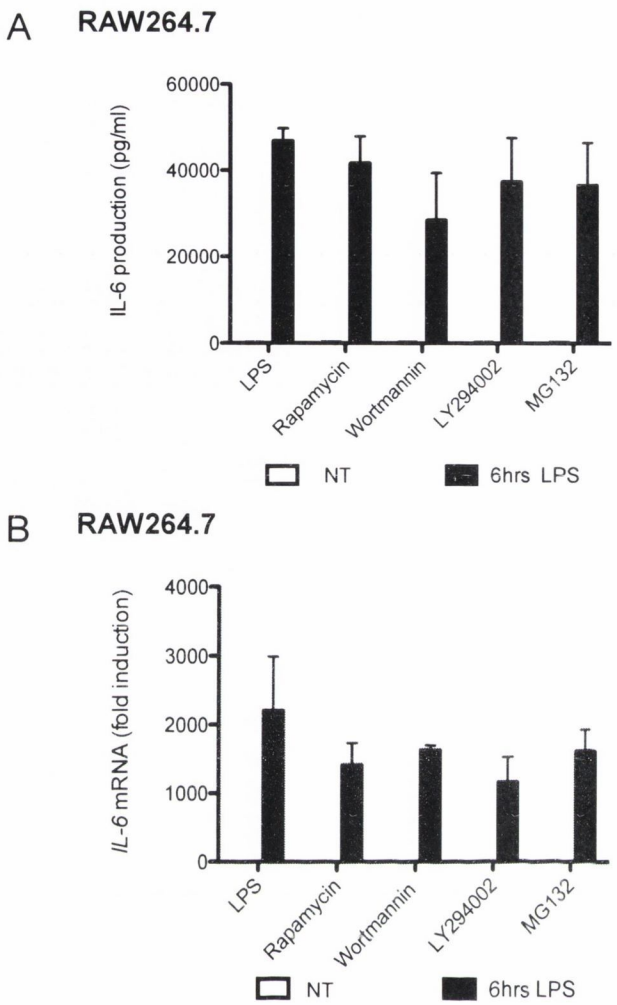


Figure 3.14 – Pre-treatment with rapamycin or PI3K inhibitors has no effect on IL-6.

RAW264.7 cells were plated at $5x10^5$ cells/mL in a 12-well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. The following day, cells were left untreated (NT) or were pretreated with 20μM LY294002, 5μM Wortmannin, 5μM MG132 or 500nM rapamycin for 30 minutes followed by 100ng/ml LPS for 6 hours. (A) IL-6 in cell supernatants was measured by ELISA. (B) mRNA was extracted and analyzed using TaqMan RT-PCR probes specific for murine IL-6 and using ΔΔCT method. Data were normalized to GAPDH mRNA expression and fold induction was calculated relative to untreated cells at 0 hours. The data shown represents the mean of three independent experiments, each carried out in triplicate ± S.E.M.

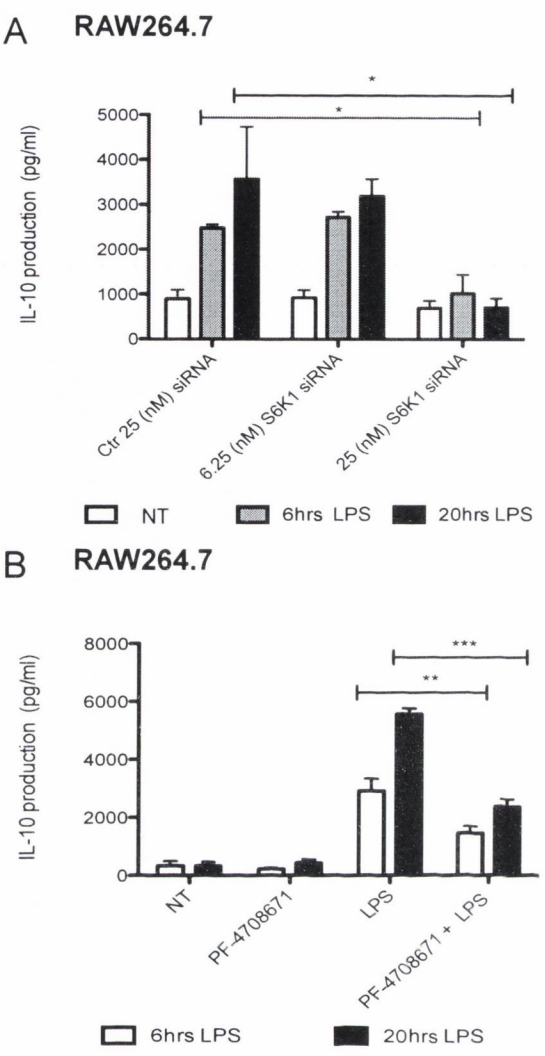


Figure 3.15 – Transfection of RAW264.7 cells with S6K1 siRNA results in inhibition of LPS-induced IL-10.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 24-well plates and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, penicillin and streptomycin (100µg/ml). (A) Cells were transfected with 6.25 nM or 25 nM siRNA targeting S6K1 or control siRNA oligonucleotide (Ctr) and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. Cells were left untreated (NT) or were stimulated with 100 ng/ml LPS for 6 or 20 hours. (B) Cells were left untreated (NT) or were pretreated with 10µM PF-47086871 or DMSO for 30 minutes followed by 100ng/ml LPS for 6 or 20 hours. After the indicated treatments IL-10 was measured in supernatants by ELISA. The data shown represents the mean of three independent experiments, each carried out in triplicate ± S.E.M.

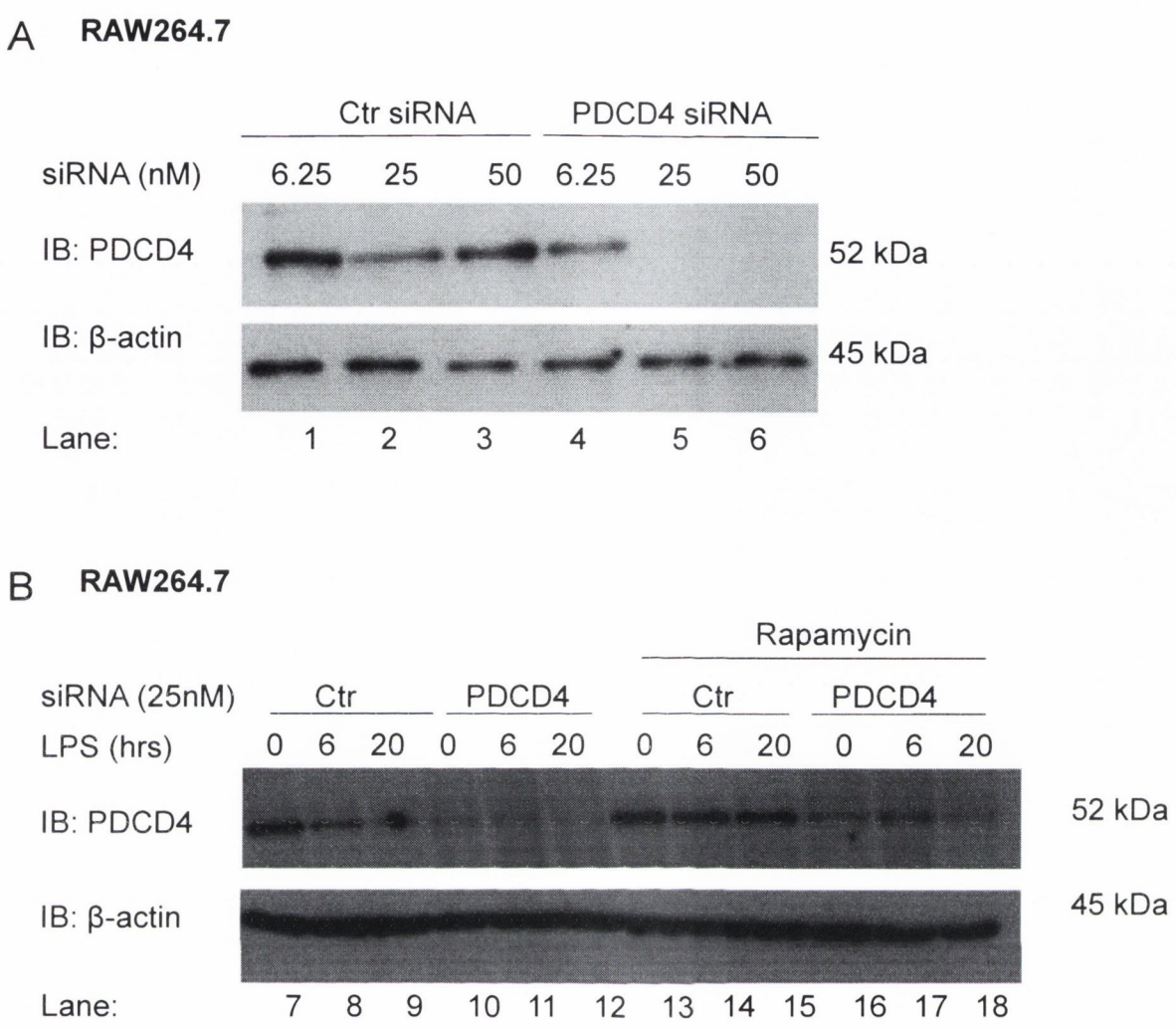
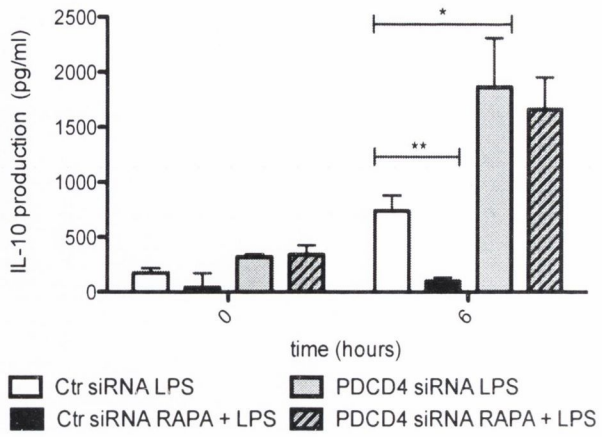


Figure 3.16 – RAW264.7 cells transfected with PDCD4 siRNA results in a knock-down of PDCD4.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 24-well plate and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, penicillin and streptomycin (100µg/ml). (A) The following day, cells were transfected with 6.25 nM or 25 nM or 50 nM siRNA targeting PDCD4 or control siRNA oligonucleotide (Ctr) and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. (B) The following day, cells were transfected with 25 nM siRNA targeting PDCD4 or control siRNA oligonucleotide (Ctr) and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. Cells were pretreated with DMSO or 500nM rapamycin for 30 minutes followed by 100ng/ml LPS for the indicated times. After stimulation protein lysates were prepared and analyzed via Western blotting followed by immuno-blotting (IB) for PDCD4. The expression of β-actin is presented as a loading control. A similar result was obtained in another independent experiment.

A **RAW264.7**



B **RAW264.7**

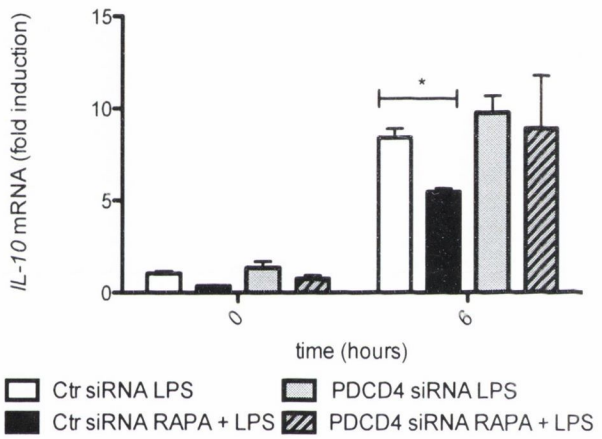
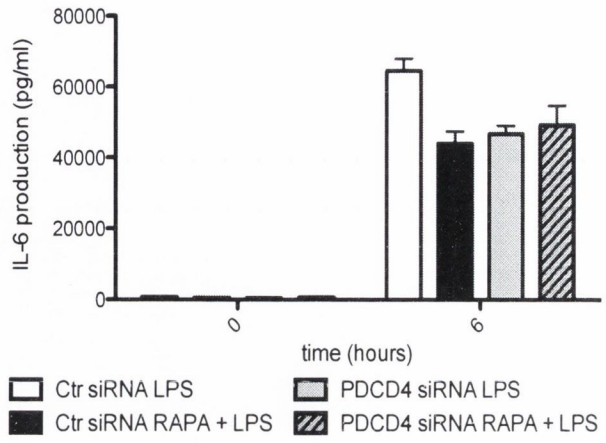


Figure 3.17 – Knock-down of PDCD4 increases LPS-induced IL-10 and prevents the inhibiting effect of rapamycin.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 24-well plates and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, penicillin and streptomycin (100µg/ml). The following day, cells were transfected with 25 nM siRNA targeting PDCD4 or control siRNA oligonucleotide (Ctr) and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. Cells were pretreated with DMSO (LPS) or 500nM rapamycin (RAPA+LPS) for 30 minutes followed by 100ng/ml LPS for 6 hours. (A) IL-10 in cell supernatants was measured by ELISA. (B) mRNA was extracted and analyzed using TaqMan RT-PCR probes specific for murine IL-10 and using ΔΔCT method. Data were normalized to GAPDH mRNA expression and fold induction was calculated relative to untreated cells at 0 hours. The data shown represents the mean of three independent experiments, each carried out in triplicate ± S.E.M. * ; $P<0.05$ **; $P<0.01$

A **RAW264.7**



B **RAW264.7**

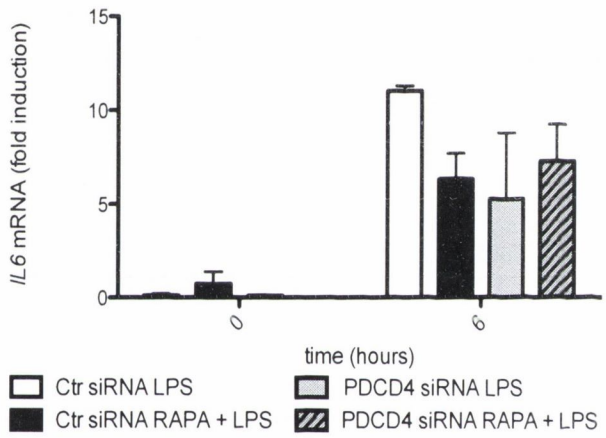


Figure 3.18 – Knock-down of PDCD4 has a slightly reduced effect on IL-6.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 24-well plates and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, penicillin and streptomycin (100µg/ml). The following day, cells were transfected with 25 nM siRNA targeting PDCD4 or control siRNA oligonucleotide (Ctr) and the medium was changed into DMEM medium supplemented with 1% FCS for 24 hours. Cells were pretreated with DMSO (LPS) or 500nM rapamycin (RAPA+LPS) for 30 minutes followed by 100ng/ml LPS for 6 hours. (A) IL-6 in cell supernatants was measured by ELISA. (B) mRNA was extracted and analyzed using TaqMan RT-PCR probes specific for murine IL-6 and using ΔΔCT method. Data were normalized to GAPDH mRNA expression and fold induction was calculated relative to untreated cells at 0 hours. The data shown represents the mean of three independent experiments, each carried out in triplicate.

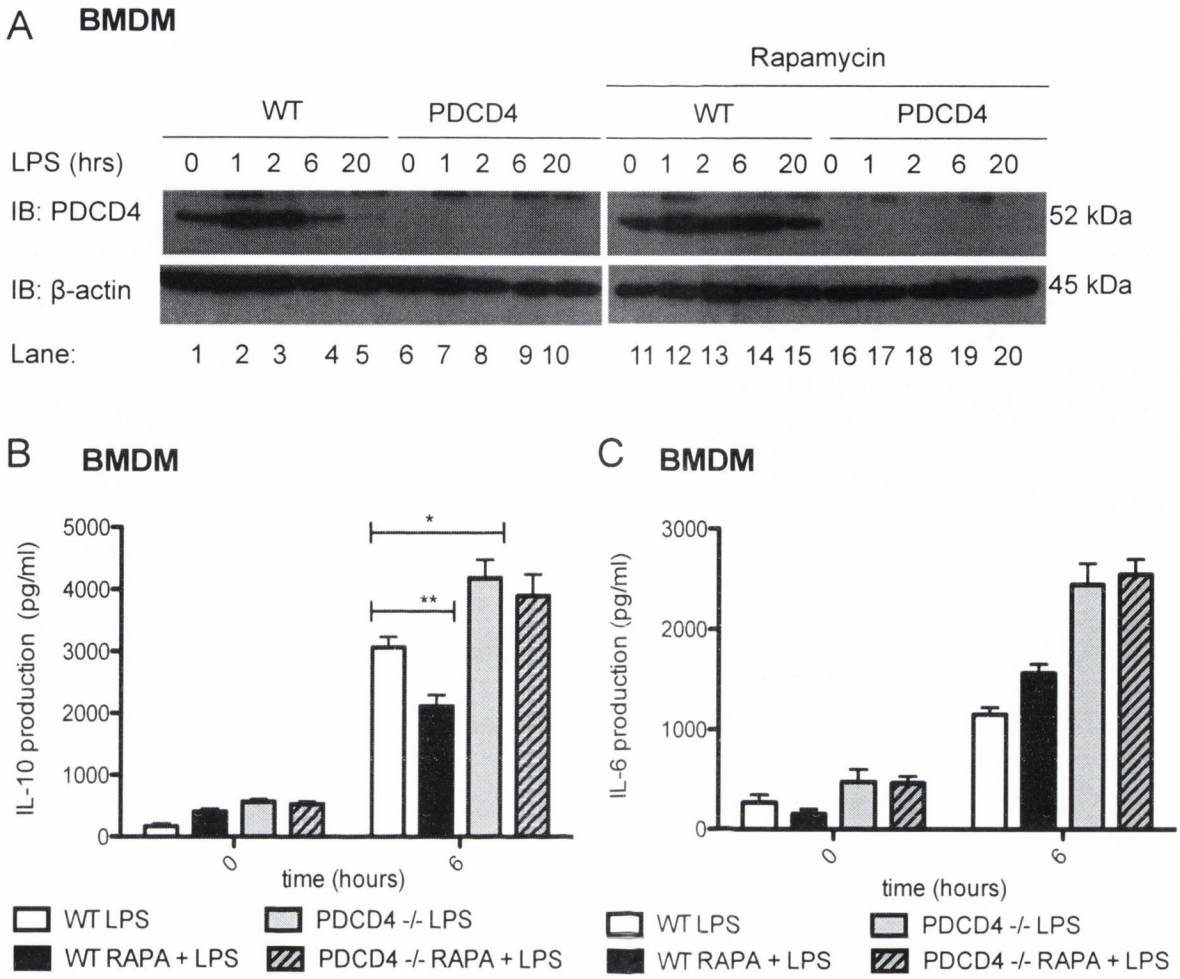


Figure 3.19 – A PDCD4 deficiency increases LPS-induced IL-10.

Wild-type BMDMs (WT) and PDCD4 deficient BMDMs (PDCD4-/-) were plated at $5x10^5$ cells/mL in 24-well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. The following day, cells were pretreated with DMSO (LPS) or 500nM rapamycin (RAPA+LPS) for 30 minutes followed by 100ng/ml LPS for the indicated time. (A) After stimulation, protein lysates were prepared and analyzed via Western blotting followed by immuno-blotting (IB) for PDCD4. The expression of β-actin is presented as a loading control. A similar result were obtained in three independent experiments. (B) After stimulation, IL-10 in cell supernatants was measured by ELISA. (C) IL-6 in cell supernatants was measured by ELISA. The data shown represents the mean of three independent experiments, each carried out in triplicate ± S.E.M. * ; $P<0.05$ **; $P<0.01$

3.3 Discussion

The initial line of investigation in this project began with the observation that LPS ligation of TLR4 results in a degradation of the protein PDCD4. PDCD4 was known to be important as a translational inhibitor through its interaction with the members of the eIF4 family of eukaryotic translation initiation factors (Yang et al., 2003, Loh et al., 2009). However previous investigations by Sheedy et al (2010) have shown that PDCD4 is important in the inflammatory response to infection, with PDCD4 deficient mice protected from the lethality of LPS (Sheedy et al., 2010). In this study it was also shown that PDCD4 is a negative regulator of IL-10, and that following LPS stimulation PDCD4 acts as a molecular switch, whereby its degradation results in increased IL-10 production (Sheedy et al., 2010).

My first aim was to examine whether the mTOR pathway and proteosomal degradation were involved in LPS signalling to IL-10. It has previously been shown that in growth factor signalling rapamycin can inhibit PDCD4 protein degradation. Dorrello et al. (2006) also showed that LY294002, a PI3K inhibitor, prevents the proteolysis of PDCD4 (Dorrello et al., 2006). Another study with LY294002 and rapamycin confirmed that treatment with the inhibitors prevented the loss of PDCD4 protein in response to growth factors in MCF7, a breast cancer cell line (Schmid et al., 2011). The results shown in this thesis are in agreement with these previous studies. In this project it has been shown that pre-treatment with the mTORC1 pathway inhibitor rapamycin followed by LPS inhibit LPS-induced PDCD4 protein degradation in macrophages.

It has been shown that stabilization of PDCD4 by the mTOR and PI3K inhibitors paralleled the lack of S6K1 phosphorylation and activation, suggesting a role for

S6K1 in the pathway. In contrast, the inhibitors of p38 and ERKs had no effect on the degradation of PDCD4 (Dorrello et al., 2006). Both rapamycin and PI3K inhibitors prevent LPS-induced PDCD4 degradation. This was further confirmed when I examined the role of S6K1. When the mTOR pathway is active in response to growth factors, it can phosphorylate S6K1 (Luo et al., 1996, von Manteuffel et al., 1997). S6K1-dependent phosphorylation has previously been shown to be critical for the phosphorylation and proteasomal degradation of PDCD4 in T98G glioblastoma cells after serum activation and in HEK293 cells activated with TPA (Dorrello et al., 2006, Schmid et al., 2008). Dorrello et al. (2006) have shown that when S6K1 is activated it phosphorylates PDCD4 Ser^{67} and that this event in turn promotes the phosphorylation of Ser^{71} and Ser^{76}. The phosphorylation of the serine sites allows βTRCP to bind and result in PDCD4 degradation (Dorrello et al., 2006). In this chapter it was determined that macrophages transfected with S6K1 specific siRNA lose the ability to induce PDCD4 degradation by LPS. However depletion with siRNA appears to only partially block LPS-induced PDCD4 degradation. This is most likely due to the incomplete knock-down of S6K1. The residual S6K1 present could still lead to partial PDCD4 degradation. Additionally another method using a S6K1 inhibitor PF-4708671 was examined to implicate whether S6K1 was involved in the degradation of PDCD4. The PF-4708671 is known as a highly specific inhibitor of S6K1. It is also known that PF-4708671-induced phosphorylation is dependent upon mTORC1 (Pearce et al., 2010). However our results have shown the novel influence of PF-4708671 on PDCD4 degradation. As expected, the inhibitor blocked LPS-induced PDCD4 degradation after 6 and 20 hours. In total the data add to the understanding of processes whereby LPS promotes PDCD4 degradation and the essential part of the mTOR pathway in this LPS-induced response.

Dorrello et al., (2006) also demonstrated by mass spectrometry that PDCD4 and βTRCP interact, suggesting that βTRCP is the ubiquitin ligase targeting PDCD4 for

degradation (Dorrello et al., 2006). The results from this chapter indicate that a process similar to that occurring in response to growth factors with regard to PDCD4 is occurring in response to LPS.

The increase of PDCD4 after rapamycin pre-treatment was a result of protein stability. As shown here using cycloheximide treatment to block protein translation, a similar result has also been shown in a previous study in MCF7 cells treated with conditioned medium and rapamycin treatment (Schmid et al., 2011). Schmid et al (2011) claimed to have identified that PI3K-mTOR-dependent proteosomal degradation as a key pathway for PDCD4 down-regulation in macrophages (Schmid et al., 2011). Their results were in agreement with the results shown in this thesis. However when cells were pre-treated with 1nM rapamycin PDCD4 degradation was inhibited. Interestingly IL-10 production was only blocked after higher doses of rapamycin. It might that the turnover blocking IL-10 only can be affected when treated with higher doses of rapamycin but the mechanism behind this remains not understood.

It has been shown many times that MAPK signalling is required for the induction of IL-10 by LPS in macrophages (Elcombe et al., 2013). However, there is growing evidence that other pathways such as the mTOR pathway are involved (Ivanov and Roy, 2013). My findings that PI3K inhibitors and rapamycin reduce induction of IL-10 by LPS in macrophages are in agreement with previous studies (Martin et al., 2003, Baker et al., 2009). It has previously been demonstrated that rapamycin has the ability to decrease IL-10 mRNA and protein in monocytes and dendritic cells treated with LPS (Haidinger et al., 2010, Weichhart et al., 2008, Baker et al., 2009, Wang et al., 2011). The LPS-induced inhibition in IL-10 production was also confirmed for the proteasome inhibitor MG132 and S6K inhibitor PF-470871 as well as S6K1 knock-down. As published before macrophages deficient in PDCD4 show more IL-10 production after LPS stimulation (Sheedy et al., 2010). When I

repeated the same experiment and confirmed an increase in IL-10 secretion I challenged the PDCD4 deficient macrophages and the PDCD4 siRNA transfected cells with rapamycin followed by LPS. Pre-treatment with rapamycin showed no effect on LPS-induced IL-10 expression in PDCD4 deficient cells, further supporting the targeting of PDCD4 by the mTOR pathway. As a control throughout this thesis IL-6 has been measured and it has been demonstrated that IL-6 mRNA and protein are not influenced by LPS-induced PDCD4 degradation. Firstly, it has been shown that TLRs can activate NF-κB through the PI3K-mTORC1 pathway (Arbibe et al., 2000). The activation of NF-κB might explain why the pro-inflammatory cytokine IL-6 is not affected in our mechanism as the activation of NF-κB may not lead to PDCD4 degradation. However, it has been shown that Twist proteins are able to bind to the p65 subunit of NF-κB and negatively regulate cytokine expression (Sosic et al., 2003). Secondly, IL-6 is unlikely to be translated through the same eukaryotic initiation complex (eIF) as it has a more simplified UTR and does not require cap-dependent translation through the eIF. It also has been shown that IL-10 requires mTOR dependent translation whereas, IL-6 induction is independent of mTOR translation.

Overall this chapter has revealed that the mTOR pathway is involved in LPS-induced IL-10 signalling, through PDCD4 degradation (Figure 3.20). Furthermore this response was shown to be rapamycin-dependent with no effect seen on IL-6. Because confocal studies suggested that PDCD4 can interact with eIF4A (Yang et al., 2003a, Goke et al., 2002), one role for PDCD4 here is to limit eIF4 and prevent translation of IL-10. As I had observed a role for PDCD4 in limiting both PDCD4 protein and mRNA I next explored this further. In the next chapter I examined the role of transcription factor Twist2, which I show to bind to PDCD4 in this pathway. I have found that Twist2 is released from PDDC4 and increases c-Maf transcription, which then promotes IL-10 production.

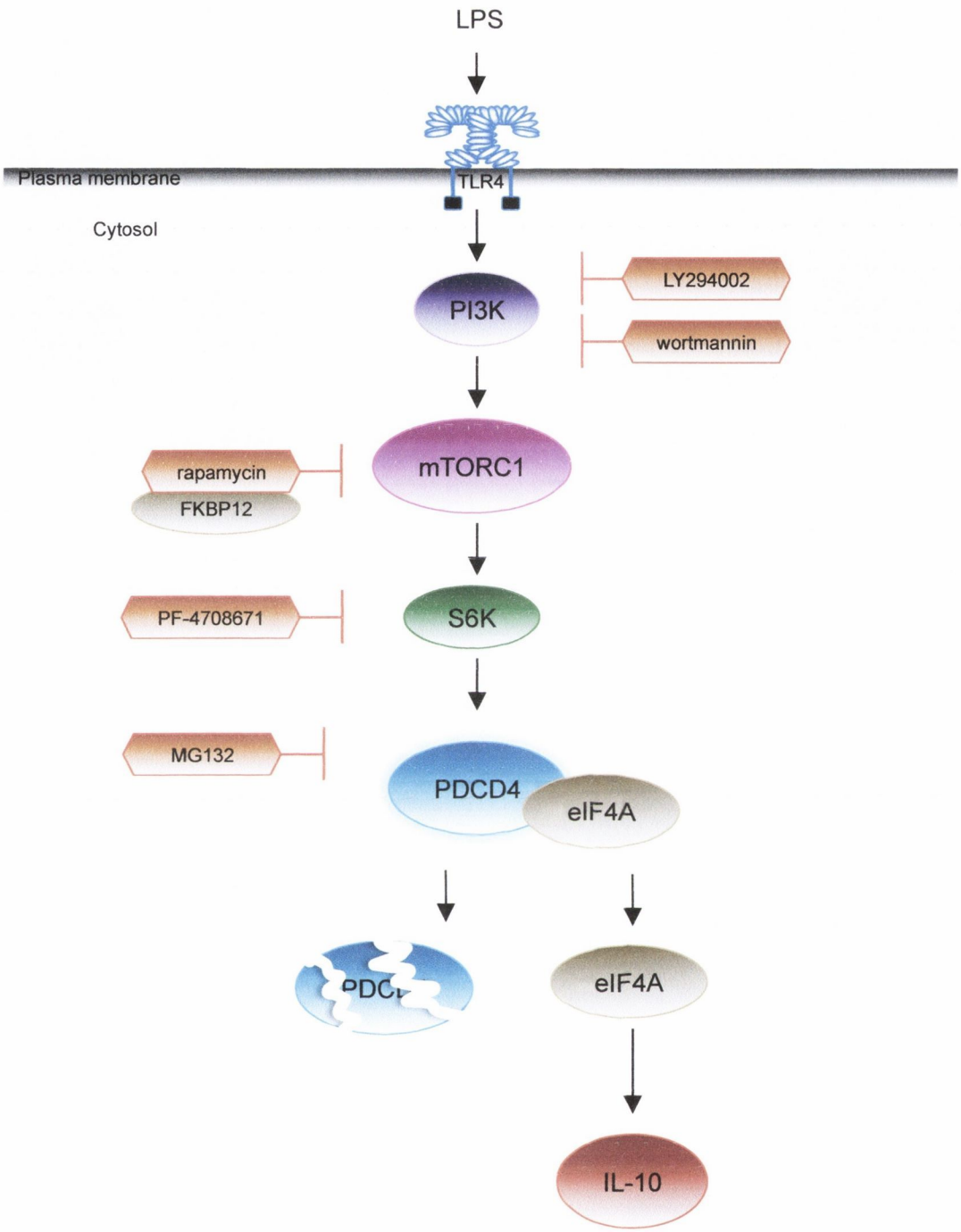


Figure 3.20 – Proposed regulation of LPS-induced IL-10 expression by mTOR, and PDCD4.

LPS activation of PI3K/mTOR signalling induces the expression of IL-10 by degradation of PDCD4. Pre-treatment with rapamycin, an inhibitor of mTOR activity can prevent LPS-induced degradation.

CHAPTER 4

PDCD4 AND TWIST2 FORM A COMPLEX

4.1 Introduction

In the previous chapter, I determined that the mTOR pathway is involved in LPS-induced signalling to IL-10 production possibly through PDCD4 degradation. PDCD4, which is known to contain two conserved MA3 domains, also found in eIF4A and eIF4G, has been shown to interact with and disrupt the function of eIF4A and eIF4G (Goke et al., 2002, Yang et al., 2003, Zakowicz et al., 2005). The crystal structures of the interactions of the two domains of PDCD4 with eIF4A and eIF4G have been solved and the interactions have been shown to occur in a 1:2:1 ratio (Loh et al., 2009, Waters et al., 2007). It is likely that PDCD4 prevents translation of IL-10 mRNA by regulating these factors.

PDCD4 has also been identified through a screen of GST-fusion proteins to be a functional partner for Twist1. This was confirmed using immunoprecipitation of PDCD4 and Twist1. Release of Twist1 lead to YB-1 expression which, is linked to various biological activities of cancer cells. (Shiota et al., 2009, Kohno et al., 2003). In the same study the authors examined different Twist1 truncated proteins to map the PDCD4 binding sites. This resulted in the discovery that the bHLH domain mediates the interaction with PDCD4 proteins. Through bioinformatics it was established that the conserved amino-terminal region of eIF4A possesses sequence homology with the bHLH region of Twist1 (Shiota et al., 2009). This indicates that both proteins bind PDCD4 in the same manner. Therefore there might be competition for the binding site of PDCD4 between these two proteins.

Twist2, a family member of Twist1 with a 95% homology in the bHLH region, has been noted to be involved in myeloid lineage development as it inhibits proliferation and differentiation of macrophages (Sharabi et al., 2008) However, in the same study,

it was indicated that the transcription factor Twist2 is involved in the production of IL-10 by regulating expression of c-Maf (Sharabi et al., 2008).

Therefore in this chapter I have examined a role for Twist2, which would be released from the degraded PDCD4 in the regulation of IL-10 production via induction of c-Maf. I have uncovered a novel mechanism for the LPS–induced IL-10 induction through the PDCD4/Twist2 complex.

4.2 Results

4.2.1 PDCD4 and Twist-2 complex

4.2.1.1 PDCD4 binds to Twist1

As described previously PDCD4 can interact with Twist1 (Shiota et al., 2009). Here I used the interaction as a control as shown in Figure 4.1 a co-immunoprecipitation (co-IP) experiment was carried out with overexpressed HA-tagged PDCD4 and FLAG-tagged Twist1. In unstimulated cells an interaction was observed between PDCD4 and Twist1, also with cells overexpressing HA-tagged PDCD4 and FLAG-tagged Twist2. (Figure 4.1 top panel lanes 6 and 7). After stimulation with LPS there was again a reduced interaction between PDCD4 and Twist2 (Figure 4.1 top panel lane 8). The reduced interaction can also be observed in PDCD4 and Twist1 (Figure 4.1 top panel lane 9).

4.2.1.2 PDCD4 binds to Twist2 and the complex is sensitive to rapamycin treatment.

First I examined Twist2 as a possible candidate for interaction and complex formation with PDCD4. A co-IP experiment was therefore carried out. As shown in Figure 4.2, in unstimulated cells an interaction between PDCD4 and Twist2 was observed (Figure 4.2 top panel lane 4). Stimulation with LPS reduced this interaction (Figure 4.2, top panel lane 5). Only a fraction of the expressed PDCD4 interacts with Twist2, since LPS did not cause a detectable decrease in the levels of total PDCD4 in the transfected cells (Figure 4.2, middle panel lane 5). When cells were pre-treated with rapamycin, the dissociation between PDCD4 and Twist2 following LPS stimulation failed to occur (Figure 4.2 top panel lanes 6 and 7). When cells were pre-treated with

rapamycin in a dose-dependent manner (Figure 4.3 top panel lanes 6,7 and 9) 500nM dose of rapamycin could block the LPS-induced dissociation between PDCD4 and Twist2 (Figure 4.3 top panel lane 8). These results show that PDCD4 and Twist2 can form a complex, which can be disrupted by LPS in a rapamycin-sensitive manner.

4.2.1.3 The effect of PI3K inhibitors and a proteasomal inhibitor on PDCD4/Twist2 binding.

After the co-IP showing that cells pre-treated with rapamycin could stabilize the interaction between PDCD4 and Twist2 following LPS stimulation, I was interested in the effect of wortmannin, LY294002 and MG132 on the interaction between PDCD4 and Twist2. Therefore a co-IP was carried out with cells overexpressing HA-tagged PDCD4 and FLAG-tagged Twist2. Untreated cells showed an interaction between PDCD4 and Twist2 (Figure 4.4 top panel lane 6) and stimulation with LPS reduced the interaction (Figure 4.4 top panel lane 5). Cells pre-treated with rapamycin, wortmannin, LY294002 or MG132 followed by 6 hours LPS stimulation inhibited this degradation, although rapamycin and wortmannin were more effective (Figure 4.4 top panel lane 6-9). The reduced HA-tagged PDCD4 construct in the cells treated with MG132 in absence of LPS-stimulation (Figure 4.4 middle panel lane 13) might have caused the reduced interaction between PDCD4 and Twist2 (Figure 4.4 top panel lane 13).

4.2.1.4 S6K-mediated phosphorylation of PDCD4 stabilizes Twist2 interaction.

I next examined the role of S6K here. As shown in Figure 4.5, overexpressed HA-tagged PDCD4 (wild-type) interacts with overexpressed FLAG-tagged Twist2 (Figure 4.5 top panel lane 6). Stimulation with LPS reduced PDCD4 and Twist2 interaction

(Figure 4.5 top panel lane 8). This interaction can also be observed in cells with overexpressed HA-tagged PDCD4 with a mutation located in the S6K phosphorylation site. The amino acid serine located at position 67 was changed for an alanine (S67A mutant) (Figure 4.5 lane 7). However, after stimulation with LPS the decrease in the interaction between this mutant and the wild-type PDCD4 was attenuated (Figure 4.5 compare lane 9 to lane 8).

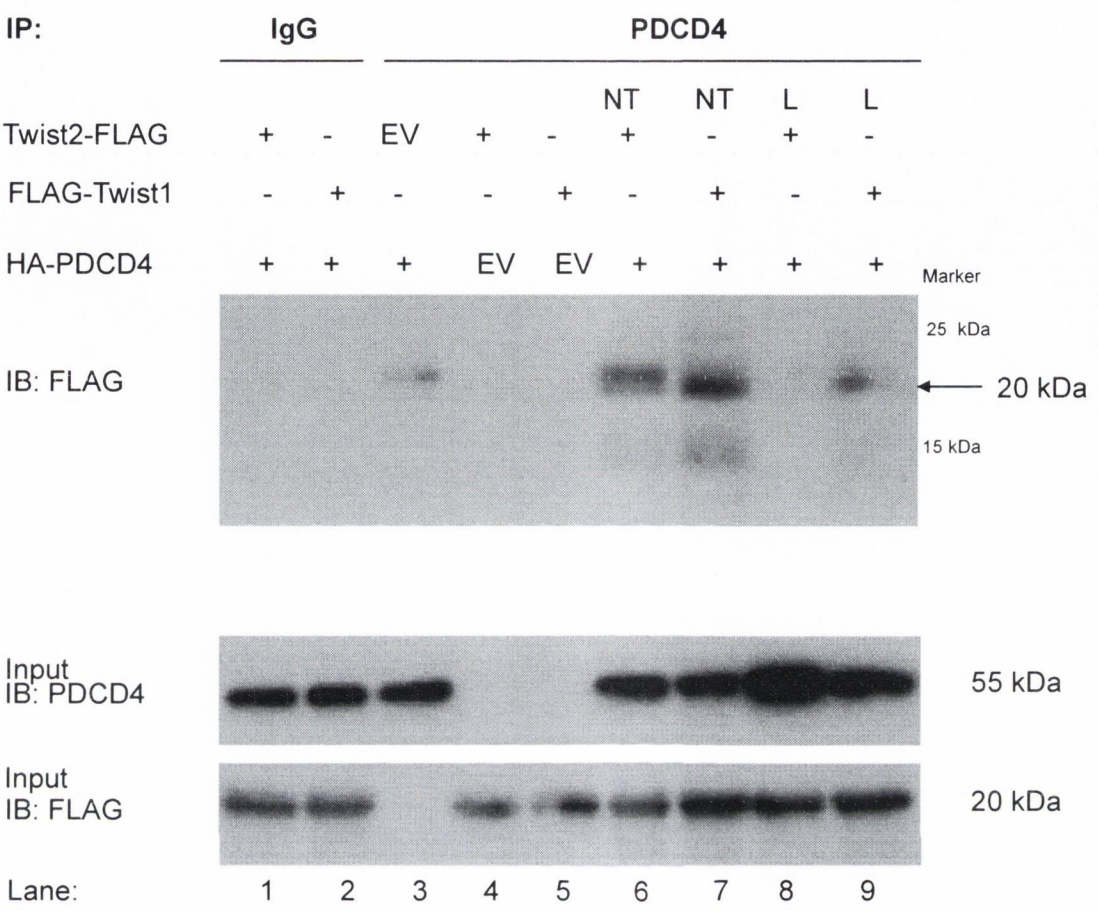


Figure 4.1 – PDCD4 binds Twist1 in HEK293-TLR4-MD2-CD14 cells.

HEK293-TLR4-MD2-CD14 cells were plated at $5x10^5$ cells/mL in 10cm dishes and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, 50mg/ml Noromycin, 100µg/ml Hydrogold and 100µg/ml Blastinomycin. The following day, cells were transfected with 2µg Twist2-FLAG, 2µg FLAG-Twist1, 2µg HA-PDCD4 or 2µg empty vector (EV) and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were stimulated with 100ng/ml LPS for 6 hours (L) indicated in lanes 8-9. Untreated samples (NT) are shown in lanes 6-7. The lysates were used for Immunoprecipitation (IP) with antibodies against IgG or PDCD4 and were prepared and analyzed via Western blotting followed by immuno-blotting (IB) with FLAG specific antibody (lane 1-9). 1:10 dilution of the whole lysate was also prepared and analyzed via Western blotting followed by immuno-blotting (IB) for PDCD4 or FLAG specific antibody. The experiment shown is representative of at least three independent experiments.

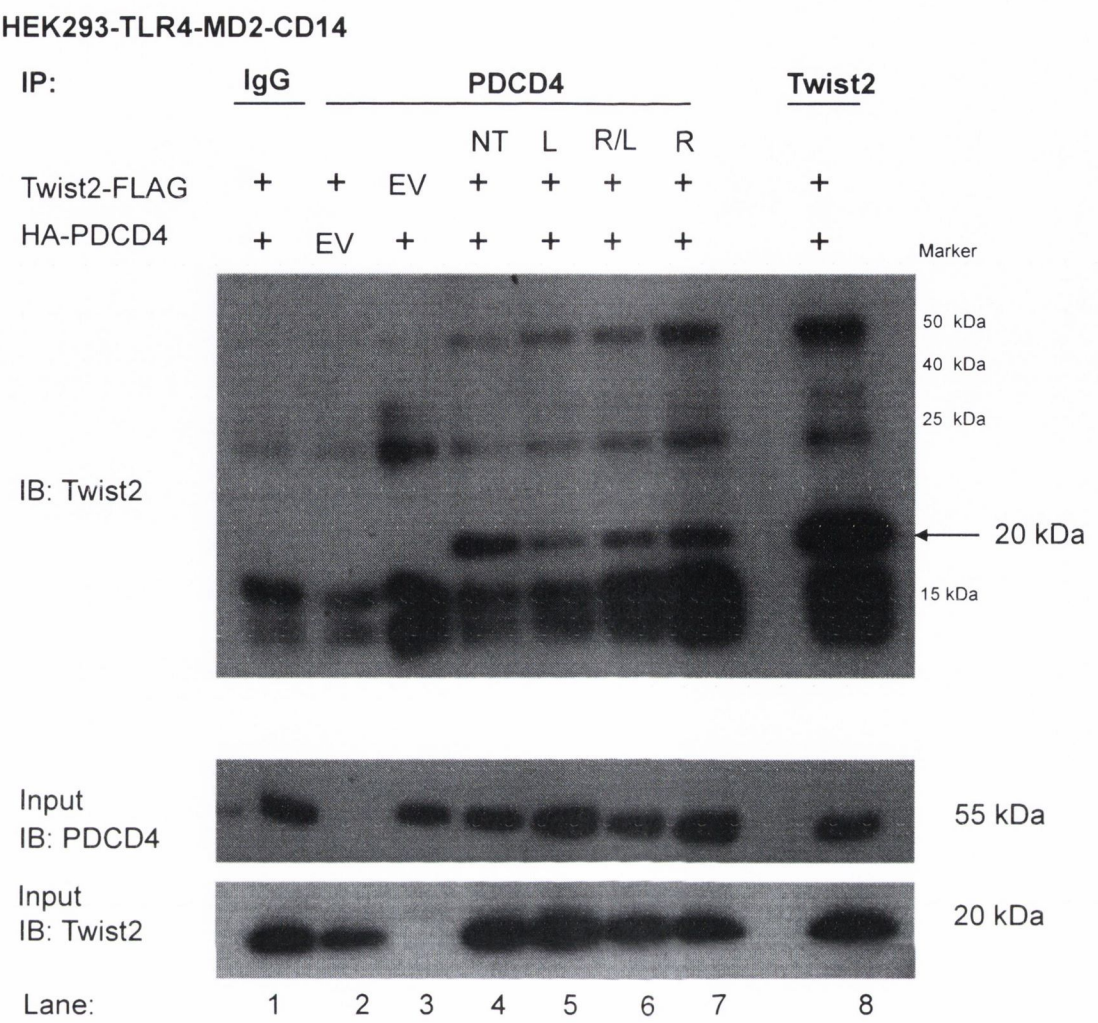


Figure 4.2 – Overexpressed PDCD4 binds to overexpressed Twist2 in HEK293-TLR4-MD2-CD14 cells, and their dissociation is sensitive to rapamycin treatment.

HEK293-TLR4-MD2-CD14 cells were plated at $5x10^5$ cells/mL in 10cm dishes and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, 50mg/ml Noromycin, 100µg/ml Hydrogold and 100µg/ml Blastinomycin. The following day, cells were transfected with 2µg Twist2-FLAG, 2µg HA-PDCD4 or 2µg empty vector (EV) and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were pre-treated for 30 minutes with DMSO control followed by 100ng/ml LPS (L) for 6 hours indicated in lanes 5 and 13. Samples from cells stimulated with rapamycin (500nM) for 30 minutes followed by 100ng/ml LPS (R/L) treatment for 6 hours are shown in lanes 6. Untreated samples (NT) are shown in lanes 4 and samples from cells stimulated with rapamycin (R) (500 nM) for 6.5 hours are shown in lanes 7. The lysates were used for Immunoprecipitation (IP) with antibodies against IgG, PDCD4 or Twist2, and were prepared and analyzed via Western blotting followed by immuno-blotting (IB) for FLAG antibody (lane 1-8). 1:10 dilution of the whole lysate was also prepared and analyzed via Western blotting followed by immuno-blotting (IB) for HA or FLAG

antibody. The experiment shown is representative of at least three independent experiments.

HEK293-TLR4-MD2-CD14

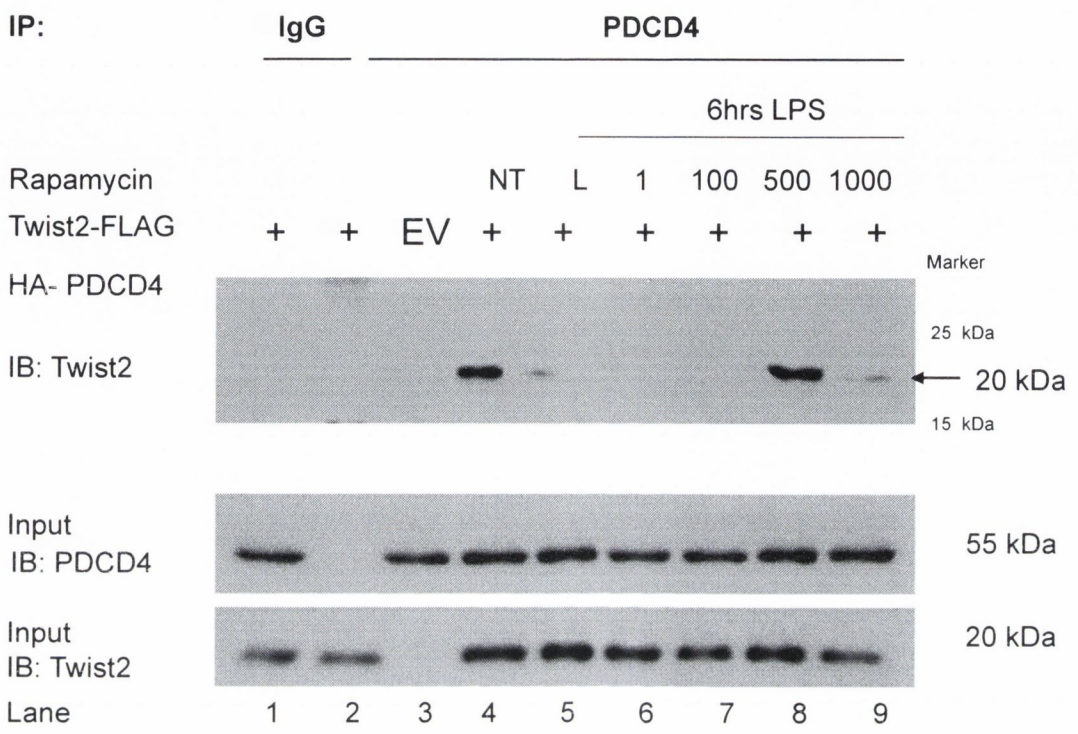


Figure 4.3 – Rapamycin inhibits the LPS-induced dissociation of the Twist2/PDCD4 complex.

HEK293-TLR4-MD2-CD14 cells were plated at $5x10^5$ cells/mL in 10cm dishes and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, 50mg/ml Noromycin, 100µg/ml Hydrogold and 100µg/ml Blastinomycin. The following day, cells were transfected with 2µg Twist2-FLAG, 2µg HA-PDCD4 or 2µg empty vector (EV) and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were stimulated with 100ng/ml LPS (L) for 6 hours indicated in lanes 5. Samples from cells stimulated with various rapamycin concentrations as indicated above each lane followed by 6 hours LPS stimulation are shown in lanes 6-9. Untreated samples (NT) are shown in lanes 4. The lysates were used for Immunoprecipitation (IP) with antibodies against IgG or PDCD4, and were prepared and analyzed via Western blotting followed by immuno-blotting (IB) with Twist2 specific antibody (lane 1-9). 1:10 dilution of the whole lysate was also prepared and analyzed via Western blotting followed by immuno-blotting (IB) for PDCD4 or Twist2 specific antibody. A similar result was obtained in another independent experiment.

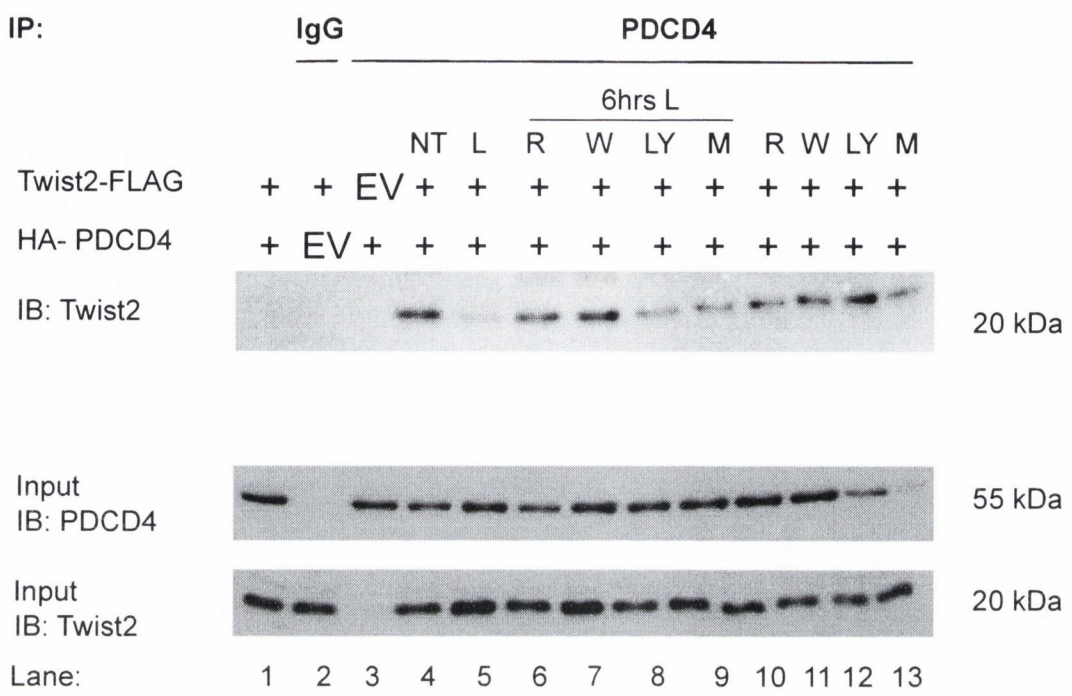


Figure 4.4 – Different mTOR signalling inhibitors stabilize PDCD4/Twist2 complex interaction in HEK293-TLR4-MD2-CD14 cells.

HEK293-TLR4-MD2-CD14 cells were plated at $5x10^5$ cells/mL in 10cm dishes and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, 50mg/ml Noromycin, 100µg/ml Hydrogold and 100µg/ml Blastinomycin. The following day, cells were transfected with 2µg Twist2- FLAG, 2µg HA-PDCD4 or 2µg empty vector (EV) and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were pre-treated with DMSO or 500nM rapamycin (R) lanes 6 and 10, 5µM Wortmannin (W) lanes 7 and 11, 20µM LY294002 (LY) lanes 8 and 12 5µM MG132 (M) lanes 9 and 13 for 30 minutes followed by 100ng/ml LPS for 6 hours (L) indicated in lanes 6-9. Untreated samples (NT) are shown in lanes 4. The lysates were used for Immunoprecipitation (IP) with antibodies against IgG or PDCD4 and were prepared and analyzed via Western blotting followed by immuno-blotting (IB) with FLAG specific antibody (lane 1-13). 1:10 dilution of the whole lysate was also prepared and analyzed via Western blotting followed by immuno-blotting (IB) for PDCD4 or Twist2 specific antibody. The experiment shown is representative of at least three independent experiments.

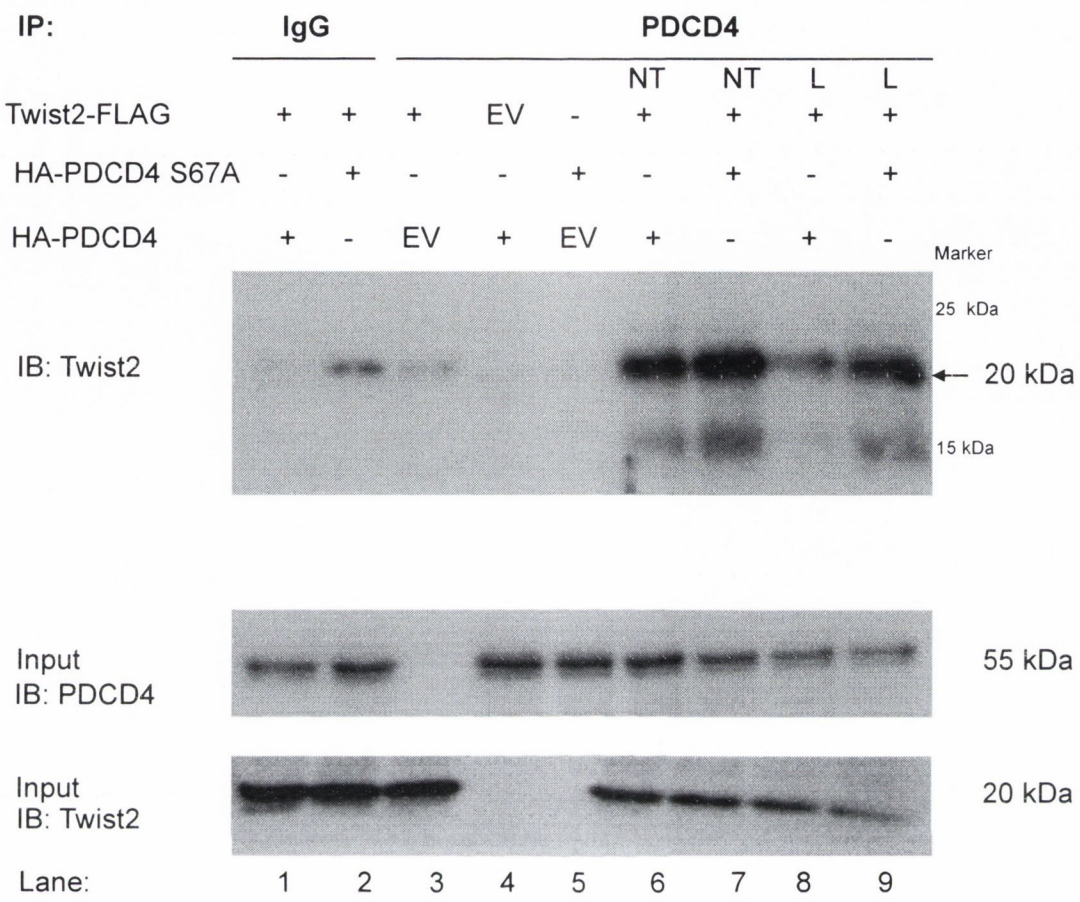


Figure 4.5 – Overexpressed PDCD4 with mutated S6K1 phosphorylation site cause continually interaction with overexpressed Twist2 in HEK293-TLR4-MD2-CD14 cells.

HEK293-TLR4-MD2-CD14 cells were plated at $5x10^5$ cells/mL in 10cm dishes and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, 50mg/ml Noromycin, 100µg/ml Hydrogold and 100µg/ml Blastinomycin. The following day, cells were transfected with 2µg Twist2-FLAG, 2µg HA-PDCD4, 2µg HA-PDCD4 S67A or 2µg empty vector (EV) and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were stimulated with 100ng/ml LPS for 6 hours (L) indicated in lanes 8 and 9. Untreated samples (NT) are shown in lanes 6 and 7. The lysates were used for immunoprecipitation (IP) with antibodies against IgG or PDCD4 and were prepared and analyzed via Western blotting followed by immuno-blotting (IB) with Twist2 specific antibody (lane 1-9). 1:10 dilution of the whole lysate was also prepared and analyzed via Western blotting followed by immuno-blotting (IB) for PDCD4 or Twist2 specific antibody. The experiment shown is representative of at least three independent experiments.

4.2.2 Twist-box and protein-domain are responsible for interaction PDCD4/Twist2 complex and DNA-domain of Twist2 responsible for IL-10 production.

4.2.2.1 Defining the domains responsible for the interaction

Having confirmed an interaction between PDCD4 and Twist, I next investigated which domains of Twist2 mediated the interaction. In Figure 4.6A the schematic representation of Twist1 and Twist2 is shown. Five Twist2 constructs from the full length FLAG-tagged Twist2 were generated as shown in Figure 4.6B. Previously it has been shown that PDCD4 interacts with Twist1 (Shiota et al., 2009) and eIF4A (Yang et al., 2003a). It was found that the amino-terminal region of eIF4A possesses sequence homology with the bHLH of Twist1 (Shiota et al., 2009) and Twist2, which is evolutionarily conserved (shown in black background), and are most likely the amino acids necessary for protein binding (Figure 4.6C). Therefore amino acids located on Ile 92 Thr, Ile 93 Ser, Tyr 113 Cys and Ile 114 Ser (I92T/I93S/Y113C/I114S mutant) in the protein binding domain (in yellow) of Twist2 were mutated (Figure 4.6B mutant Twist-FLAG I92T/I93S/Y113C/I114S). To find a mechanistic function for Twist2 the amino acids located on Thr 79 Ala and Ser 81 Ala (T79A/S81A mutant) in the DNA binding domain (in red) were mutated (Figure 4.6B mutant Twist-FLAG T79A/S81A). The amino acids at position on 79 and 81 are located in a conserved phosphorylation site and are described as important for DNA binding and dimer selectivity for the different bHLH transcription factors (Figure 4.6D) (Firulli and Conway, 2008).

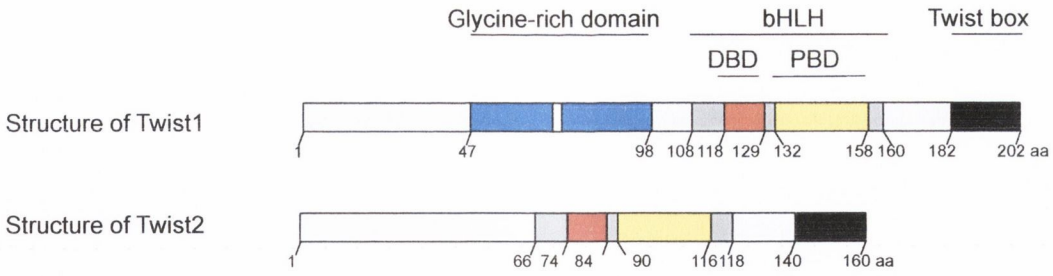
Figure 4.7 illustrates the results obtained from interaction analysis with the constructs. Lane 4 (top panel, top band) shows the interaction between PDCD4 and Twist2 as previously demonstrated in Figure 4.2. The higher percentage gel (18%) revealed a non- specific band running faster than Twist2. The results suggest that

both the Twist Box and the bHLH domain are required for the interaction as removal of either domains abolished the interaction (Figure 4.7, top panel lanes 5, 6 and 8). The mutations generated in the DNA binding domain (Figure 4.7, top panel lane 7) did not affect the interaction between PDCD4 and Twist2, however mutations in the protein-binding domain appeared to reduce the interaction (Figure 4.7 lane 9).

4.2.2.2 Influence of the domains on IL-10 production.

These results led us to investigate the importance of the different domains for IL- 10 production. As shown in Figure 4.8A, in RAW264.7 cells transfected with constructs lacking a functional Twist-Box and protein-binding domain (Twist2 I92 Stop) or mutated protein-binding domain (Twist2-FLAG I92T/I93S/Y113C/I114S), there was an induction of IL-10 production (white bars). LPS was able to induce IL-10 in cells transfected with a plasmid encoding Twist2-FLAG WT (Black bar, Twist2-FLAG WT). Transfection with a plasmid encoding Twist2 with the mutated DNA binding domain (Black bar Twist2-FLAG T79A/S81A) decreased this response, whilst transfection with a plasmid encoding the construct lacking the Twist-Box and protein binding domain (Twist2 I92 Stop) or mutated protein-binding domain (Black bar Twist2-FLAG I92T/I93S/Y113C/I114S) both showed a potentiated response. None of the constructs affected the induction of IL-6 by LPS (Figure 4.8B, black bars). These results suggest that the Twist-box and protein- binding domain in the bHLH domain are involved in the interaction with PDCD4, and their absence allows Twist2 to increase IL-10 production, most likely due to an inability to be sequestered by PDCD4. The DNA-binding domain in Twist2 is therefore needed for induction of IL-10.

A



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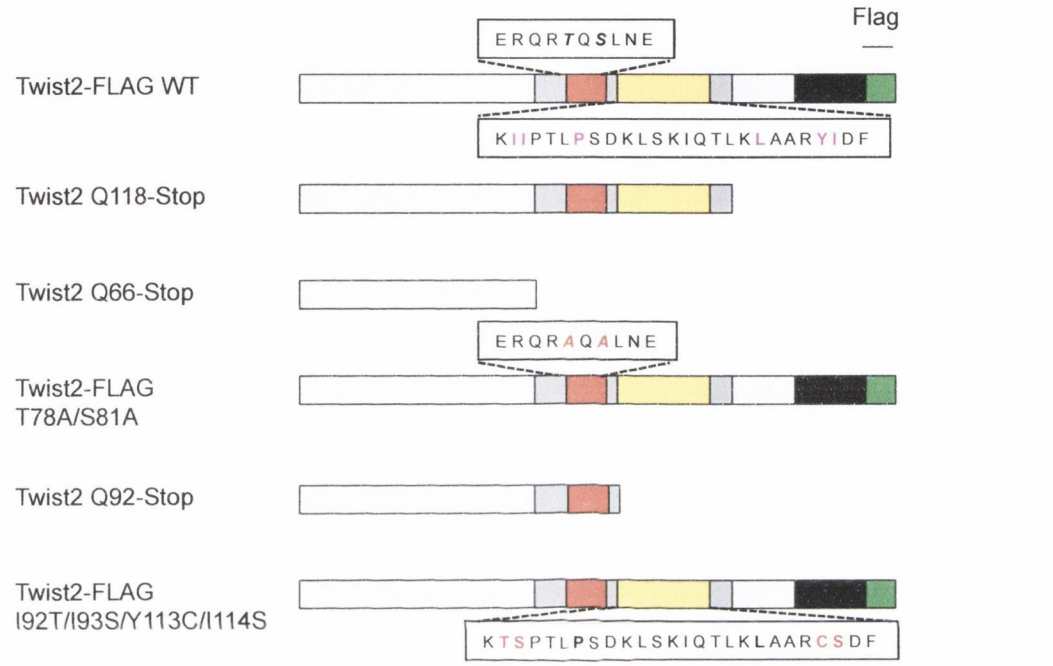


C

	aa	Amino acid sequence	aa	pI
Human_Twist2	91	KIIPTLPSDKLSKIQTLKLAARYIDF	116	9.82
Human_Twist1	133	KIIPTLPSDKLSKIQTLKLAARYIDF	158	9.82
Mouse_Twist2	91	KIIPTLPSDKLSKIQTLKLAARYIDF	116	9.82
Mouse_Twist1	137	KIIPTLPSDKLSKIQTLKLAARYIDF	162	9.82
Drosophila_Twist	387	QIIPTLPSDKLSKIQTLKLATRYIDF	412	9.53
Human_eIF4A1	153	HIIVGTPGRVFDMLNRRYLSPKYIKM	178	10.43

D

	aa	Amino acid sequence	aa	pI
Mouse_Twist2	68	ILANVR ERQRTQSINE AFAALR	70	11.52
Mouse_Twist1	114	VMANVR ERQRTQSINE AFAALR	70	11.52
Mouse_Hand1	96	GSGPKK ERRRTESINS AFAELR	98	10.83
Mouse_Hand2	101	GTANRK ERRRTQSINS AFAELR	94	11.83
Mouse_Paraxis	72	QAANAR ERDRTQSVNT AFTALR	102	11.52
Mouse_Scleraxis	80	HTANAR ERDRTNSVNT AFTALR	105	11.52

Figure 4.6 – Twist2 schematic structure

(A) Schematic representation of Twist1 and Twist2. Twist1 with 202 amino acids (aa) is bigger then Twist2 with 160 aa. Twist1 contains a glycine-rich domain (blue). (B) Schematic representation of Twist2-FLAG deletion mutants and Twist2-FLAG with mutations in the DNA binding domain (DBD) and protein binding domain (PBD) in the basic helix-loop-helix (bHLH). (C) Sequences of the amino acids in the protein binding domain can be found in the bHLH region of the proteins. The isoelectric point (pI) of each sequence was estimated using ExPASy Bioinformatics Resource Portal. Black background; identical residues and most likely amino acids necessary for protein binding domain. Grey background; identical residues. Bolt; similar residues (D) Sequences of the amino acids in the DNA binding domain can be found in the bHLH region of the proteins. The pI of each sequence was estimated using ExPASy Bioinformatics Resource Portal. Black background; identical residues and most likely amino acids involved in dimer choice and DNA-binding site. Grey background; marked as DNA-binding site.

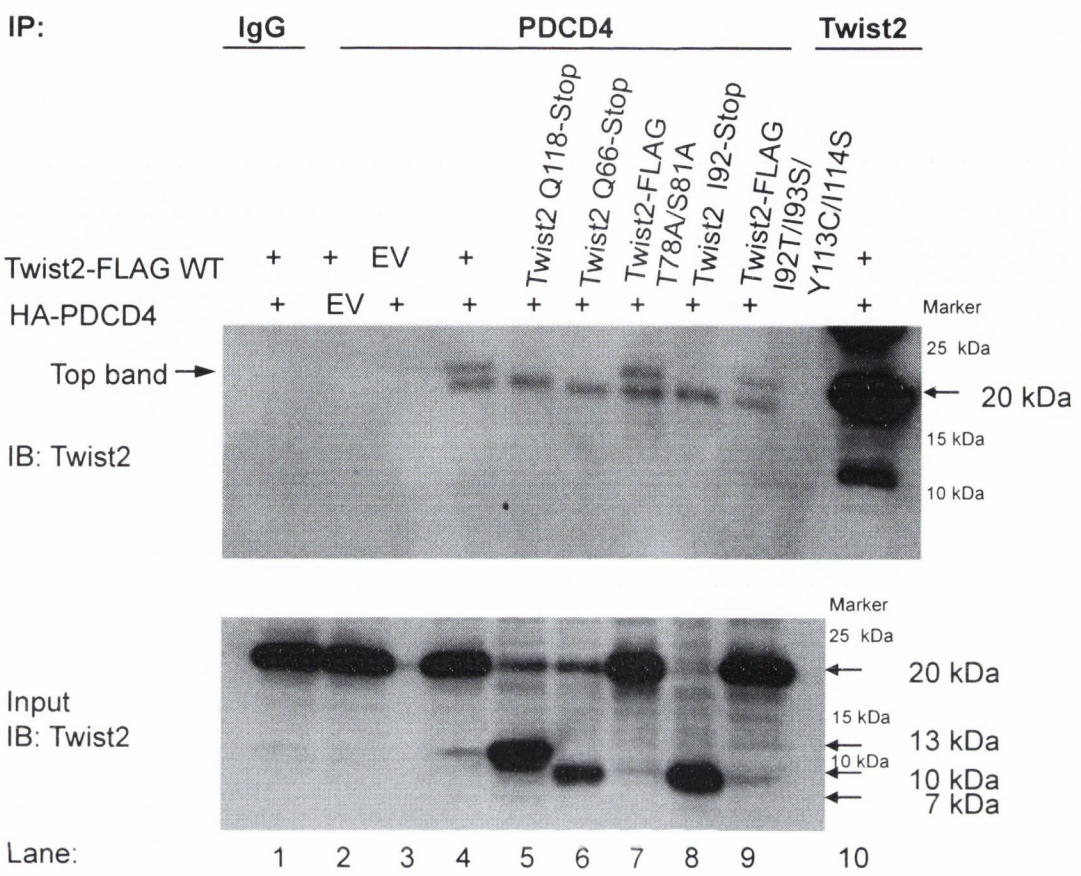
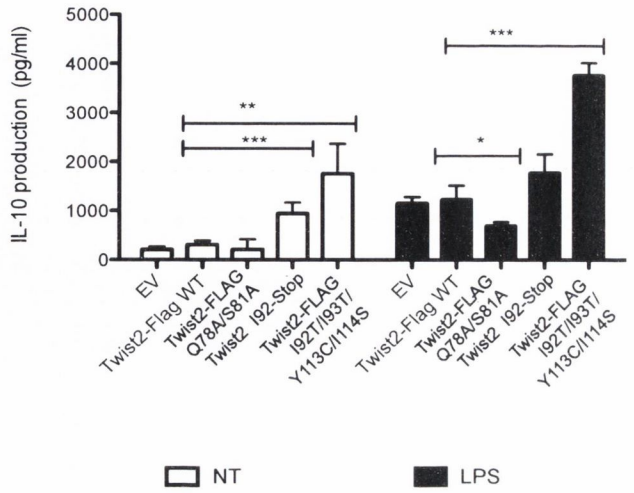


Figure 4.7 – Defining the domains responsible for the interaction between PDCD4 and Twist2 in HEK293-TLR4-MD2-CD14 cells.

HEK293-TLR4-MD2-CD14 cells were plated at $5x10^5$ cells/mL in 10cm dishes and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, 50mg/ml Noromycin, 100µg/ml Hydrogold and 100µg/ml Blastinomycin. The following day, cells were transfected with 2µg FLAG-Twist2, Twist2 N118, Twist2 N66, Twist2-FLAG M79/81, Twist2 N92, Twist2-FLAG M92/93/113/114, 2µg HA-PDCD4 or 2µg empty vector and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. The lysates were used for Immunoprecipitation (IP) with antibodies against IgG, PDCD4 or Twist2, and were prepared and analyzed via Western blotting followed by immuno-blotting (IB) for Twist2 antibody (lane 1-10). 1:10 dilution of the whole lysate was also prepared and analyzed via Western blotting followed by immuno-blotting (IB) for Twist2 antibody. The experiment shown is representative of at least three independent experiments.

A **RAW264.7**



B **RAW264.7**

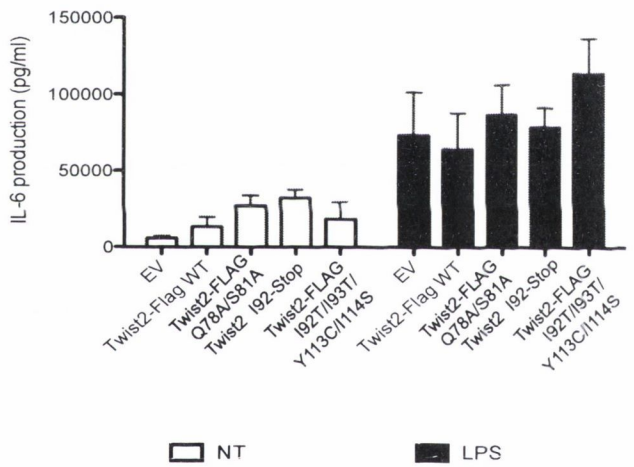


Figure 4.8 – Overexpressed Twist2 with mutations in the protein-binding domain increases IL-10 production in RAW264.7 cells.

RAW264.7 cells were plated at $5x10^5$ cells/mL in three 15 cm dishes per condition and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS. The following day, cells were transfected with 4µg FLAG-Twist2, Twist2 N118, Twist2 N66, Twist2-FLAG M79/81, Twist2 N92, Twist2-FLAG M92/93/113/114, 4µgHA-PDCD4 or empty vector (EV) and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were stimulated with 100ng/ml LPS (LPS) for 6 hours or left untreated (NT). (A) After stimulation, IL-10 in cell supernatants were measured by ELISA. (B) IL-6 in cell supernatants were measured by ELISA. Data shown are the mean ± S.D. from three separate experiments all carried out in triplicate *; P<0.05, **; P<0.01, ***; P<0.001.

4.2.3 Twist2 binds to a highly conserved site in the c-Maf promoter after LPS stimulation.

4.2.3.1 Bioinformatics identified a conserved domain in the c-Maf promoter

My previous data suggested that Twist2 might be released from PDCD4 in order to bind to the c-Maf promoter in response to LPS. The mouse gene of c-Maf can be found on chromosome 8 (Figure 4.9A). Using UCSC, a program for aligning sequences, the c-Maf promoter was found to contain a highly conserved region with several E-box formations of CAGCTG (Figure 4.9B labeled in red). The two E-box formations shown are conserved in a variety of animals and are predicted to be recognised by Twist2. These E-Box formations were important in designing Chromatin Immunoprecipitation (ChIP) primers and oligonucleotide pull down.

4.2.3.2 Oligonucelotide pull down showed binding of Twist2 to the c-Maf promoter

RAW264.7 cells were transfected with Twist2-FLAG and used for an oligonucleotide pull down technique to investigate Twist2 binding to the E-Box of c-Maf promoter. Figure 4.10A shows a schematic diagram of the region to which the oligonucleotide containing the specific Twist2 E-Box binds. Figure 4.10B shows an interaction between Twist2 and the E-Box of the c-Maf promoter after LPS stimulation (Figure 4.10B lane 2) but there was no such interaction in unstimulated cells (Figure 4.10 lane 1) or cells pre-treated with rapamycin (Figure 4.10 lanes 3 and 4). This result confirmed binding of Twist2 to the c-Maf promoter, which increased with LPS stimulation. This process was inhibited by rapamycin.

4.2.3.3 Twist2 binding to the c-Maf promoter

Sharabi et al. (2008) demonstrated a role for Twist2 in the induction of the transcription factor c-Maf, which subsequently regulates IL-10 (Sharabi et al., 2008). They demonstrated that Twist2 binds basally to the promoter of c-Maf. In order to establish a functional consequence of the PDCD4-Twist2 complex I hypothesized that PDCD4 may inhibit IL-10 expression by preventing Twist2 from binding to the c-Maf promoter. LPS-induced degradation of PDCD4 would thereby permit Twist2 to bind the c-Maf promoter, allowing c-Maf expression and subsequent IL-10 production. In order to investigate this hypothesis a ChIP in RAW264.7 cells transfected with Twist2-FLAG was carried out. Figure 4.11A shows a schematic diagram of the regions to which the ChIP PCR primers were designed. The antibody used for ChIP is the Twist antibody reported by Sharabi et al. (2008) as a Twist2 antibody. However when the protein sequences were aligned the reported Twist antibody would be predicted to recognise Twist1 as the alignment was 100% identical to Twist1 (Figure 4.11B). Therefore I performed the ChIP with this antibody and ran a western blot after the pull-down. RAW264.7 cells with overexpressed HA-tagged PDCD4 or FLAG-tagged Twist2 were used and the western blot was probed with the antibody specific for Twist1 and Twist2 (Figure 4.11C). Twist1 protein was detected after the pull down (Figure 4.11C, lanes 1 and 2) as well in the lysates (Figure 4.11 lanes 3 and 4). With a specific antibody for Twist2 there were bands detected for the overexpressed FLAG-tagged Twist2 in the lysates (Figure 4.11C lanes 7 and 8) but more importantly also in the pull down (Figure 4.11C lanes 5 and 6). These results show that this Twist antibody can be used for the investigation of Twist2, which can subsequently bind on the c-Maf promoter. Figure 4.12A shows the positive control, the β-actin promoter, demonstrating the binding of RNA Polymerase II (RPOL) to this promoter in the presence or absence of rapamycin followed by LPS stimulation (Figure 4.12A black bars). As an additional control it can be seen that Twist2 does not bind to this

promoter, and the negative control IgG antibody does not pull down any DNA (Figure 4.12A grey and white bars). Another negative control for ChIP was generated investigating the binding at the negative control site called the Twist2 non binding site of the c-Maf promoter. There is no binding evident at this region of the promoter for RNA Polymerase II, Twist2 or IgG when pulled down with the specific antibodies, as shown in Figure 4.12B. To confirm binding to the promoter site compare the pull down with Twist2 antibody to the negative control IgG antibody (Figure 4.12C grey bars compare to white bars). As the negative IgG antibody can be seen as background signal and therefore signals that are higher then background can be seen as binding of the protein to the promoter site.

As shown in Figure 4.12C the anti-Twist2 antibody immuno-precipitated Twist2 bound to the c-Maf promoter in unstimulated RAW264.7 cells (Figure 4.12C grey bars). Stimulation with LPS for 6 hours increased the amount of Twist2 binding on the c-Maf promoter. When cells were pre-treated with rapamycin and stimulated with LPS little binding was seen (Figure 4.12C grey bars). Additionally, there are increased levels of RNA Polymerase II bound at 6 hours after LPS stimulation in the presence or absence of rapamycin, implying increased transcriptional activity (figure 4.12C black bars).

Another technique to show binding utilised agarose gel electrophoresis as shown in Figure 4.12D. Amplified DNA, using PCR, with the same primers used for ChIP, were loaded on an agarose gel. In Figure 4.12D the agarose gel showed an increase in signal when cells were stimulated with LPS and immuno-precipitated with Twist2 antibody, indicating again that Twist2 binds to the c-Maf promoter. There is also a band visible in the unstimulated cells, suggesting basal binding of Twist2 to the c-Maf promoter. However, cells pre-treated with rapamycin show decreased or no binding. Additionally, there are increased levels of RNA Polymerase II bound after 6 hours

LPS stimulation in the presence or absence of rapamycin, implying again increased transcriptional activity. This result clearly indicates that Twist2 binds to the c-Maf promoter, an effect increased by LPS stimulation and inhibited by rapamycin.

4.2.3.4 PI3K inhibitors and proteasome inhibitors inhibit binding of Twist2 to c-Maf promoter.

The previous results led us to investigate Twist2 binding to the c-Maf promoter in the presence of the PI3K inhibitors wortmannin, LY294002 and MG132 inhibitors in RAW264.7 cells. RAW264.7 cells with overexpressed HA-tagged PDCD4 and FLAG-tagged Twist2 were pre-treaded with wortmannin (Figure 4.13), LY294002 (Figure 4.14) or MG132 (Figure 4.15) following 6 hours LPS stimulation. Figure 4.13A, 4.14A and 4.15A demonstrates the binding of RNA Polymerase II to the β-actin promoter in the presence or absence of the inhibitor followed by LPS stimulation (Figure 4.13A, 4.14A and 4.15A black bars). As an additional control Twist2 does not bind to this promoter indicated by the negative control IgG (4.13A, 4.14A and 4.15A grey and white bars). There is no binding evident at the negative control site called the non Twist2 binding site of the c-Maf promoter for RNA Polymerase II, Twist2 or IgG when pulled down with the specific antibodies, as shown in Figure 4.13B, 4.14B, 4.15B. Again to confirm binding to the promoter site compare the pull down with Twist2 antibody to the negative control IgG antibody (Figure 4.13C, 4.14C, 4.15C grey bars compare to white bars). As the negative IgG antibody can be seen as background signal and therefore signals that are higher then background can be seen as binding of the protein to the promoter site. As shown in Figure 4.13C, 4.14C, 4.15C there is binding of Twist2 to the c-Maf promoter in unstimulated RAW264.7 cells as well as an increased Twist2 binding after 6 hours LPS stimulation (grey bars). However when cells were pre-treatment with the inhibitors followed by 6 hour LPS stimulation there

was reduced binding seen of the Twist2 protein to the c-Maf promoter. Also the agarose gel electrophoresis (Figure 4.13D, 4.14D, 4.15D) shows the same trend with the different inhibitors when cells were pre-treated with rapamycin as shown in Figure 4.12D. The agarose electrophoresis gels also showed an increase in signal after LPS stimulation, indicating increased Twist2 binding to the c-Maf promoter. The lower bands visible in Figure 4.13D are most likely the primers used in the PCR as the agarose gel used was higher in percentage. These results together indicate that Twist2 binds to the c-Maf promoter, an effect increased by LPS stimulation and inhibited by PI3K inhibitors wortmannin and LY294002 and protesomal inhibitor MG132.

4.2.3.5 The mutated DNA-binding domain and protein-binding domain of Twist2 show decreased Twist2 binding to the c-Maf promoter after LPS stimulation.

As previously demonstrated different constructs were generated with mutations in the protein-binding site (Twist-FLAG I92T/I93S/Y113C/I114S mutant) and DNA-binding site (Twist-FLAG T79A/S81A mutant) of Twist2. I also tested Twist2-mutated constructs and their ability to bind to the c-Maf promoter. Firstly, Figure 4.16 shows the positive control of RNA Polymerase II binding to the β-actin promoter in the absence (Figure 4.16A black bars) or presence of LPS (Figure 4.16B black bars). As a negative control, primers for a non-Twist2 binding site were used in both non-stimulated (Figure 4.17A) and 6 hours LPS-stimulated cells (Figure 4.17B) to show that no binding to non-Twist2 binding site located on the c-Maf promoter of the the was evident for RNA Polymerase II, Twist2 or IgG.

Figure 4.18 shows the binding of overexpressed wild type Twist2 to the c-Maf promoter in unstimulated cells (Figure 4.18A grey bars). This binding is increased

after LPS stimulation (Figure 4.18B grey bars). To confirm that this binding is specific, an IgG antibody was used as a negative control (Figure 4.18A and B white bars). The IgG antibody can be seen as a background signal and therefore signals that are higher than this background can be considered as binding of the Twist2 protein to the c-Maf promoter site.

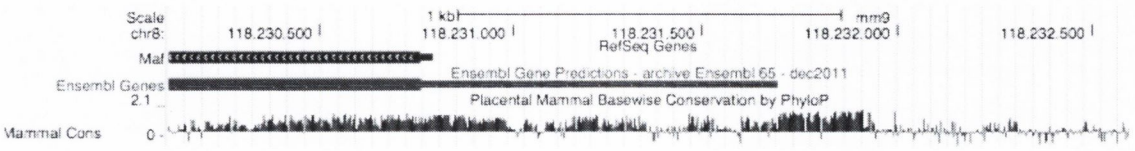
As shown in Figure 4.18 the cells transfected with plasmid encoding the Twist2 protein with the mutated DNA-binding domain (Twist-FLAG T79A/S81A) showed a slight decrease in binding to the c-Maf promoter compared to the wild type Twist2 protein in unstimulated cells (Figure 4.18A, grey bars). There is also a decrease in binding of the mutant Twist-FLAG T79A/S81A protein to the c-Maf promoter in LPS-stimulated cells (Figure 4.18A, grey bars). This result was as expected because the mutation in the DNA-binding site would block the binding of Twist2 to the c-Maf promoter when Twist2 is released from the PDCD4/Twist2 complex.

The other overexpressed constructs used lacked a functional Twist-Box and protein-binding domain (Twist2 Q92-Stop), or contained a mutation in the protein-binding domain (Twist-FLAG I92T/I93S/Y113C/I114S). These constructs would in theory show increased binding to the c-Maf promoter because they are not able to be in the PDCD4/Twist2 complex. However, the constructs lacking a functional Twist-Box and protein-binding domain (Twist2 Q92-Stop) or containing a mutated protein-binding domain (Twist-FLAG I92T/I93S/Y113C/I114S) showed the same basal binding to the c-Maf promoter in unstimulated cells as can be seen using the wild type Twist2 (Figure 4.18A grey bars) unlike wild type Twist2. However, no increase was seen in the ability of the protein-binding domain mutant constructs to bind the c-Maf promoter (Figure 4.18B grey bars). Therefore, these results suggest that the different mutations do not influence the binding of Twist2 to the c-Maf promoter in unstimulated cells, and there is no increase in binding in response to LPS.

4.2.3.6 Twist2 does not bind to the IL-10 promoter

After showing that Twist2 can bind to the c-Maf promoter by recognising the E-box formations in the c-Maf promoter regions I also investigated if Twist2 was able to bind directly to IL-10 promoter regions with the ChIP technique. The IL-10 promoter can be found on chromosome 8 in mouse (Figure 4.19A). The promoter contains two E-box formations that Twist2 potentially could recognise (Figure 4.19 labelled in red). Therefore two different primer sets were designed containing the E-box regions in the IL-10 promoter as shown schematically in Figure 4.20A. RAW264.7 cells overexpressing HA-tagged PDCD4 and FLAG-tagged Twist2 were pre-treated in the presence or absence of rapamycin followed by LPS stimulation, or left untreated. There is no binding on the IL-10 promoter site when Twist2 antibody pull down is compared to the negative control IgG antibody for both primer sets shown in Figure 4.20B and 4.20C, grey bars compare to white bars. This is because the negative IgG antibody in the same graph can be seen as background signal (Figure 4.20B and 4.20C, white bars). Only the signals higher then the background can be determined as binding. The negative control with a non-Twist2 binding site on the IL-10 promoter was shown in Figure 4.20D.

A



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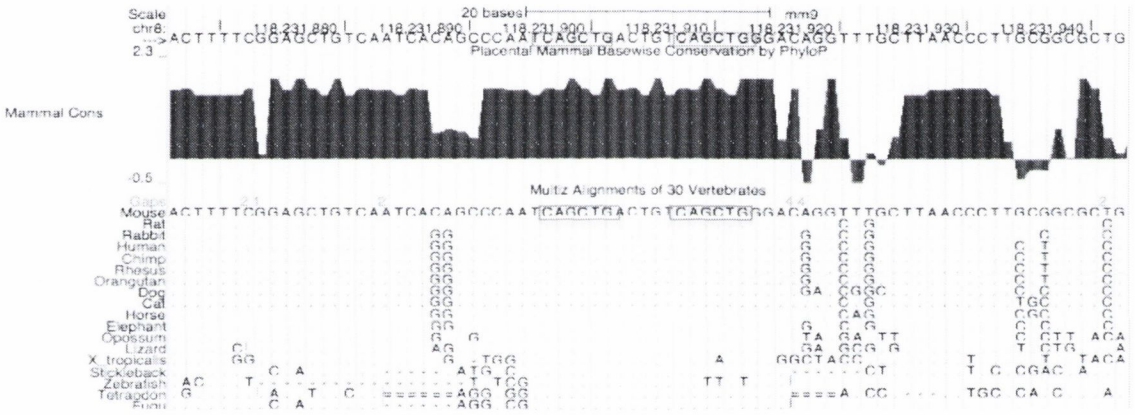


Figure 4.9 – Schematic illustration of the conserved E-box formations in the promoter of c-Maf.

(A) The predicted location of the c-Maf gene (top blue bar) is shown, generated with the genome USCS program. C-Maf can be found on chromosome 8 in the *mus musculus* (mouse). Mammal cons show the sequence conserved in mammal. The blue spikes show conserved nucleotides, higher spikes are relative to higher conserved sequences between different mammals. (B) Zoom in from (A) of the promoter region of c-Maf with a highly conserved region containing two E-box formations CAGCTG labeled in red. The E-boxes are conserved in range of animals and are predicted to be recognised by Twist2.

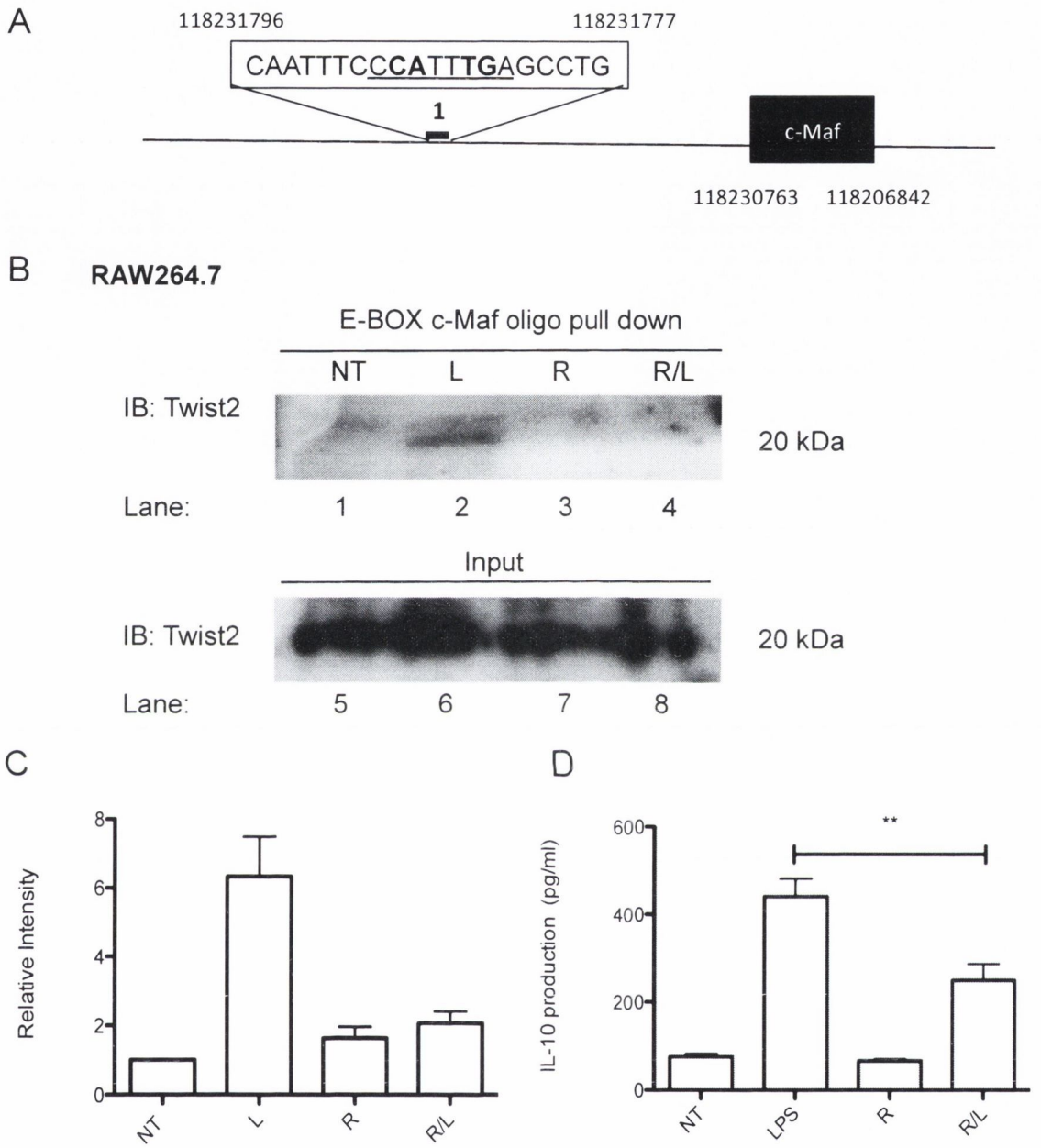


Figure 4.10 – In response to LPS, overexpressed Twist2 directly binds to an E-Box located on the promoter of c-Maf in Raw264.7 cells.

(A) Schematic diagram of the c-Maf promoter region and the location of the c-Maf gene with (1) indicating sequence used for oligonucleotide pull down. The specific sequence of the E-box used is underlined; nucleotides recognising Twist2 are bold. RAW264.7 cells were plated at $5x10^5$ cells/mL in 10cm dishes, and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS. The following day,

cells were transfected with 5µg FLAG-Twist2 plasmid and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were left unstimulated (NT) or cells were stimulated with 500nM rapamycin (R) for 6 hours. Other cells were pre-treated with DMSO and stimulated for 6 hours with 100ng/ml LPS (L). Finally, cells were pre-treated with 500nM rapamycin for 30 minutes followed by 100ng/ml LPS (R/L) treatment for 6 hours. (B) The whole lysates where used for oligonucleotide pull down experiments. The oligonucleotides used were specific for the E-box in c-Maf promoter region, located as shown in (A). Samples were prepared and analyzed via Western blotting followed by immuno-blotting (IB) with Twist2 antibody (lanes 1-8). A 1:10 dilution of the lysate was also analyzed via Western blotting followed by immuno-blotting with Twist 2 antibody (lane 5-8). (C) The bands from the oligonucleotide pull down were quantified with the program image lab from Biorad and samples are normalized against the lysates. (D) After stimulation, mouse IL-10 cytokine secretion into cell supernatants was measured by ELISA, relative to a standard curve. The data shown represents the mean of three independent experiments, each carried out in triplicate ± S.E.M. **; $P<0.01$

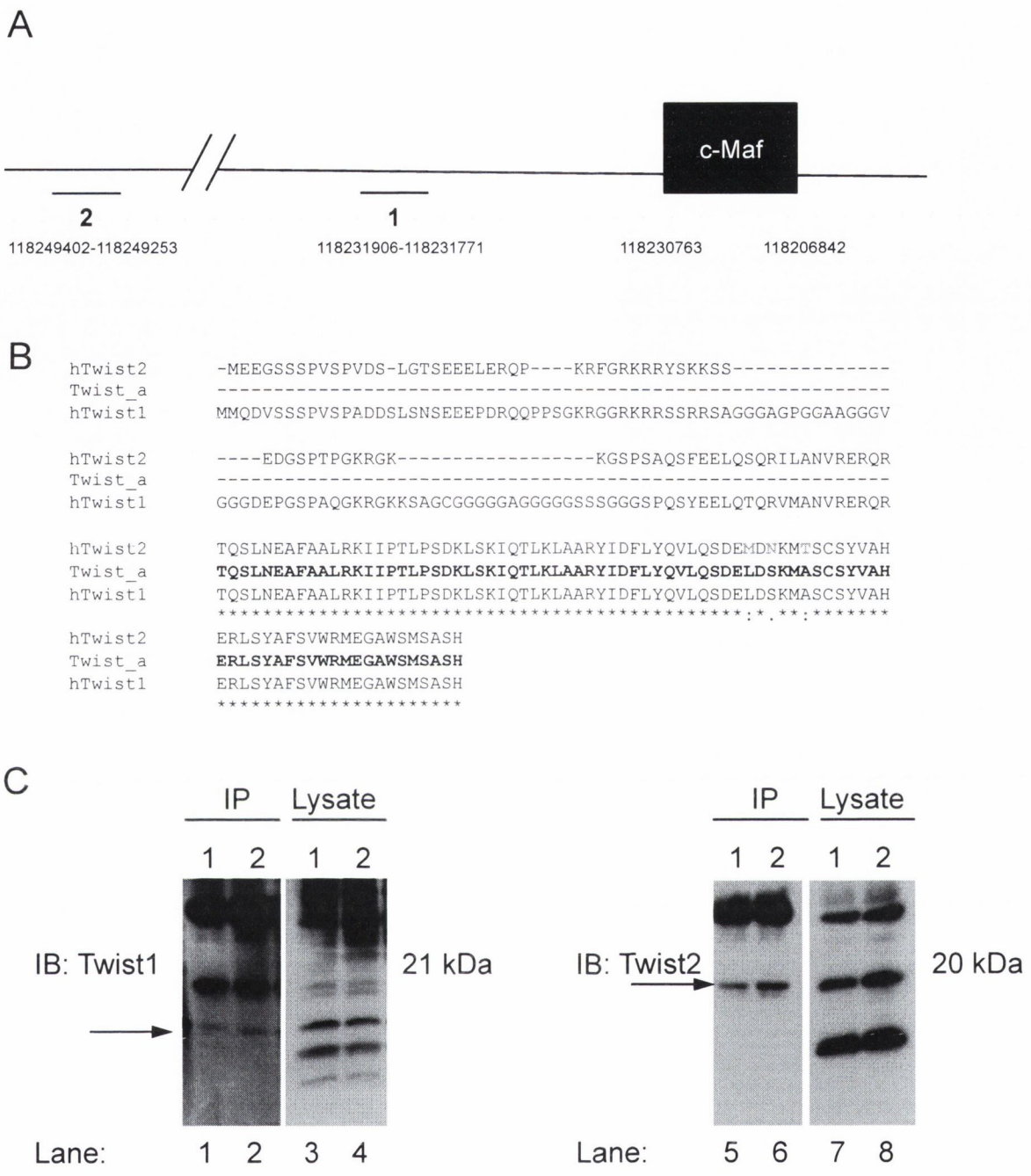


Figure 4.11 – Schematic diagram of primers used for ChIP and alignment of Twist2 and Twist1 with the ChIP Twist antibody

(A) Schematic diagram of primers recognizing sequences of the c-Maf gene (1) Primer set recognizing a highly conserved region of the c-Maf promoter containing E-box sites and (2) Primer set recognizing a non promoter region of genomic DNA approximately 20 kb upstream of the c-Maf promoter. The primer sets are used for ChIP assay. (B) Alignment of Twist2 and Twist1 with the Chip Twist antibody with the program CLUSTAL O(1.2.0). The three amino acids in red are the difference between

Twist1 and Twist2. (C) RAW264.7 cells were plated at $5x10^5$ cells/mL in 3x 15 cm dishes per condition and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS. The following day, cells were transfected with 5µg FLAG-Twist2 plasmid and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were left untreated. The lysates were used for ChIP using an anti-Twist antibody. After ChIP pull down the samples were prepared and analyzed via Western blotting followed by immuno-blotting (IB) with Twist1 specific antibody (lane 1-4) or Twist2 antibody (5-8). 1:10 dilution of the whole lysate was also analyzed via Western blotting followed by immuno-blotting (IB) (lane 3-4 and 7-8). A similar result was obtained in another independent experiment.

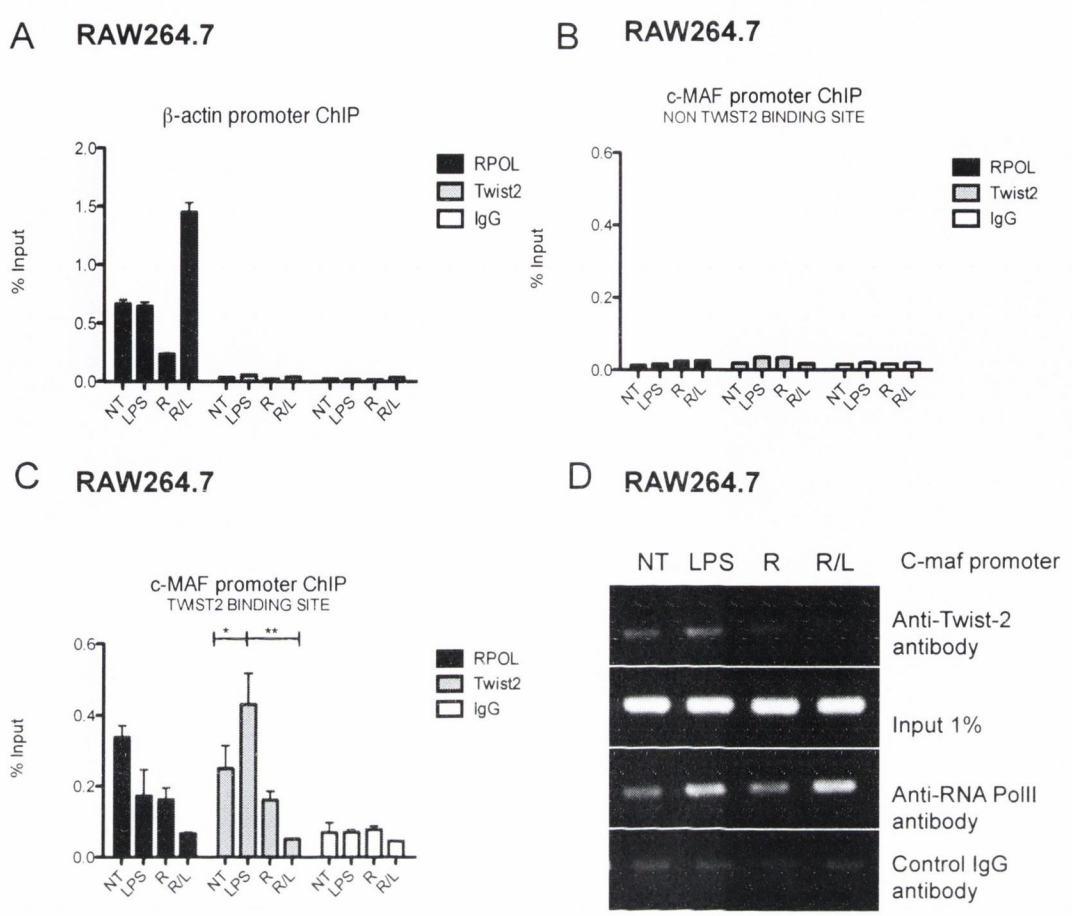


Figure 4.12 – Overexpressed Twist2 directly binds to the promoter of c-Maf in Raw264.7 cells and is sensitive to rapamycin treatment.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 3x 15 cm dishes (per sample) and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS. The following day cells were transfected with 5μg FLAG-Twist2 plasmid and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were pretreated with DMSO for 30 minutes followed by 100ng/ml LPS (L) for 6 hours. Cells were also pre-treated with rapamycin (500nM) for 30 minutes followed by 100ng/ml LPS (R/L) treatment for 6 hours, or cells were left untreated (NT) or with rapamycin alone (R). The lysates were used for ChIP assays using an anti-Twist2 antibody (grey bars) probing for c-Maf promoter DNA pull down, or an anti-RNA poll (positive control: black bars) or an IgG antibody (negative control: white bars). All data were normalized to input. (A) DNA pull down purified and measured using Real time PCR with specific primers designed for the β-actin promoter. (B) Primers designed for a non-Twist2 binding site (C) Primers for the Twist2 binding site of interest. (D) DNA pull down purified and measured using PCR and a DNA agarose gel with specific primers designed in (A). Binding events were measured as percent of input. Data are means of 3 measurements, with error bars representing S.D. Statistical analysis was carries out using Student's t test. *; $P<0.05$, **; P,0.01. Values are representative for three separate experiments.

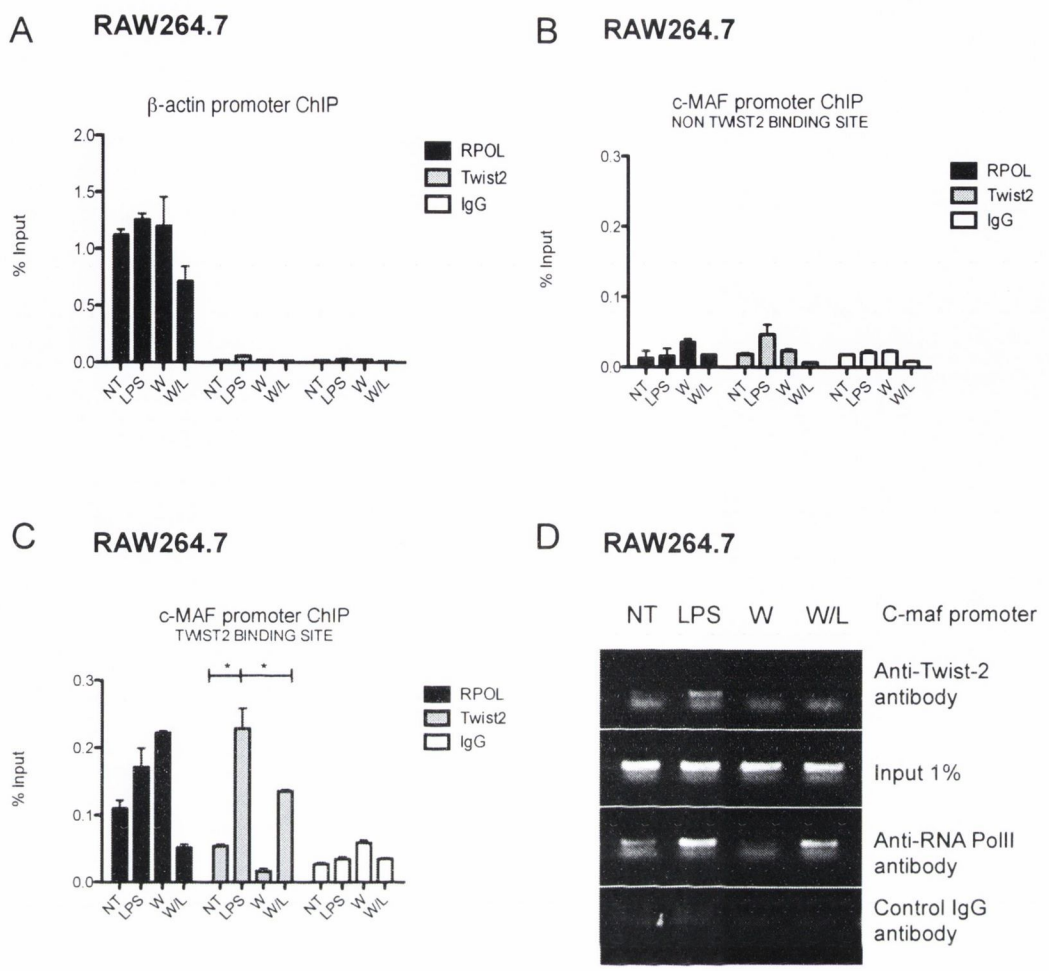


Figure 4.13 – Overexpressed Twist2 directly binds to the promoter of c-Maf in Raw264.7 cells and is sensitive to wortmannin treatment.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 3x 15 cm dishes (per sample) and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS. The following day, cells were transfected with 5μg FLAG-Twist2 plasmid and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were pretreated with DMSO for 30 minutes followed by 100ng/ml LPS (L) for 6 hours. Cells were also pre-treated with wortmannin (5μM) for 30 minutes followed by 100ng/ml LPS (W/L) treatment for 6 hours, or cells were left untreated (NT) or with wortmannin alone (W). The lysates were used for ChIP assays using an anti-Twist2 antibody (grey bars) probing for c-Maf promoter DNA pull down, or an anti-RNA pol (positive control: black bars) or an IgG antibody (negative control: white bars). All data were normalized to input. (A) DNA pull down purified and measured using Real time PCR with specific primers designed for the β-actin promoter. (B) Primers designed for a non-Twist2 binding site (C) Primers for the Twist2 binding site of interest. (D) DNA pull down purified and measured using PCR and a DNA agarose gel with specific primers designed in (A). Binding events were measured as percent of input. Data are means of 3 measurements, with error bars representing S.D. Statistical analysis was carries out using Student's t test. *; $P<0.05$. Values are representative for three separate experiments.

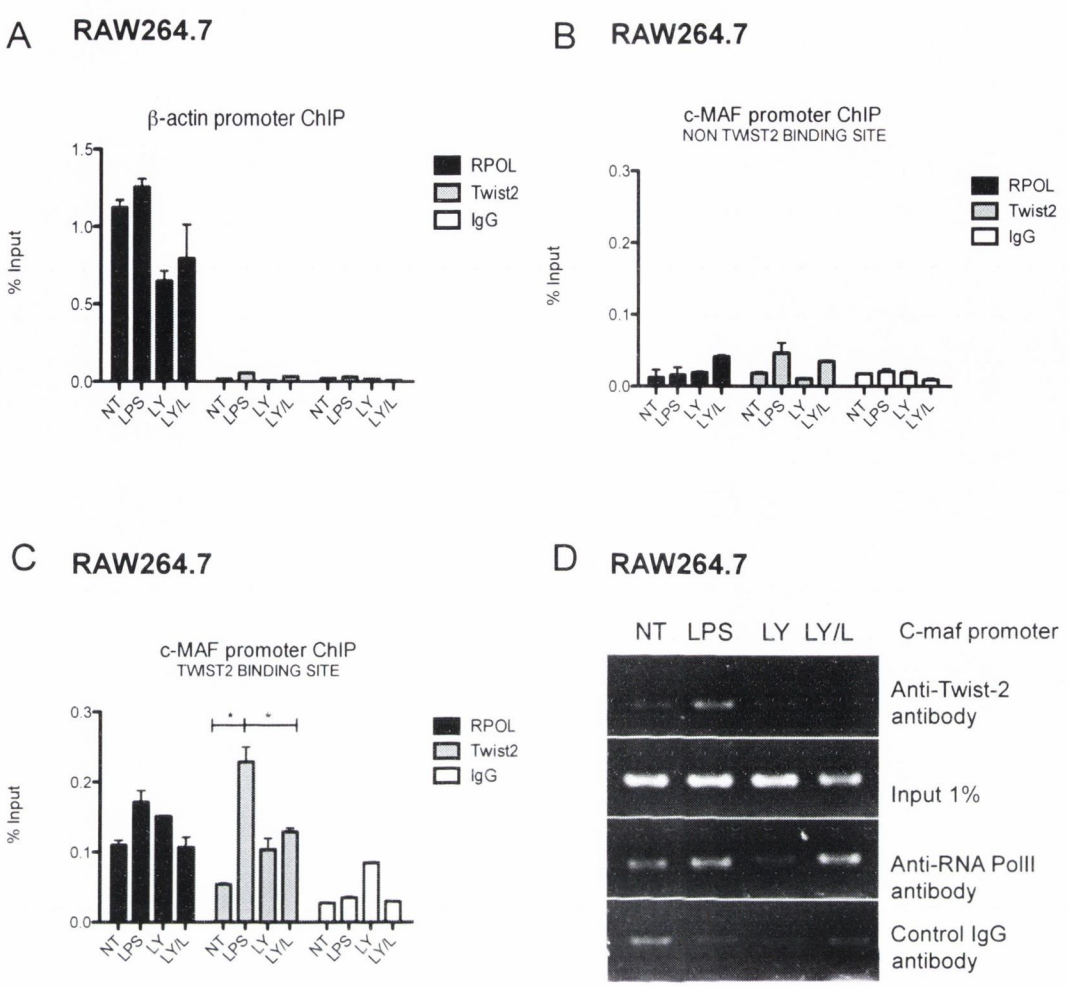


Figure 4.14 – Overexpressed Twist2 directly binds to the promoter of c-Maf in Raw264.7 cells and is sensitive to LY294002 treatment.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 3x 15 cm dishes (per sample) and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS. The following day, cells were transfected with 5µg FLAG-Twist2 plasmid and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were pretreated with DMSO for 30 minutes followed by 100ng/ml LPS (L) for 6 hours. Cells were also pre-treated with LY294002 (20µM) for 30 minutes followed by 100ng/ml LPS (LY/L) treatment for 6 hours, or cells were left untreated (NT) or with LY294002 alone (LY). The lysates were used for ChIP assays using an anti-Twist2 antibody (grey bars) probing for c-Maf promoter DNA pull down, or an anti-RNA pol (positive control: black bars) or an IgG antibody (negative control: white bars). All data were normalized to input. (A) DNA pull down purified and measured using Real time PCR with specific primers designed for the β-actin promoter. (B) Primers designed for a non-Twist2 binding site (C) Primers for the Twist2 binding site of interest. (D) DNA pull down purified and measured using PCR and a DNA agarose gel with specific primers designed in (A). Binding events were measured as percent of input. Data are means of 3 measurements, with error bars representing S.D. Statistical analysis was carries out using Student's t test. *; $P<0.05$. Values are representative for three separate experiments.

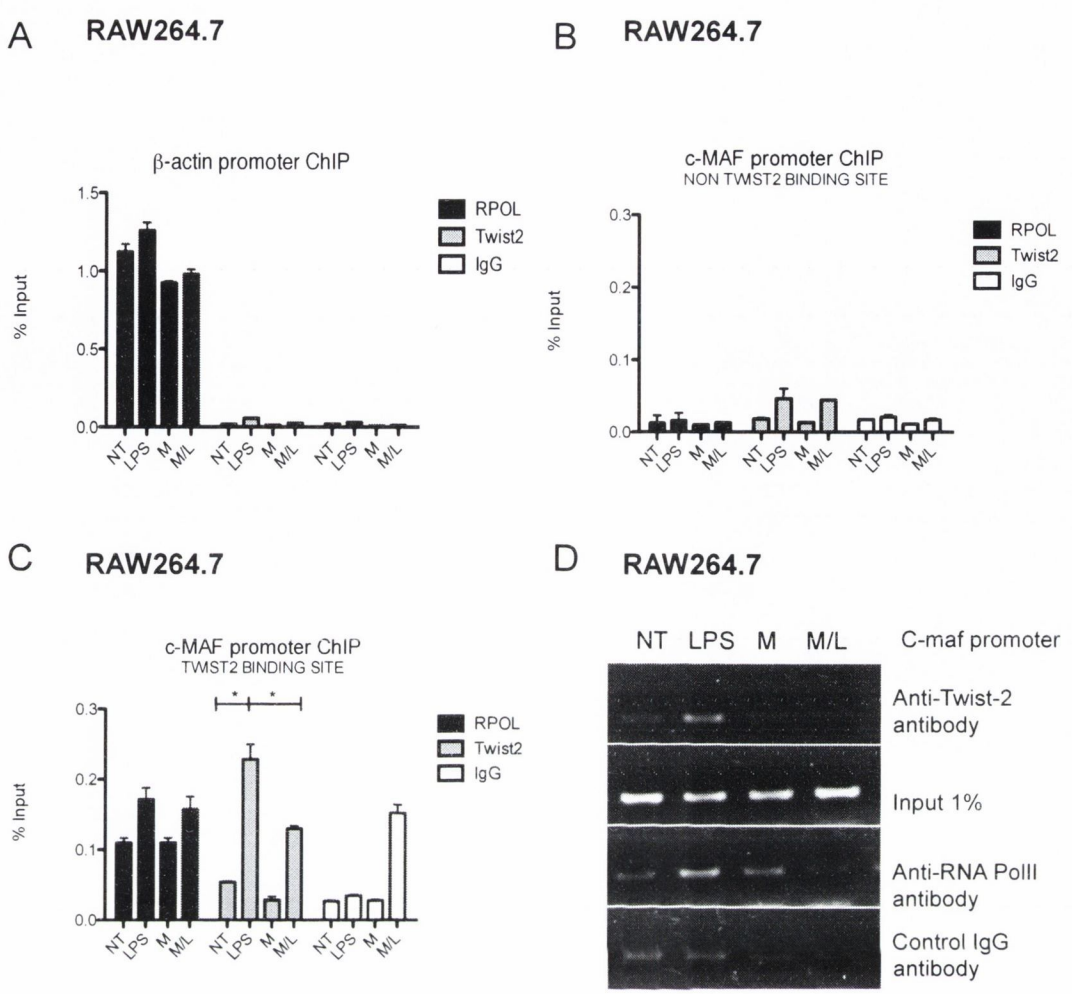


Figure 4.15 – Overexpressed Twist2 directly binds to the promoter of c-Maf in Raw264.7 cells and is sensitive to proteasome inhibitor MG132

RAW264.7 cells were plated at $5x10^5$ cells/mL in 3x 15 cm dishes (per sample) and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS. The following day, cells were transfected with 5μg FLAG-Twist2 plasmid and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were pretreated with DMSO for 30 minutes followed 100ng/ml LPS (L) for 6 hours. Cells were also pre-treated with MG132 (5μM) for 30 minutes followed by 100ng/ml LPS (M/L) treatment for 6 hours, or cells were left untreated (NT) or with MG132 alone (M). The lysates were used for ChIP assays using an anti-Twist2 antibody (grey bars) probing for c-Maf promoter DNA pull down, or an anti-RNA poll (positive control: black bars) or an IgG antibody (negative control: white bars). All data were normalized to input. (A) DNA pull down purified and measured using Real time PCR with specific primers designed for the β-actin promoter. (B) Primers designed for a non-Twist2 binding site (C) Primers for the Twist2 binding site of interest. (D) DNA pull down purified and measured using PCR and a DNA agarose gel with specific primers designed in (A). Binding events were measured as percent of input. Data are means of 3 measurements, with error bars representing S.D. Statistical analysis was carries out using Student's t test. *; $P<0.05$. Values are representative for three separate experiments.

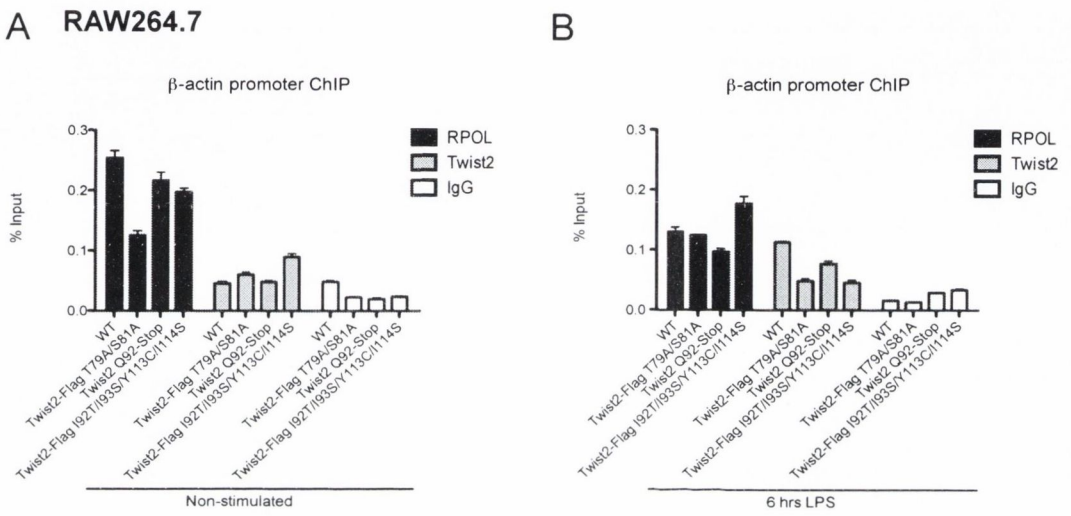


Figure 4.16 – Positive ChIP control RNA Polymerase II binds to β-actin promoter in RAW246.7 cells overexpressing Twist2 and PDCD4.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 3x 15 cm dishes per condition and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS. The following day, cells were transfected with 4µg FLAG-Twist2, Twist2 N118, Twist2 N66, Twist2-FLAG M79/81, Twist2 N92, Twist2-FLAG M92/93/113/114 and 4µg HA-PDCD4 and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation cells were stimulated with 100ng/ml LPS (L) for 6 hours (B) or left untreated (A). The lysates were used for ChIP assays using an anti-Twist2 antibody probing for c-Maf promoter DNA pull down, or an anti-RNA poll (positive control) or an IgG antibody (negative control). All data were normalized to input. DNA pull-down was purified and measured using Real time PCR with specific primers designed for the β-actin promoter. Binding events were measured as percent of input. Data are means of three measurements, with error bars representing S.D. Values are representative for three separate experiments.

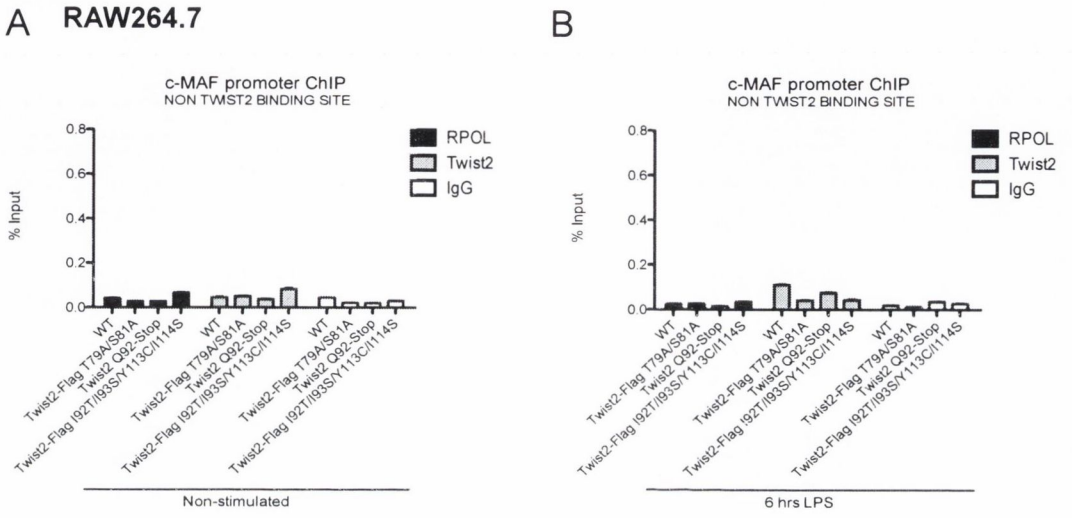


Figure 4.17 – Twist2 does not bind to the non Twist2 binding site in RAW264.7 cells overexpressing Twist2 and PDCD4.

RAW264.7 cells were plated at $5x10^5$ cells/mL in 3x 15 cm dishes per condition and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS. The following day, cells were transfected with 4µg FLAG-Twist2, Twist2 N118, Twist2 N66, Twist2-FLAG M79/81, Twist2 N92, Twist2-FLAG M92/93/113/114 and 4µg HA-PDCD4 and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation cells were stimulated with 100ng/ml LPS (L) for 6 hours (right panel) or left untreated (left panel). The lysates were used for ChIP assay using an anti-Twist2 antibody probing for c-Maf promoter DNA pull-down, or an anti-RNA poly (positive control) or an IgG antibody (negative control). All data were normalized to input. A DNA pull down was purified and measured using Real time PCR with specific primers designed for the non Twist2 binding site. Binding events were measured as percent of input. Data are means of three measurements, with error bars representing S.D. Values are representative for three separate experiments.

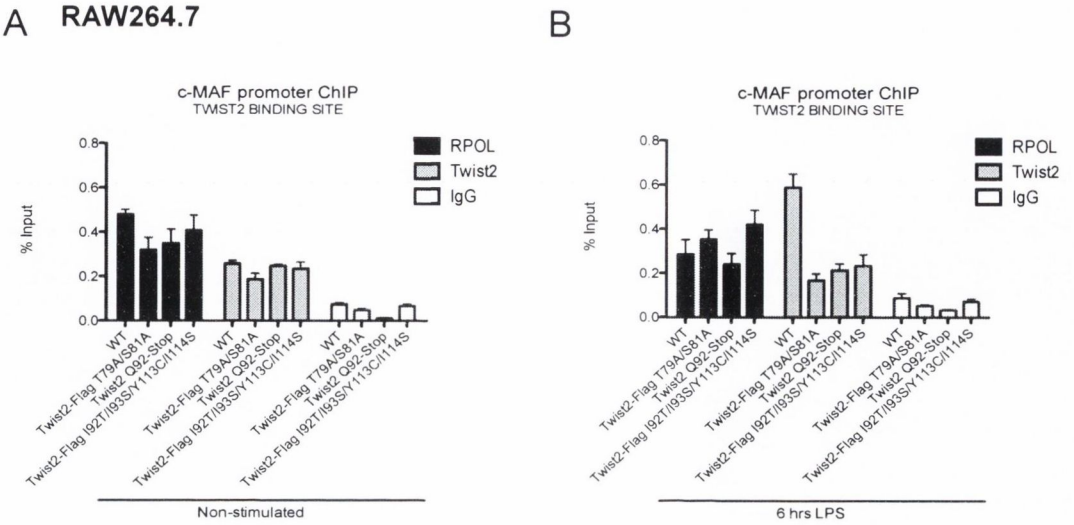
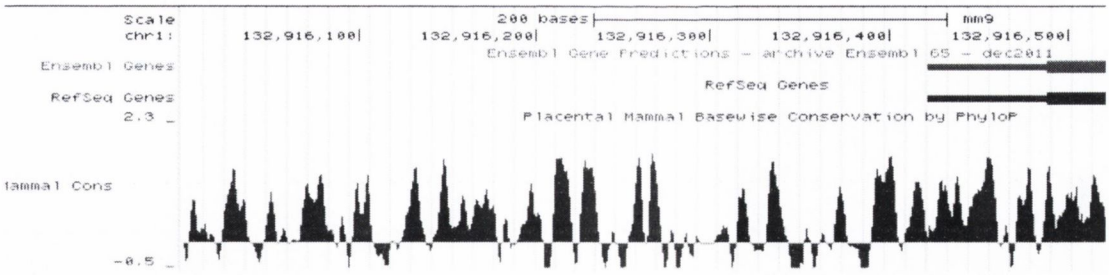


Figure 4.18 – Increased binding of overexpressed wild type Twist2 to the promoter of c-Maf however protein- and DNA-binding site mutations in Twist2 reduces binding in LPS-treated Raw264.7 cells.

RAW264.7 cells were plated at $5x10^5$ cells/mL in three 15 cm dishes per condition and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS. The following day, cells were transfected with 4µg FLAG-Twist2, Twist2 N118,Twist2 N66, Twist2-FLAG M79/81, Twist2 N92, Twist2-FLAG M92/93/113/114 and 4µg HA-PDCD4 and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were stimulated with 100ng/ml LPS (L) for 6 hours (right panel) or left untreated (left panel). The lysates were used for ChIP assays using an anti-Twist2 antibody probing for c-Maf promoter DNA pull down, or an anti-RNA poly (positive control) or an IgG antibody (negative control). All data were normalized to input. A DNA pull down was purified and measured using Real time PCR with specific primers for the Twist2 binding site of interest. Binding events were measured as percent of input. Data are means of three measurements, with error bars representing S.D. Values are representative for three separate experiments.

A



B

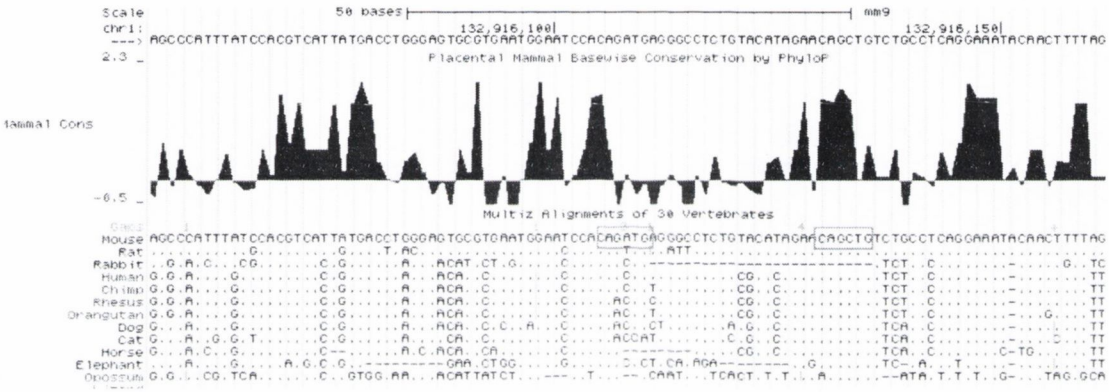


Figure 4.19 – Schematic illustration of the E-Box formations of the IL-10 promoter.

A) The predicted location of the IL-10 promoter is shown (top blue and red bar) generated with the genome USCS program. IL-10 can be found on chromosome 8 in the *mus musculus* (mouse). Mammal cons show the sequence conserved in mammal. The blue spikes show conserved nucleotides, higher spikes are relative to higher conserved sequences between different mammals. (B) Zoom in from (A) of the promoter region of IL-10 with a conserved region containing an E-box formation CAGCTG labeled in red. The E-box is conserved in range of animals and is predicted to be recognised by Twist2.

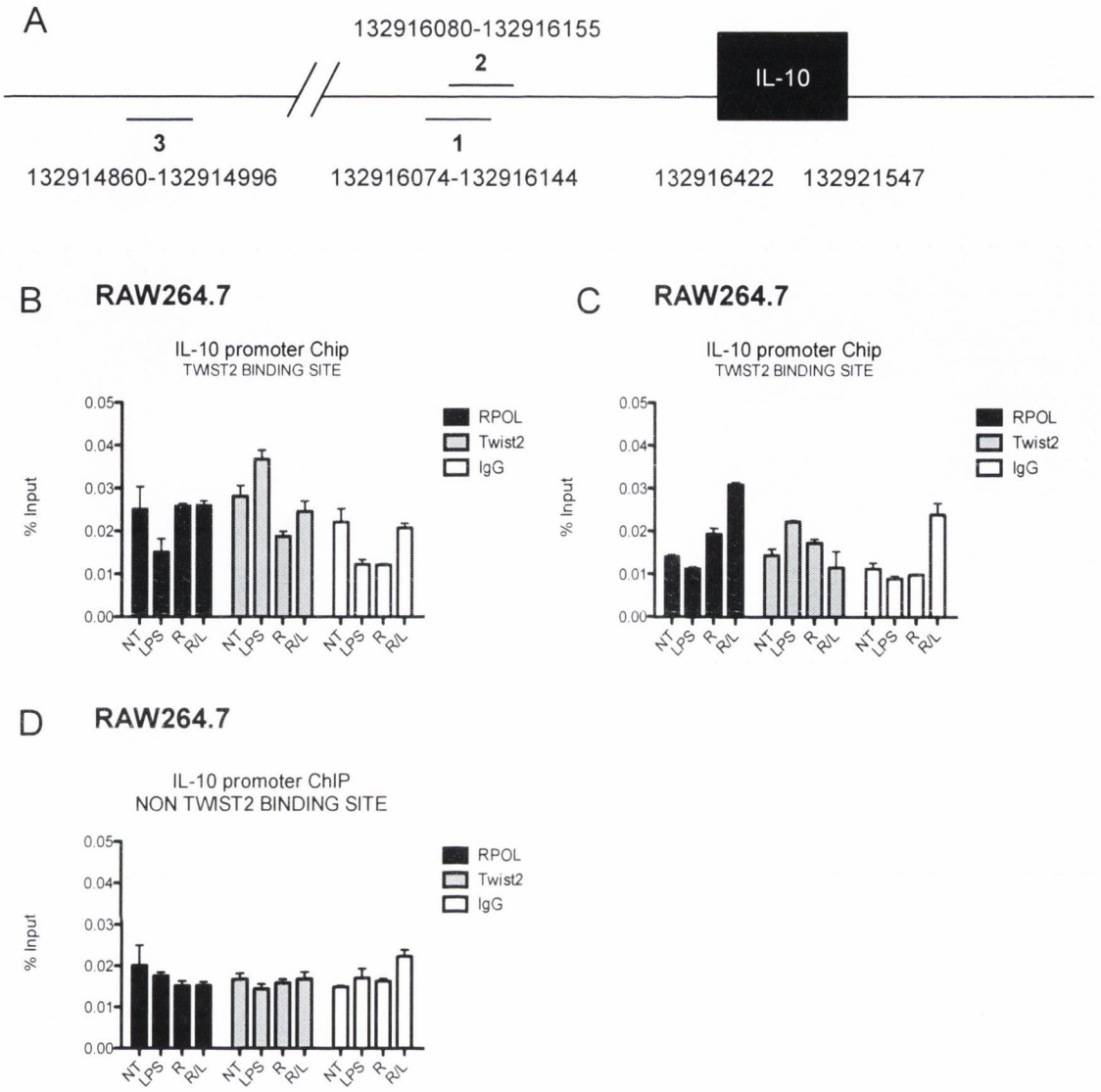


Figure 4.20 Overexpressed Twist2 does not bind to the IL-10 promoter in RAW264.7 cells.

(A) Schematic diagram of the IL-10 promoter region and the location of the IL-10 gene with two different primer sets used. The primers recognize the following regions (1 and 2) a highly conserved region of the IL-10 promoter containing E-box sites. (3) non-promoter region of genomic DNA approximately 20 kb upstream of the IL-10 promoter. The primer sets are used for ChIP assays. (B-D) RAW264.7 cells were plated at $5x10^5$ cells/mL in 3x 15 cm dishes per condition and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, The following day, cells were transfected with 4µg HA-PDCD4 and 4µg FLAG-Twist2 plasmid and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. After incubation, cells were stimulated with 100ng/ml LPS (L) for 6 hours. Cells were also pre-treated with rapamycin (500nM) for 30 minutes followed by 100ng/ml LPS (R/L)

treatment for 6 hours, or cells were left untreated (NT), or with rapamycin alone (R). The lysates were used for ChIP assay using an anti-Twist2 antibody (grey bars) probing for IL-10 promoter DNA pull down, or an anti-RNA poll (positive control: black bars) or an IgG antibody (negative control: white bars). All data were normalized to input. Primers for β-actin promoter are shown in figure 4.12. (B) DNA pull down purified and measured using Real Time PCR with primers set 1 designed for the Twist2 binding site on the IL-10 promoter. (C) Primer set 2 designed for a Twist2 binding site on the IL-10 promoter. (D) Primers designed for a non-Twist2 binding site on the IL-10 promoter. Data shown are the mean ± S.D. from three separate experiments all carried out in triplicate.

4.2.4 The effect of the PDCD4/Twist2 complex on c-Maf

4.2.4.1 The effect of inhibitors on c-Maf expression.

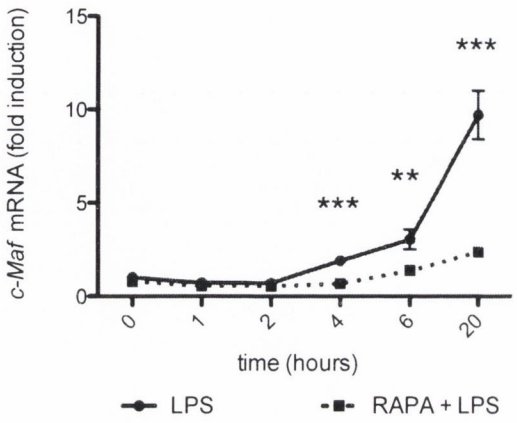
A study with c-Maf-deficient cells showed that c-Maf is essential for IL-10 transcription (Cao et al., 2005). Previous data suggested that IL-10 expression was inhibited by rapamycin, I therefore tested if rapamycin also inhibited the transcription factor c-Maf. As shown in Figure 4.21A, induction of c-Maf expression by LPS was inhibited when cells were pre-treated with rapamycin (dotted line). After this result I was interested if the PI3K inhibitors wortmannin, LY294002 and the protesomal inhibitor MG132 would generate the same effect if these inhibitors also blocked IL-10 expression (Figure 3.12). RAW264.7 cells were pre-treated with the different inhibitors followed by 6 hours LPS stimulation. Figure 4.21B shows increased c-Maf expression after 6 hours LPS stimulation. However, when RAW264.7 cells were pre-treated with LY294002, Wortmannin, MG132 or rapamycin before 6 hours of LPS stimulation, there was a significant reduction in c-Maf expression in all cases. These data indicate that inhibition of IL-10 by rapamycin, PI3K and protesomal inhibitors also involves c-Maf expression.

4.2.4.2 PDCD4 deficiency increases LPS-induced c-Maf expression.

I next determined if PDCD4 targeting is important for c-Maf induction by LPS as it is known to be a transcription factor for IL-10 and I have shown that Twist-2 can bind to the c-Maf promoter. An induction of c-Maf mRNA by LPS was shown in Figure 4.22, and this was enhanced in PDCD4 deficient BMDM (Figure 4.22, 6 hours LPS treated samples compare grey bar with white bar). When the cells were pre-treated with rapamycin before LPS stimulation, the wild-type BMDM show a significant decrease in IL-10 after 6 hours (Figure 4.22 6 hours LPS treated samples, compare black bar

with white bar). But unlike wild-type BMDM this induction was not inhibited by rapamycin (Figure 4.22B, 6 hours LPS treated samples compare grey hatched bar with grey bar). This points to PDCD4 targeting as a important transcription factor for c-Maf induction by LPS.

RAW264.7



RAW264.7

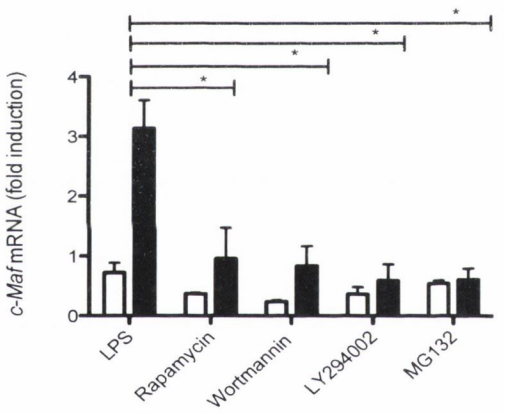


Figure 4.21 – The effect of inhibitors on c-Maf mRNA expression in RAW264.7 cells

Cells were plated at $5x10^5$ cells/mL in 24-well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. (A) The following day, RAW264.7 cells were pre-treated with DMSO (LPS) or 500nM rapamycin (RAPA+LPS) for 30 minutes followed by 100ng/ml LPS for the indicate time. (B) Cells were pre-treated with DMSO (LPS) or with 20μM LY294002, 5μM Wortmannin, 5μM MG132 or 500nM rapamycin for 30 minutes followed by 100ng/ml LPS for 6 hours. (A+B) After stimulation, RNA was extracted and analyzed using TaqMan RT-PCR probes specific for murine c-Maf using ΔΔCT method. Data were normalized to GAPDH mRNA expression and fold induction was calculated relative to untreated cells at 0 hours.The data shown represents the mean of three independent experiments, each carried out in triplicate ± S.E.M. * ; $P<0.05$ **; $P<0.01$

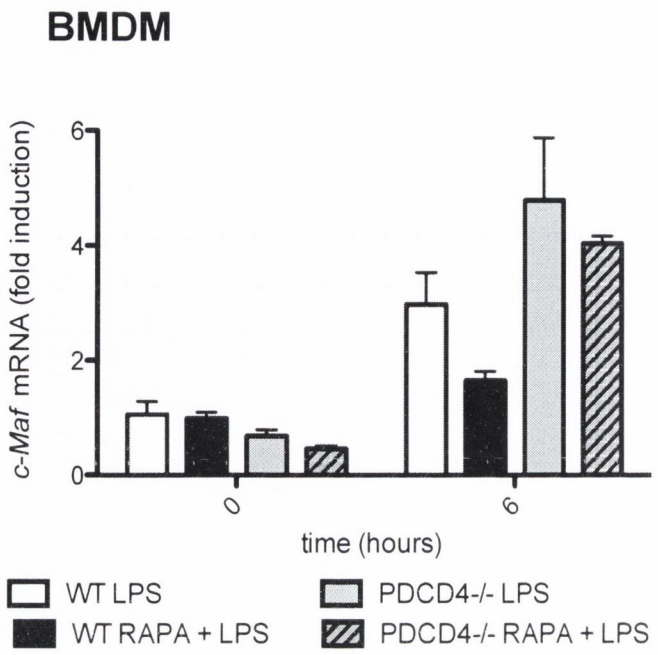


Figure 4.22 – A lack of PDCD4 increases LPS-induced c-Maf expression

(A) Wild-type BMDMs (WT) and PDCD4-deficient BMDMs (PDCD4-/-) were plated at $5x10^5$ cells/mL in 24-well plates and incubated overnight at 37°C in DMEM medium supplemented with 1% FCS. The following day, cells were pre-treated with DMSO (LPS) or 500nM rapamycin (RAPA+LPS) for 30 minutes followed by 100ng/ml LPS for the indicated time. (B) RAW264.7 cells were plated at $5x10^5$ cells/mL in 24-well plates and incubated overnight at 37°C in DMEM medium supplemented with 10% FCS, penicillin and streptomycin (100µg/ml). The following day, cells were transfected with 25 nM siRNA targeting PDCD4 or control siRNA oligonucleotide (Ctr) and the medium was changed to DMEM medium supplemented with 1% FCS for 24 hours. Cells were pre-treated with DMSO (LPS) or 500nM rapamycin (RAPA+LPS) for 30 minutes followed by 100ng/ml LPS for 6 hours. mRNA was extracted and analyzed using TaqMan RT-PCR probes specific for murine c-Maf using ΔΔCT method. Data were normalized to GAPDH mRNA expression and fold induction was calculated relative to untreated cells at 0 hours. The data shown represents the mean of three independent experiments, each carried out in triplicate ± S.E.M.

4.3 Discussion

PDCD4 has been described as an inhibitor of cap-dependent translation of mRNA, including IL-10 mRNA, through interaction via MA3 domains with eukaryotic initiation factors eIF4a and eIF4G (Yang et al., 2003, Loh et al., 2009). However I have found more evidence for a link between PDCD4 and IL-10 regulation through Twist2, a novel finding. My hypothesis was that PDCD4 could regulate Twist2. After LPS-induced PDCD4 degradation Twist2 would be free to induce IL-10, either directly or indirectly via c-Maf, and this became the next focus of the project.

Twist2 was examined initially because of a study showing that PDCD4 can interact with eIF4A (Yang et al., 2003a). Shiota et al, (2009) predicted homology between eIF4 and another Twist family member Twist1. They predicted that Twist1 would be another PDCD4 binding protein. With immunoprecipitation was demonstrated that PDCD4 could form a complex with Twist1, which was in agreement with the results I found. However the interaction between PDCD4 and Twist1 inhibited Twist1 dependent transcription of Y-box binding protein 1(YB-1) (Shiota et al., 2009). YB-1 has been reported to have a critical role in various biological activities in cancer cells including apoptosis and differentiation (Kohno et al., 2003). It has also been shown that YB-1 is a downstream target of Twist1 and is involved in cell growth (Shiota et al., 2008). It was found that the interaction between Twist1 and PDCD4 occurs through the bHLH DNA binding domain of Twist1 (Shiota et al., 2009). A family member of Twist1 was shown to be 95% similar in the bHLH binding domain region and was called Twist2 (Franco et al., 2011). Although these Twist transcription factors have some common similarities, their functions as gene regulators are unique.

It has been shown that Twist2-deficient mice up-regulate transcripts for pro-inflammatory cytokines TNF-α and IL-1β, suggesting a role for Twist2 in the negative

regulation of these cytokines (Sosic et al., 2003). Sosic and Olson, (2003) proposed that there is a negative feedback loop in which inflammatory cytokines activate NF-κB, leading to the expression of both Twist1 and Twist2. In turn, both proteins can block the transactivation activity of NF-κB in a promoter-specific matter. Sharabi et al. (2008) showed that Twist2 controls myeloid lineage development and function by regulating the transcription factors Runx1 and C/EBPα (Sharabi et al., 2008). Other studies have shown that Twist2 can bind to E-box consensus sites present in target gene promoters, or can directly bind transcription factors such as MEF2 and NF-κB (Spicer et al., 1996, Yang et al., 2004b, Sosic et al., 2003). These studies led us to question whether Twist2 could bind to PDCD4, and whether in response to LPS, it would be released to regulate IL-10 via c-Maf. The release of Twist2 from PDCD4 would be another mechanism for Twist2 to regulate pro-inflammatory cytokine responses acting as an inducer of IL-10 production. It is known that IL-10 JAK/STAT3 signalling can inhibit pro-inflammatory cytokine production. IL-10 can also block NF-κB nuclear translocation by inhibiting IKK activity as well blocking p65/p50 heterodimer when NF-κB is bound to the DNA (Clarke et al., 1998, Driessler et al., 2004, Asadullah et al., 2003). As described in Sosic et al., (2003) Twist2 can also act as an inhibitor of NF-κB and therefore the release of Twist2 from PDCD4 might have inhibitory effects on pro-inflammatory cytokine expression. However clearly LPS can increase IL-6 production and the release of Twist2 is unable to impact on IL-6. This could be because in this system Twist2 does not affect NF-κB. Similarly, eIF4 is unlikely to be involved in IL-6 production, since rapamycin did not block the induction of IL-6 in spite of stabilising PDCD4.

In this project Twist2 was identified as another binding partner of PDCD4 by using immunoprecipitation. The interaction was shown to occur via the bHLH and Twist box as mutants with a truncation of bHLH and Twist-box showed no interaction. LPS caused a decrease in this interaction most likely through PDCD4 degradation. Pre-

treatment with a concentration of 500nM rapamycin prevented this degradation, thus prolonging this interaction. The prolongation of the interaction between PDCD4 and Twist2 was also shown in the PI3K inhibitors wortmannin and LY294002 and the proteasomal inhibitor MG132. These, and previously shown results, suggested that S6K was involved in PDCD4 degradation and therefore reduced PDCD4 interaction with Twist2. To show more evidence that S6K phosphorylation was involved, a S6K mutant of PDCD4, which cannot be phosphorylated was used. The S6K mutant displayed a constant interaction with Twist2 after LPS treatment, showing again the involvement of S6K-ted phosphorylation of PDCD4 leading to its degradation. As it was previously described that PDCD4 could also interact with Twist1, I established an interaction between PDCD4 and Twist1 (Shiota et al., 2009). LPS caused a decreased interaction between PDCD4 and Twist1. This again was most likely due to the degradation of PDCD4.

The amino acids responsible for recognising E-BOX formation (CANNTG) in promoters of target genes in Twist2 related transcription factors are found through bioinformatics alignment. Using these alignments two specific amino acids were shown to be responsible, namely threonine 79 and serine 81, and these were conserved throughout different species (Firulli and Conway, 2008). It has previously been shown that these two specific amino acids can be phosphoregulated by the actions of protein kinase A, protein kinase C and B56δ-containing protein phosphatase 2A; however this only has been shown with Twist1 (Firulli et al., 2005, Firulli et al., 2003). Studies have shown that the charge on these conserved residues affects the dimerization affinities that Twist family members have for their potential bHLH partners (Castanon et al., 2001). It has been shown that phosphorylation mutants of Twist1 affect the dimerization characteristic of Twist1 as a homodimer but also as a heterodimer with E-proteins and Hand2 (Firulli et al., 2005). Furthermore, in the autosomal dominant disease Saethre-Chotzen syndrome, characterised by

mutations in the *TWIST1* gene, the protein kinase A site is mutated and is poorly phosphorylated (Firulli et al., 2005). Mutations within the phosphorylation site of Twist1 have also been shown by electrophoretic mobility shift assay, to affect DNA binding in a *cis*-element-dependent manner (Firulli et al., 2007). Because Twist1 is a close family member of Twist2, the two specific and conserved amino acids were point mutated and used to establish a functional role for the PDCD4/Twist2 complex. Therefore, the DNA-binding site (containing threonine 79 and serine 81) in Twist2 was mutated and as a result, this mutant was transfected in RAW264.7 cells and showed decreased IL-10 production in response to LPS. This demonstrated that the DNA-binding domain in Twist2 was required for IL-10 induction.

The amino acids in the bHLH responsible for protein-binding in Twist2 were also mutated by the previous knowledge that both Twist1 and eIF4A1 both interact with PDCD4. Bioinformatic alignment showed that mainly six amino acids located in the bHLH are involved during interaction. Through mutagenesis, four of the six amino acids were mutated and showed reduced interaction with PDCD4 and increased IL-10 production. This reiterates that release of Twist2 from the PDCD4/Twist2 complex is correlated with expression of IL-10.

Sharabi et al., (2008) demonstrated in Twist2 deficient mice, that there was decreased IL-10 cytokine secretion and c-Maf mRNA after LPS stimulation. Furthermore, a study with c-Maf deficient cells showed that c-Maf is an essential transcription factor for IL-10 gene expression in macrophages activated with LPS (Cao et al., 2005). Sharabi, et al., (2008) demonstrated that Twist2 could bind to the c-Maf promoter suggesting that Twist2 is able to promote the expression of IL-10 via direct activation of c-Maf transcription. This project confirmed the binding of Twist2 to the E-box of the c-Maf promoter in unstimulated cells. I also demonstrated that LPS

treatment increased Twist2 binding to the c-Maf promoter in a PI3K- and rapamycin-sensitive manner using ChIP and oligonucleotide pull down experiments. This increase of Twist2 binding to the c-Maf promoter might be due to the induction or activation of binding partners of Twist2 to enhance binding to the c-Maf promoter. However there was no increase seen in IL-10 expression in the cells transfected with the wild type Twist2 compared to cells containing the empty vector. This might show that there is enough Twist2 activation available in the system to drive IL-10.

However, when c-Maf binding of the protein-binding site mutant was examined, the results were slightly unexpected. I hypothesized that the mutation in the protein-binding site of Twist2 would reduce the interaction between PDCD4 and mutant Twist2 as seen in the co-immunoprecipitation assay, and that this would therefore lead to more Twist2 binding to the c-Maf promoter. However, no increase in binding of the Twist2 protein-binding site mutant to the c-Maf promoter was seen. It is possible that due to the mutation in the protein-binding domain another bHLH binding partner is needed for DNA binding and cannot be recruited and therefore less Twist2 binding to the c-Maf promoter is evident. It has been shown that bHLH transcription factors act different with differently binding partners (Firulli et al., 2005). Another possibility for the lack of enhanced binding to c-Maf might be that Twist2 mainly acts as a homodimer in this situation and is unable to bind to another Twist2 protein as both the protein-binding site and the DNA-binding site are needed for full Twist2 binding activity. Further research will be required to determine the underlying cause for a lack of this binding to c-Maf.

The previous data suggested that the DNA- phosphorylation-binding site is required for IL-10 induction as the mutations in the sites of Twist2 (which in Twist1 are required for DNA binding) reduce IL-10 expression. However, when the cells were transfected with the DNA-binding domain there was an unexpected basal binding of

the DNA-binding mutant of Twist2 to the c-Maf promoter. The DNA-binding activity of Twist2 might not be affected by only mutating the phosphorylation site in the DNA-binding domain as this might be only be the case for Twist1 (Firulli et al., 2007). Firulli et al (2007) showed that these conserved phosphorylation sites are essential for DNA-binding of Twist1. Therefore, I hypothesized that Twist2 would have the same effect on DNA binding as Twist1. However my results show that although Twist2 is a close family member of Twist1 with the same conserved phosphorylation residues, its DNA-binding activity might not only be depended on threonine 79 and serine 81, and that further mutations in the DNA-binding site would be needed to prevent DNA binding. IL-10 expression in cells transfected with the DNA-binding Twist2 mutant was reduced. This might be because the DNA-binding Twist2 mutant is unable to be activated when located on the promoter. Another possibility is that the mutant is unable to bind to form a homo- or heterodimer to enhance binding to the c-Maf promoter. The ChIP experiments with the mutant forms of Twist2 have limitations. Expression levels of each mutant were not confirmed and I did not test whether the phosphorylation site mutants were not undergoing phosphorylation or that the interaction with possible partner proteins was impaired. Further experiments would have to examine these aspects. Notwithstanding the protein-binding domain mutant showed reduced binding to PDCD4. Further work would be needed to examine regulation of DNA-binding and Twist2 interaction partners in this system.

Even though the data suggest that Twist2 regulates IL-10 induction through c-Maf the question remains if Twist2 would be able to regulate IL-10 directly by binding to its promoter. Through bioinformatics I established one predicted conserved E-box formation that Twist2 could recognise. However when this was examined with ChIP there was no direct binding seen of Twist2 to the IL-10 promoter.

Furthermore, to provide more evidence that c-Maf is involved in the regulation of IL-10 through the PDCD4/Twist2 complex, c-Maf expression was examined. The data showed that rapamycin, wortmannin, LY294002 and MG132 all inhibited the induction of c-Maf mRNA. c-Maf mRNA was also enhanced in PDCD4 deficient BMDM suggesting again that PDCD4 targeting is important for c-Maf induction by LPS.

Overall this chapter has revealed a new complex to be important for LPS-mediated induction of IL-10 (Figure 4.23). PDCD4 in complex with eIF4A or Twist2 therefore controls IL-10 production in response to LPS.

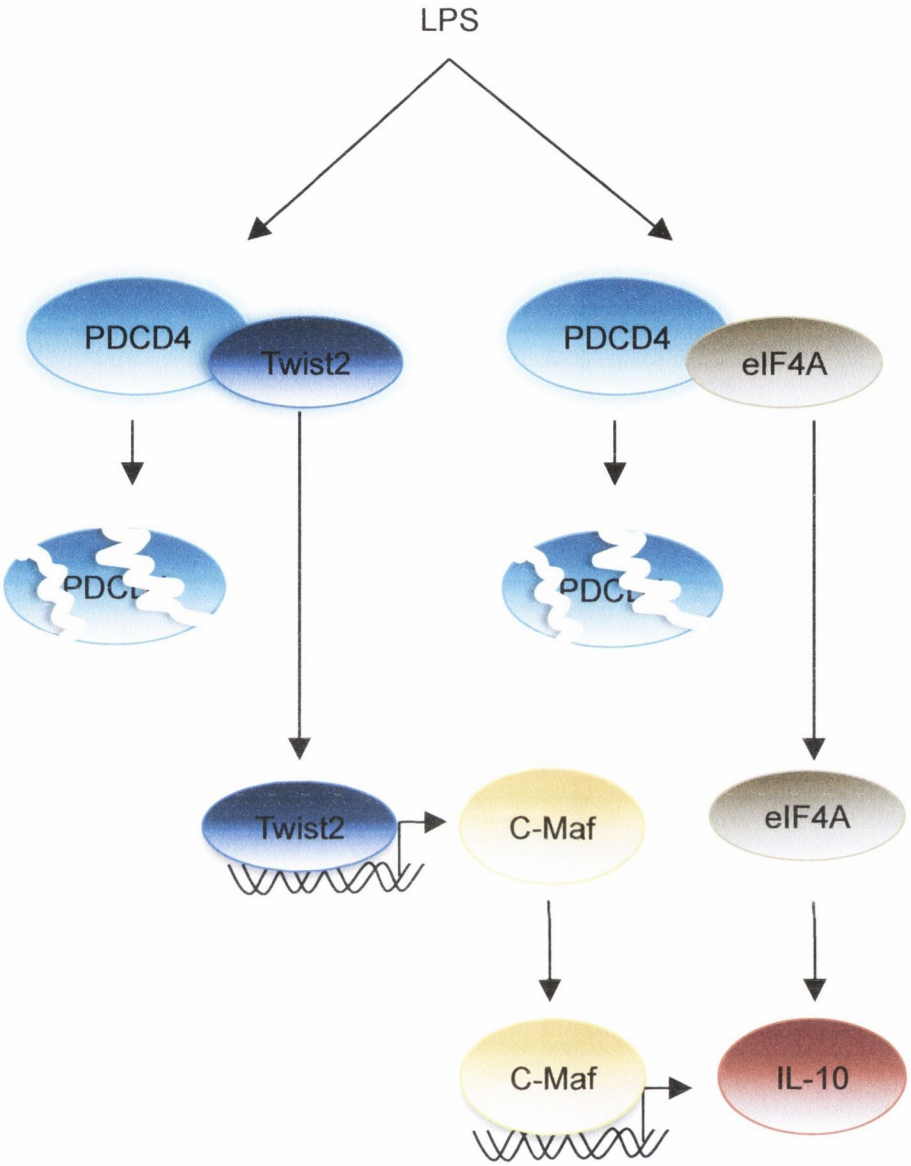


Figure 4.23 – Proposed regulation of LPS-induced IL-10 induction through the PDCD4/ Twist2 complex.

LPS activation of PI3K/mTOR signalling induces the expression of c-Maf and IL-10 by degradation of PDCD4. The degradation of PDCD4 allows Twist2 protein to bind to the c-Maf promoter. c-Maf is a well-known transcription factor of the anti-inflammatory cytokine IL-10. Degradation of PDCD4 also releases eIF4 which is known to be involved in IL-10 translation.

CHAPTER 5

FINAL DISCUSSION

This study uncovers for the first time an important process in the induction of IL-10 by LPS. In particular I have found how PDCD4 degradation plays an important role in the regulation of IL-10. This finding has added new understanding to the mechanism by which LPS-TLR4 acts to control gene expression and highlights the importance of transcriptional regulation through different signalling pathways. My investigation into the degradation of PDCD4 following the LPS response uncovered a novel complex, with PDCD4 binding to Twist2. The study of PDCD4 in the context of TLR signalling adds more complexity to the regulation of anti-inflammatory genes as PDCD4 has now been shown to be important for IL-10 transcription through the transcription factor c-Maf. PDCD4 is already known for its function during IL-10 translation. My result therefore reveal a novel way in which TLR4 regulates innate immune signalling through PDCD4 and contributes to our understanding of pathogen recognition and molecular responses to infection.

My results have highlighted the area of transcriptional control of IL-10 gene expression in the immune response. Thus far, our understanding of how TLRs activate and promote the transcription of many genes through the activation of transcription factors such as NF-κB and the IRFs is well established. My results have shown that TLR4 also activates the mTOR-signalling pathway, resulting in an increase of IL-10 production through regulating PDCD4 degradation.

IL-10 is known to play a role in determining the response *in vivo* to LPS (Berg et al., 1995). In *in vitro* studies of cells, IL-10 is known to be produced at later time points than the pro-inflammatory cytokines IL-6 and TNF-α. It is most likely that PDCD4 degradation serves a key control mechanism in IL-10 translation, through eIF4, but also its transcription to promote IL-10 production in response to LPS. This may have a broader immunosuppressive effect and may explain the sensitivity of PDCD4 deficient mice to lymphoma (Hilliard et al., 2006). IL-10 is known to play a key role in

immunosuppression and in the development and expansion of malignant B-cells as IL-10 deficient mice have been shown to display resistance to B-cell lymphoma (Czarneski et al., 2004).

However a question for discussion is that rapamycin and IL-10 are both known to suppress the immune system. By decreasing IL-10 production an opposite effect to immunosuppression by rapamycin would be expected. Inhibition of IL-10 products is however, not the only effect of rapamycin treatment. Rapamycin is generally used after organ transplantation to activate cell death of large number of effector T cells and favours the induction of tolerance (Katholnig et al., 2013b). IL-10 and rapamycin might suppress the immune system in different ways depending on tissue type, dose of rapamycin used and duration of treatment. However, the effects of IL-10 as an immunosuppressive signal, while important, are complex and are not fully understood.

Rapamycin is known to block the production of several cytokines, maturation of macrophages, and cell expansion by blocking mTOR activity in macrophages. As rapamycin is an artificial agent it might have off-targets effects when used in patients. It has been found that when post-transplantation patients displaying inflammatory symptoms were treated with rapamycin they showed an increase of IL-6 and TNF-α expression in the peripheral blood, which was speculated, could be due to a decrease in IL-10 (Buron et al., 2013). Buron et al (2013) suggested that the mTOR inhibition triggers a destabilization of the inflammatory cytokine balance in transplanted patients (Buron et al., 2013). A recently published paper suggested that rapamycin unbalances the polarization of human macrophages to M1 macrophages (Mercalli et al., 2013). Macrophages can be classed in response to environmental signals into classically (M1) and alternatively activated macrophages (M2). Under normal conditions M1 macrophages develop after LPS stimulation. When M1 macrophages

are treated with rapamycin they express IL-6, IL1-β and TNF-α and release lower levels of IL-10. *In vitro* data using peripheral blood mononuclear cells of 12 transplant patients pre-treated with rapamycin before LPS stimulation showed a shift towards M1 macrophages (Mercalli et al., 2013). These results are consisted with inhibition of IL-10 by rapamycin.

The regulation of PDCD4 has been shown to be post-transcriptionally regulated by miR-21 (Sheedy et al., 2010). miR-21 can target PDCD4 mRNA, as it has been demonstrated that LPS leads to the degradation of PDCD4, which was shown to be mediated by the induction of miR-21. miRNAs are 19-22 nucleotides long, and these noncoding small RNAs are found in all eukaryotic cells. They regulate approximately 30% of the human genome, primarily through translational repression. miRNAs were linked with immune responses in a study in which miRNA expression profiling was performed in a monocytic cell line treated with LPS (Taganov et al., 2006). miR-21 was identified as one of the first mammalian miRNAs as its sequence is strongly conserved throughout evolution (Roy and Sen, 2011). miR-21 was initially described as an 'oncomir' and is known as a common inflammation-inducible miRNA (Roy and Sen, 2011). The degradation of PDCD4 as well as PDCD4 deficiency resulted in an increase in IL10 production (Sheedy et al., 2010), as also can be seen in my study. However in the study from Sheedy et al., (2010) it was suggested that regulation of PDCD4 would have more of a translational effect on IL-10, as it has been demonstrated that PDCD4 acts as an inhibitor of cap-dependent translation of complex mRNAs through its interaction with eIF4A and eIF4G (Yang et al., 2003, Loh et al., 2009). Gene products inhibited by this mechanism include growth factors and cytokines, including IL-10 (Sheedy et al., 2010). However, there is evidence in other published data as well as my study that PDCD4 can act as a transcriptional regulator of IL-10 expression. A recent publication demonstrated that macrophages phagocytosing dead cells, trigger the release of IL-10 in a miR-21 –dependent

mechanism via PDCD4, after LPS stimulation (Das et al., 2014). Efferocytosis is known as an important reparative anti-inflammatory process in the resolution phase after wound injury. A study of efferocytosis in macrophages suggests that induction of miR-21 results in PDCD4 degradation and increasing IL-10 production is mediated via a PDCD4-JNK-AP1 pathway. PDCD4 is known to block c-Jun activation by inhibiting the expression of JNK (Wang et al., 2012). The Jun/AP-1 proteins are known to be involved in transcriptional activation of IL-10 in monocytic cells after LPS response (Wang et al., 2005). Das et al., (2014) demonstrated that an inhibitor for JNK as well as a siRNA knockdown of cellular c-Jun results in a significant downregulation of IL-10 protein expression. They also show that efferocytosis or delivery of a miR-21 mimic to monocyte derived macrophages induces the transcriptional activity of AP-1. To further establish their role in c-Jun/AP-1 pathway, knockdown of PDCD4 shows increased phospo-Jun levels. The study from Das et al., (2014) and the study in this thesis show that PDCD4 can also regulate IL-10 transcriptionally and through different transcription factors. This shows the complexity of IL-10 regulation in the LPS response.

Additionally the study by Das et al., (2014) also suggests that miR-21, through targeting PTEN, can block NF-κB activity and TNF-α production (Das et al., 2014). It has been shown before that PTEN is a target gene of miR-21 (Roy et al., 2009, Meng et al., 2007). In PTEN deficient macrophages, LPS-induced TNF-α production was reduced (Das et al., 2014). However, recent published data have also shown that myeloid PTEN deficiency results in the development of M2 type macrophages, which respond to ischemia reperfusion (tissue damage by lack of oxygen) -induced innate immune stimulation by producing a regulatory inflammatory response with high levels of IL-10, but lower levels of TNF-α and IL-6 (Yue et al., 2014). PTEN is a dual protein-lipid phosphatase that dephosphorylates the second messenger (PIP3) produced by PI3K and interrupts the downstream activation of Akt (Rosivatz, 2007, Maehama and

Dixon, 1999, Georgescu, 2010). Thus, downregulation of PTEN activity results in the activation of the PI3K/Akt pathway, resulting in an increase of IL-10. This effect seen in the PTEN deficient cells could potentially be through the PDCD4/Twist2 complex as described in this thesis.

Furthermore, Akt deficient macrophages and TSC1 deficient macrophages have been shown to produce increased levels of pro-inflammatory cytokines after LPS stimulation. Additionally, TSC1 deficient mice have shown that TSC1 negatively controls the TLR response by enhancing mTORC1 activation but also JNK activation after LPS stimulation. This effect is not specific to the TLR response, as NOD- and RIG-I induced innate responses are also reduced in TSC1-deficent macrophages following LPS stimulation (Pan et al., 2012). It has been shown that deficiency in S6K1 drastically decreases TLR or virus-induced IFN-α production in dendritic cells. In contrast, deficiency in 4E-BP1 in this system results in enhanced IFN-α and IFN-β production in vitro and in vivo due to the increased IRF7 transcription. In this thesis it was demonstrated that a specific inhibitor of S6K1, and siRNA specific to S6K1, can decrease IL-10 production in response to LPS. Although these observations have suggested critical roles for mTOR signalling in the innate immune response, the mechanism and the importance of mTOR regulation in innate immunity are not fully understood.

The mechanism for IL-10 production through the PDCD4/Twist2 complex as demonstrated in this study might also be common to different TLR pathways, i.e. not only in TLR4 pathway signalling, as it have been shown in innate immune cells that multiple TLRs can recruit and activate PI3K (Arbibe et al., 2000, Sarkar et al., 2004, Santos-Sierra et al., 2009). The PDCD4/Twist2 complex could be involved in stimulation of monocytes with *Porphyromonas gingivalis (P. gingivalis)* LPS. It was demonstrated that *P. gingivalis* LPS stimulation results in the selective activation of

the PI3K/Akt pathway via TLR2. The activation of this TLR results in the *P. gingivalis* LPS-induced production of IL-10, IL-12 p40, and IL-12 p70. Inhibition of this pathway results in an abrogation of ERK1/2 phosphorylation, whereas the activation of p38 and JNK were unaffected. Inhibition of PI3K led to the suppression of IL-10, whereas IL-12 was enhanced. Furthermore, the ability of the PI3K-Akt pathway to modulate IL-10 and IL-12 production appears to be mediated by the selective suppression of ERK1/2 activity, as the MEK1 inhibitor PD98059 closely mimicked the effects of wortmannin and LY294002 to differentially regulate IL-10 and IL-12 production by *P. gingivalis* LPS-stimulated monocytes (Martin et al., 2003). Although, it was not shown in this study that PDCD4 was regulated by *P. gingivalis,* it is possible that the PDCD4/Twist2 complex could be involved in the regulation of IL-10 in this response as p38 phosphorylation was not affected. p38 is know to activate MK2 that in its turn can phosphorylate TSC and block its inhibitory function on mTORC1 (Katholig et al., 2013a). Another example of the mTOR pathway controlling the inflammatory responses has been shown in TLR3 signalling, but this was observed in non-myeloid keratinocytes (Zhao et al., 2010). In the study of Zhao et al., (2010) it was shown that TLR3 signalling can induce the cytokine secretion of IL-1β and TNF-α. Treatment with rapamycin led to both an anti-inflammatory activity by lowering IL-1β and TNF-α secretion, as well as a pro-inflammatory response by enhancing IL-12p70, suggesting that the dominant effect of mTOR on keratinocytes is to modify the inflammatory responses (Zhao et al., 2010). In this study the authors could not detect Poly I:C - induced IL-10 protein expression in keratinocytes with or without rapamycin treatment. However, It was shown that rapamycin inhibited the phosphorylation of S6K1 and 4E-BP1(Zhao et al., 2010), suggesting that PDCD4 degradation was inhibited. It is known that Twist2 is upregulated in keratinocytes and that Twist2 influenced positive regulation of proliferation in keratinocytes (Tsuji et al., 2008). Therefore it might be the case that the PDCD4/Twist2 complex is involved in this

pathway. However, in keratinocytes Twist2 may regulate other genes through the complex as there was no IL-10 expression found in response to Poly IC in these cells.

Recently it has been shown that four homozygous nonsense mutations in the *Twist2* gene cause Setleis syndrome (Girisha et al., 2014). Setleis syndrome is an inherited development disorder classified as a focal facial dermal dysplasia type III (FFDD III). The phenotypes of the patients are additional facial features, including absent eyelashes on both lids or multiple row on the upper lids, absent Meibomian glands, slanted eyebrows and cleft chin (Tukel et al., 2010). Notably, Setleis syndrome patients and Twist2 knockout mice have similar facial features, indicating the gene's conserved role in mammalian development. In mice it has been indicated that Twist2 has different roles in immunity, as Twist2-deficient mice show high levels of pro-inflammatory cytokines and decreased levels of IL-10, and dysregulate c-Maf expression and NF-κB activity (Sharabi et al., 2008, Sosic et al., 2003). However to date, there are no studies published showing that Setleis syndrome patients having an increased inflammatory responses. Therefore it would be interesting for future studies to determine if Setleis syndrome patients suffer from autoimmune diseases related to a decreased IL-10 response.

Furthermore, the PDCD4/Twist2 pathway might affect the ability of cells to discriminate between pathogenic bacteria and non-pathogenic bacteria or PAMPs such as LPS. A study from Ivanov and Roy, 2013 has shown that in macrophages infected with *Legionella pneumophila* (a pathogenic bacteria) the PI3K-mTOR pathway is suppressed instead of activated, as evident with LPS (non-pathogenic) (Ivanov and Roy, 2013). They further show that when a mutant of *Legionella pneumophila*, which is non-pathogenic, was used and IL-10 was measured, there was an increase compared to the pathogenic *Legionella pneumophila*. However, rapamycin treatment of the cells infected with the mutant *Legionella pneumophila*

decreased IL-10 levels to a similar extent as in cells infected with wild-type *Legionella pneumophila*, suggesting that there are differences in the regulation of mTOR signalling in response to pathogenic or non-pathogenic bacteria, and free PAMPs such as LPS (Ivanov and Roy, 2013). These recent findings reveal that pathogenic bacteria can somehow suppress IL-10 induction in response to LPS. One possibility is that pathogenic bacteria somehow interfere with the PDCD4/Twist2 pathway.

Given the influence of IL-10 as a potent anti-inflammatory cytokine the discovery of PDCD4 interacting with Twist2 may have important therapeutic implications. IL-10 plays a critical role in preventing excessive inflammation and autoimmune diseases as it has been shown that IL-10 deficiency can lead to the development of inflammatory bowel disease and other auto-immune diseases (Sellon et al., 1998). Therefore, the mechanisms that regulate IL-10 expression are critical to developing therapeutic strategies. In my study I have shown that IL-10 expression can be regulated by the PDCD4/Twist2 complex through the PI3K-mTOR pathway, uncovering an important mechanism of IL-10 regulation (van den Bosch et al., 2014). There are several studies showing that mTOR inhibition can suppress autoimmune diseases, particularly type 1 diabetes, nephritis associated with systemic lupus erythematosus (SLE) and arthritis (Iyer and Cheng, 2012). It has also been shown that rapamycin combined with IL-10 blocks the incidence of type 1 diabetes and induces long-term tolerance without chronic immunosuppression in non-diabetic mice (Thomson et al., 2009). Further studies should focus on the combination treatment of rapamycin and IL-10 and the consequences of limiting mTOR activity and cytokine release in these diseases. However, as rapamycin is a well-known mTOR inhibitor, and has been shown to activate mTORC2 of the mTOR pathway, this might result in side effects when rapamycin is used as a treatment. Therefore, it will be important to define in further studies other functional roles of the PDCD4/Twist2 complex as this

may lead to specific agents for blocking or inducing IL-10 production through the binding or non-binding state of Twist2.

Taken together, the data shown in this thesis indicate that rapamycin, by inhibiting mTORC1 activity, can prevent LPS-induced PDCD4 degradation. Rapamycin allows the PDCD4/Twist2 complex to remain stable, thereby preventing Twist2 from binding to the c-Maf promoter, limiting c-Maf expression. This in turn will limit IL-10 induction by LPS (Figure 5.1). Overall I have provided a novel insight into the important anti-inflammatory role of the mTOR/PDCD4/Twist2/c-Maf pathway in LPS-induced signalling, this signalling process leading to IL-10 induction.

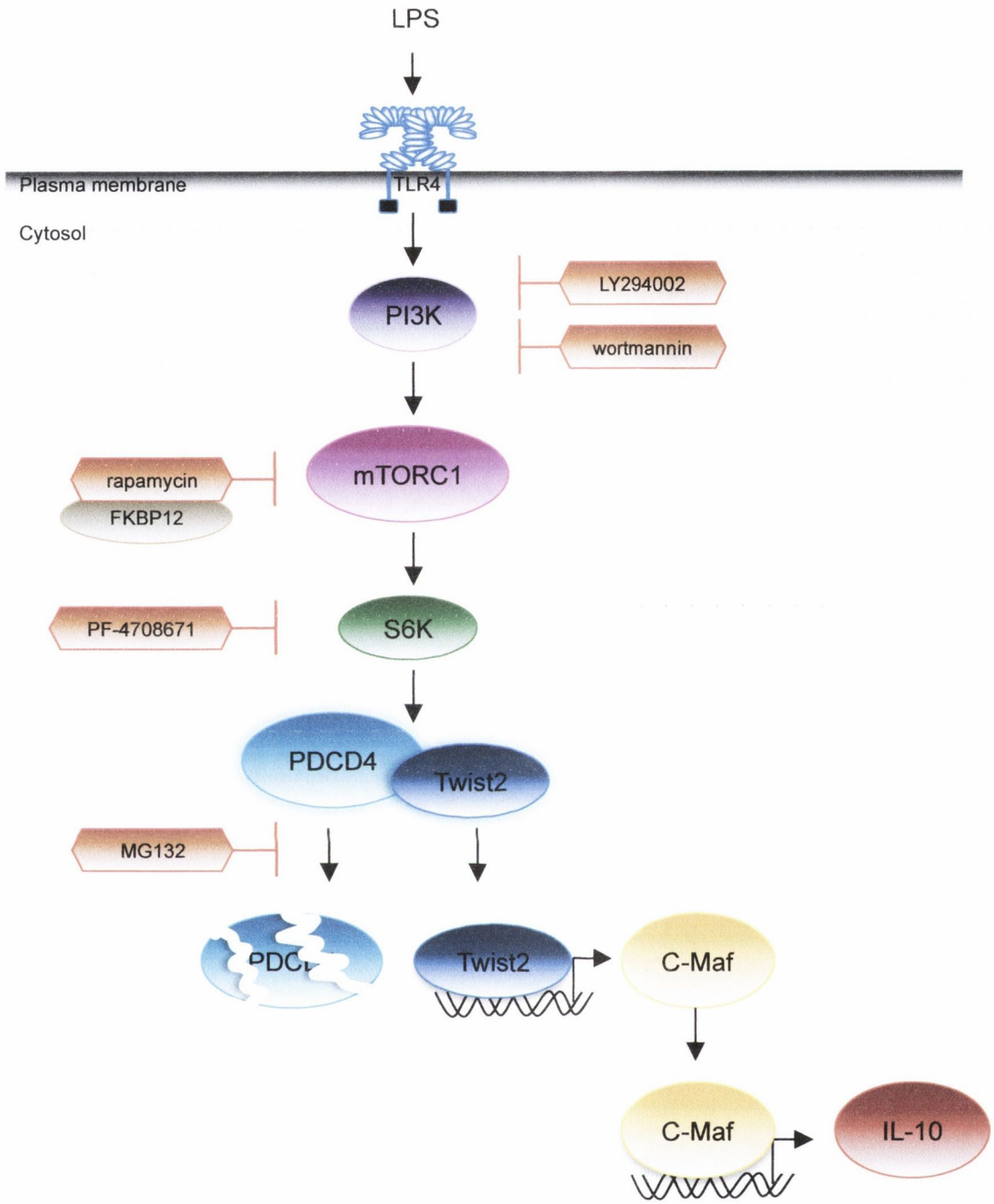


Figure 5.1 – Proposed regulation of LPS-induced cytokine expression by mTOR, and PDCD4/ Twist2 complex.

LPS activation of PI3K/mTOR signalling induces the expression of c-Maf and IL-10 by degradation of PDCD4. The degradation of PDCD4 allows Twist2 protein to bind on the c-Maf promoter. C-Maf is a well-known transcription factor of the anti-inflammatory cytokine IL-10. Pretreatment with rapamycin, an inhibitor of mTOR activity can prevent LPS-induced degradation. Therefore PDCD4/Twist2 complex remains intact and inhibits the Twist2-driven transcription of c-Maf and indirect inhibition of IL-10.

CHAPTER 6

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CHAPTER 7

APPENDICES

7.1 Abstract in Dutch/ Samenvatting in het Nederlands

Ongepaste of ongecontroleerde activatie van cytokines kan leiden tot infecties en ontstekingsziekten. Van de anti-inflammatoire en ontstekingsremmende cytokine IL-10 is bekend dat het de productie van pro-inflammatoire cytokines zoals TNF-α, IL-6 en IL-12 kan regelen. De regulatie van IL-10 is gecompliceerd maar recent is gebleken dat PDCD4 de expressie van IL-10 op een negatieve manier kan regelen. Na een LPS stimulatie werkt PDCD4 als een moleculaire schakelaar. Wanneer PCDC4 wordt afgebroken resulteert dit in een verhoogde IL-10 productie. In deze studie heb ik in detail onderzocht hoe PDCD4 wordt gereguleerd in LPS gestimuleerde macrofagen. Tevens heb ik bewezen dat de mTOR signalling en de proteosomale degradatie betrokken zijn bij de LPS gestimuleerde PDCD4 afbraak. Dit doormiddel van gebruik te maken van de mTOR remmer rapamycin en de proteosomale remmer MG132, deze blokkeren namelijk beide de PDCD4 afbraak. Het blokkeren van de PDCD4 afbraak door het gebruik van rapamycin leverde een verminderde expressie van IL-10 en c-Maf op. C-Maf is een transcriptie factor die nodig is voor de IL-10 expressie. In deze studie heb ik ook bewijs gevonden doormiddel van de techniek immuno-precipitatie dat er een interactie plaats vindt tussen PDCD4 en Twist2. Tevens heb ik doormiddel van chromatine immuno-precipitatie (ChIP) en oligonucleotide pull down technieken een nieuwe rol voor PDCD4 en Twist2 gevonden. Wanneer macrofagen worden gestimuleerd met LPS verbreekt de PDCD4/Twist2 interactie door PDCD4 afbraak en daarmee is Twist2 vrij om in hogere mate op de c-Maf promotor te binden. Dit leidt tot een verhoogde productie van IL-10. Maar wanneer het PDCD4/Twist2 complex in stand wordt gehouden heeft dit een negatief effect op expressie van c-Maf en IL-10. Samenvattend heb ik doormiddel van deze studie bewijs gevonden dat er nieuwe mechanisme is voor de regulatie van het ontstekingsremmende cytokine IL-10.

7.2 Published Articles

Van Den Bosch, M. W., Palsson-Mcdermott, E., Johnson, D. S. & O'Neill, L. A. 2014. LPS induces the degradation of PDCD4 to release Twist2, activating c-Maf transcription to promote IL-10 production. *J Biol Chem*.

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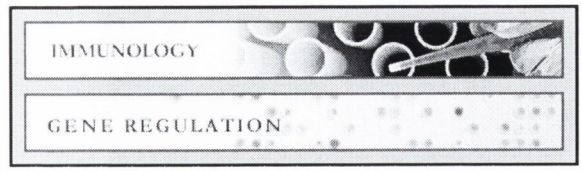
LPS Induces the Degradation of Programmed Cell Death Protein 4 (PDCD4) to Release Twist2, Activating c-Maf Transcription to Promote Interleukin-10 Production



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LPS Induces the Degradation of Programmed Cell Death Protein 4 (PDCD4) to Release Twist2, Activating c-Maf Transcription to Promote Interleukin-10 Production*

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Background: LPS-induced PDCD4 degradation leads to IL-10 induction.
Results: LPS-induced PDCD4 degradation results in release of Twist2, resulting in c-Maf induction and IL-10 production.
Conclusion: The PDCD4/Twist2 interaction has an important anti-inflammatory role in LPS signaling.
Significance: This study reports the mechanism of PDCD4/Twist2 interaction and provides a new insight of IL-10 production via suppression of PDCD4/Twist2 interaction.

Programmed cell death protein 4 (PDCD4) is a tumor suppressor and has also been shown to suppress production of the immunomodulatory cytokine IL-10. The precise role of PDCD4 in IL-10 induction in macrophages is still not fully understood. Incubation of macrophages with inhibitors of PI3K and mTOR blocked LPS-stimulated PDCD4 degradation and expression of c-Maf and IL-10 production. PDCD4 and the transcription factor Twist2 were shown to form a complex in untreated cells. LPS disrupted the complex allowing Twist2 to bind to the c-Maf promoter. PI3K and mTOR inhibitors prevented this disruption by stabilizing PDCD4 and thereby decreased Twist2 binding to the c-Maf promoter and induction of c-Maf mRNA. These results indicate a regulatory role for PDCD4 and Twist2 in LPS-induced IL-10 production in macrophages. LPS promotes PDCD4 degradation via a pathway involving PI3K and mTOR, releasing Twist2, which induces IL-10 via c-Maf.

The Gram-negative bacterial product lipopolysaccharide (LPS) is a potent inducer of the proinflammatory response. LPS is also able to induce multiple negative feedback regulators of inflammation, notably the immunomodulatory cytokine interleukin-10 (IL-10). IL-10 limits the production of proinflammatory cytokines such as TNF, IL-6, and IL-12 (1). The induction of IL-10 by LPS requires activation of Toll-like receptor 4 (TLR4)[2] signaling through p38 mitogen-activated protein kinase (p38); however, the mammalian target of rapamycin (mTOR) signaling pathway has also been shown to play an important role (2). The mTOR system comprises two different complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 comprises mTOR in a complex with mLST8 (mammalian lethal with SEC13 protein 8), PRAS40 (proline-rich AKT substrate of 40 kDa) and RAPTOR (regulatory-associated protein of mTOR). mTORC1 is sensitive to inhibition by rapamycin. mTORC2 comprises mTOR, RICTOR (rapamycin-insensitive companion of mTOR), mTOR-associated proteins Sin1 and mLST8, and is rapamycin-insensitive (3).

The activation of mTORC1 by LPS results in the activation of S6 kinase (S6K1) (4). S6K1 has multiple substrates, a notable example in the context of IL-10 being programmed cell death protein 4 (PDCD4). Following phosphorylation by S6K1, PDCD4 undergoes degradation by β-TRCP ubiquitin ligases (5, 6). PDCD4 was first described as a tumor suppressor (7). It inhibits translation, interacting via its MA3 domains with the eukaryotic translation-initiation factors eIF4a and eIF4G, targeting cap-dependent translation of mRNA, which include those encoding IL-10 and IL-4 (8–11). The induction of IL-10 by LPS has been shown to require degradation of PDCD4, allowing translation to occur (6), providing one mechanism whereby PDCD4 might suppress IL-10 production.

PDCD4 has been shown to interact with other proteins, a notable example being Twist1 (12). It has been shown that PDCD4 inhibits the transcriptional function of Twist1. In mammals, there are two Twist family members: Twist1 and Twist2 (also known as Dermo-1) (13, 14). Basic helix-loop-helix (bHLH) transcription factors such as Twist2 act as a homo- or heterodimer through their HLH motifs to form a DNA-binding domain. Twist2 has been shown to regulate c-Maf, via c-Maf IL-10 expression after LPS-induced stimulation (15).

In this study, we have addressed whether the inhibitory effect of PDCD4 on IL-10 production involves Twist2. We have found that PDCD4 interacts with Twist2 and that PDCD4 degradation results in the release of Twist2, allowing it to bind to the c-Maf E-box and resulting in c-Maf induction, leading to enhanced IL-10 production. Our study therefore provides a further function for PDCD4 in the regulation of IL-10, whereby it acts to limit c-Maf induction by sequestering Twist2.

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[2] The abbreviations used are: TLR4, Toll-like receptor 4; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; PDCD4, programmed cell death protein 4; bHLH, basic helix-loop-helix; DMSO, dimethyl sulfoxide.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—The HA-tagged PDCD4 expression vectors was kindly provided by Dr. Michele Pagano and subcloned into pc.DNA3.1. Dr. Toschi and Dr. Kohno kindly provided the HA-tagged PDCD4 S67A and the FLAG-tagged Twist1 expression vectors respectively. The Myc-DDK-tagged (FLAG tag) ORF clone of Twist2 was cloned into pCMV6 and was purchased from OriGene Technologies. LPS from *Escherichia coli*, serotype 0111:B4, was from Alexis. Rapamycin (R-5000) was from LC Laboratory. MG132 (M7449), wortmannin (W1628), LY294002 (L9908), PF-4708671 (PZ0143), and cycloheximide (C7698) were all purchased form Sigma. Oligonucleotides and SYBR primers were from Eurofins, and Taqman probes were from Applied Biosystems. SMARTpool siRNAs specific for mouse PDCD4 and S6K1 and negative control were from Dharmacon. Rabbit anti-Twist2 (ab66031) antibody was from Abcam or from Santa Cruz Biotechnology (H81 sc-15393), mouse anti-β-actin (AC-74), anti-HA (H6908), anti-FLAG (F7425), and rabbit IgG (I5006) antibodies were from Sigma. The antibodies PDCD4 (9535) and S6K (9202) were purchased from Cell Signaling. Protein A/G-plus agarose beads were from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Lipofectamine 2000™ was from Invitrogen. IL-10 (DY-417) and IL-6 (DY-406) ELISA Duoset® kits were purchased from R&D Systems.

Cell Culture—The human embryonic kidney 293 TLR4-MD2-CD14 (HEK293-TLR4-MD2-CD14) cell line was obtained from Invivogen and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated FCS, 50 mg/ml noromycin, 100 μg/ml blasticidin, and 100 μg/ml HygroGold obtained from Invivogen. RAW264.7 cells were obtained from the European Cell Culture Collection and cultured in DMEM containing 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin. Bone marrow from wild type and PDCD4$^{-/-}$ were a kind gift from Dr. Derek Johnson and were differentiated for 7 days in DMEM supplemented with 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin, and 20% (v/v) M-CSF (L929 mouse fibroblast supernatant). For experiments RAW264.7, BMDM, and HEK293-TLR4-MD2-CD14 cells were seeded at 5×10^5 cells/ml.

RNA Isolation and Real Time PCR—Cells (primary bone marrow derived macrophages ((BMDM) or RAW264.7) were plated and serum-starved for 16 h prior stimulation. Cells were stimulated with LPS, rapamycin, MG132, wortmannin, or LY294002 as indicated in the figure legends. Total RNA was extracted using an RNeasy kit (Qiagen), modified to obtain small RNA species. cDNA for mRNA analysis was prepared for 5–100 ng/ml total RNA using the High Capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions, and incorporating TaqMan primers for mouse c-Maf, mouse IL-6, mouse IL-10, mouse GAPDH, and mouse 18S (Applied Biosystems). mRNA expression were measured on the 7900HT RT-PCR system (Applied Biosystems), and fold changes in expression were calculated by $\Delta\Delta C_t$ method using mouse GAPDH and mouse 18S as an endogenous control for mRNA expression. All fold changes are expressed normalized to the non-stimulated control for each cell type.

Enzyme-linked Immunosorbent Assay—For cytokine measurements, BMDM and RAW264.7 cells were seeded at 5×10^5 cell/ml in a 12-well plate and stimulated in triplicate for each experiment. Supernatants were removed and analyzed for murine IL-10 and IL-6 (R&D Systems) using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

Protein Expression—Differentiated BMDM or RAW264.7 cells were seeded at 5×10^5 in six-well plates and stimulated with LPS rapamycin, MG132, wortmannin, or LY294002 as indicated in the figure legends. Cells were lysed in low stringency lysis buffer complete with protease inhibitors. Protein concentration was then determined using the Coomassie Bradford reagent (Pierce). Lysates were resolved on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membrane. Membranes were blocked in 5% (w/v) dried milk in TBS-T (50 mM Tris/HCl, pH 7.6, 150 mM NaCl, and 0.1% (v/v) Tween 20) before being immunoblotted with anti-PDCD4, anti-total S6K1, anti-HA, anti-FLAG, or anti-β-actin antibodies (1:1000 or 1:10,000 respectively) in 5% (w/v) dried milk in TBS-T at 4 °C overnight or at room temperature for at least 2 h. Membranes used for Twist2 determination were blocked in 5% (w/v) bovine serum albumin (Sigma) before being immunoblotted with anti-Twist2 antibody. Membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody diluted 1:2000 in 5% (w/v) dried milk in TBS-T for 1 h. Blots were developed by enhanced chemiluminescence according to the manufacturer's instructions (Cell Signaling Technology, Inc.).

Co-immunoprecipitation Assay—HEK293-TLR4-MD2-CD14 were seeded at 4×10^5 cells/ml in 10-cm dishes. 24 h later, cells were transfected with a total of 4 μg of the indicated plasmids using GeneJuice® and serum-starved cultured for a further 24 h before 6 h stimulation with LPS rapamycin, MG132, wortmannin, or LY294002 as indicated in the figures. After treatment, cells were lysed in low stringency lysis buffer complete with protease inhibitors, and 50 μl of whole cell lysate was kept for analysis. Co-immunoprecipitations were performed with A/G-plus agarose beads and with an IgG or PDCD4 antibody. Cell lysates were centrifuged at $2.200 \times g$ for 15 min before incubation with the beads and antibodies at 4 °C for 16 h. Following this, the lysate and beads were centrifuged at $80 \times g$ for 2 min at 4 °C, the supernatant was removed, and the beads were washed three times in 1 ml of low stringency lysis buffer. Immune complexes were eluted by the addition of 50 μl of SDS-Laemmli buffer and boiling the samples. Co-immunoprecipitations were analyzed by SDS-PAGE and Western blotting.

Chromatin Immunoprecipitation—RAW264.7 cells were seeded at 4×10^5cell/ml in three 15-cm dishes per sample, 24 h later, cells were transfected with a total of 4 μg of the indicated plasmids using GeneJuice® and serum-starved cultured for a further 24 h before a 6-h stimulation with LPS rapamycin, MG132, wortmannin, or LY294002 as indicated in the figures. After treatment, cells were fixed by adding a final concentration of 1% formaldehyde to each culture dish and were incubated for 10 min at room temperature. A 1/20 volume of 2.5 M glycine was then added to each dish and allowed to set at room temper-

ature for 5 min prior to washing in PBS and resuspension in 6 ml of ChIP lysis buffer (SDS lysis buffer with leupeptin, aprotinin, and PMSF) and immediately snap frozen in liquid nitrogen. The samples were thawed and resuspended in SDS:Triton buffer and then sonicated at 22% intensity, 10 × 30 s per sample, placing on ice in between pulses. 10 μl of whole cell lysate was kept for analysis. Lysates were incubated overnight with primary antibodies: anti-Twist2 (H81) (Santa Cruz Biotechnology, sc15393) and anti-IgG (Sigma, I5006). On the following day, cell lysates were incubated for 1 h at 4 °C with preblocked A/G-plus agarose beads. Next, the lysate and beads were centrifuged at 80 × *g* for 2 min at 4 °C, the supernatant was removed, and the beads were washed three times in 1 ml of ChIP lysis buffer. Quantitative RT-PCR was carried out using primers for either the c-Maf promoter consensus Twist2 binding site (forward, 5′-tgacgtcaggctcaaatgggaa-3′, reverse; 5′-agtcagctgattgggctgtgat-3′) or a non-Twist2 binding site (forward, 5′-ttcccagttcacattcagccct-3′; reverse, 5′-acgcatcatcattcctggct-3′). Data are presented as a percentage of input.

Affinity Purification with Biotinylated Oligonucleotides—Oligonucleotides for the terminal Twist2 binding site on the c-Maf promoter were annealed at 90–95 °C for 3–5 min, and then the heat block was allowed to cool to room temperature (forward, 5′BIO-caggctcaaatgggaaattg-3′; reverse, 5′-caatttccatttgagcctg-3′). RAW264.7 cells were seeded at 4 × 10^5 cell/ml and transfected with a total of 4 μg of Myc-DDK-tagged Twist2 plasmid using GeneJuice® and serum-starved and cultured for a further 24 h before a 6-h stimulation with LPS rapamycin. Cells were then lysed in 100 μl of oligonucleotide buffer (25 mM Tris, 5% glycerol, 50 mM EDTA, 5 mM NaF, Nonidet P-40 1%, 1 mM DTT, 150 mM NaCl, and protease and phosphatase inhibitors) and snap-frozen. Samples were then thawed on ice and diluted with a further 900 μl of oligonucleotide buffer containing no NaCl. A 10-μl sample of lysate was kept to which 40 μl of 5× SDS buffer was added. Remaining lysates were then precleared with 20 μl of prewashed streptavidin-agarose beads, rotating at 4 °C for 15 min before centrifuging at 80 × *g* for 5 min at 4 °C. Supernatants were removed to a fresh tube with 30 μl prewashed streptavidin-agarose beads and 30 μg of 5′-biotinylated oligonucleotide. Binding was performed for 2 h at 4 °C, and samples were rotated. Samples were centrifuged for 5 min at 4 °C to pellet the beads, which were washed three times before 50 μl of 5× SDS sample buffer was added to the beads. Samples were loaded on a 15% gel and Twist2 binding detected by Western blotting as described previously.

Site-directed Mutagenesis of Twist2—The QuikChange site-directed mutagenesis kit (Stratagene) was use to mutate or delete certain basis in the Twist2 gene. The manufacturer's instructions were followed using primers incorporating the desired mutations and deletions. To find a mechanistic function for Twist2, the amino acids located on 79 and 81 in the DNA binding domain were mutated. Previously, it has been shown that PDCD4 interacts with Twist1 (12) and eIF4A (7). It was found that the amino-terminal region of eIF4A possesses sequence homology with the bHLH of Twist1 (12) and Twist2 (data not shown), which is evolutionarily conserved. Therefore, amino acids located on 92, 93, 113, and 114 in the protein binding domain of Twist2 were mutated in the protein binding domain. Primers used were as follows: for Twist2 Gln-118-stop, 5′-catagacttcctctactaggtcctgcagagcga-3′ (forward) and 5′-tcgctctgcaggacctagtagaggaagtctatg-3′ (reverse), Twist2 Gln-66-stop, 5′-ggagctgcagagctagcgcatcctggc-3′ (forward) and 5′-gccaggatgcgctagctctgcagctcc-3′ (reverse); Twist2-FLAG T78A/S81A, 5′-agcgccagcgcgcccaggcgctcaacgag-3′ (forward) and 5′-ctcgttgagcgcctgggcgcgctggcgct-3′ (reverse); Twist2 Ile-92-stop, 5′-gcgctgcgcaagatctagcccacgctgccctct-3′ (forward) and 5′-agagggcagcgtgggctagatcttgcgcagcgc-3′ (reverse); Twist2-FLAG I92T/I93S/Y113C/I114S (I92T/I93S), 5′-cgcggcgctgcgcaagaccagccccacgct-3′ (forward) and 5′-agcgtggggctggtcttgcgcagcgccgcg-3′ (reverse); and Y113C/I114S, 5′-aagctggccgccaggtgcagagacttcctctacc-3′ (forward) and 5′-ggtagaggaagtctctgcacctggcggccagctt-3′ (reverse).

RESULTS

LPS-induced PDCD4 Degradation Requires mTOR Signaling—We first studied the effect of rapamycin on LPS induced PDCD4 degradation. As shown in Fig. 1*A*, in mouse primary BMDMs and in Fig. 1*B* in RAW264.7cells, LPS stimulation resulted in higher expression of PDCD4 protein after 1 h, followed by a gradual decrease after 6 and 20 h. In both BMDM and RAW264.7 cells, PDCD4 was almost completely degraded at 20 h (*lane 6* in both Fig. 1, *A* and *B*). Pretreatment of cells with rapamycin inhibited LPS-induced PDCD4 protein degradation (compare *lanes 4–6* and *lanes 10–12* shown in Fig. 1, *A* and Fig. *B*). The residual degradation of PDCD4 occurring at 6 h and 20 h in BMDM in the presence of rapamycin is likely to be due to miR-21, as shown previously (6). The inhibitory effect of rapamycin was evident at 1 nM (Fig. 1*C*, *lane 3* compared with *lane 2*) with higher doses increasing PDCD4 levels above basal level (Fig. 1*C*, *lanes 4–7*). These results confirmed those seen previously (16). We observed that pretreatment with rapamycin followed by 6 h of LPS stimulation almost completely prevented PDCD4 degradation in a dose-dependent manner (Fig. 1*C*). To verify that the increase in PDCD4 protein in response to inhibitors of the PI3K-mTOR pathway was a result of increased protein stability, we next determined the effect of cycloheximide on the rapamycin effect. We observed again a decrease in PDCD4 protein levels in RAW264.7 cells in the absence of rapamycin but stimulated with LPS (Fig. 1*D*, compare *lane 2* with *lane 1*). The decrease in PDCD4 was still evident in cells pretreated with cycloheximide for various times followed by LPS (Fig. 1*D*, *lanes 6–8*). Rapamycin was still able to stabilize PDCD4 in LPS-treated cells that had been pretreated with cycloheximide (Fig. 1*D*, *lanes 9–11*). Although cycloheximide does cause a slight decrease in overall PDCD4 levels, as can be seen in Fig. 1*D* (*lane 4*). This result indicates that the increase in PDCD4 after rapamycin pretreatment was a result of protein stability. This has also been shown in MCF7 cells treated with conditioned medium and rapamycin pretreatment (17). The PI3K inhibitors LY294002 (Fig. 1*E*) and wortmannin (Fig. 1*F*) also inhibited the effect of LPS on PDCD4 (compare *lanes 3* and *4* and *7* and *8* in Fig. 1, *E* and *F*). The 26S proteasome inhibitor MG132 also inhibited the degradation of PDCD4 (Fig. 1*G*, compare *lanes 3* and *6*), indicating that the degradation is mediated by the proteasome. It has previously been shown that phosphorylation by S6K1 is critical for the proteasomal degradation of

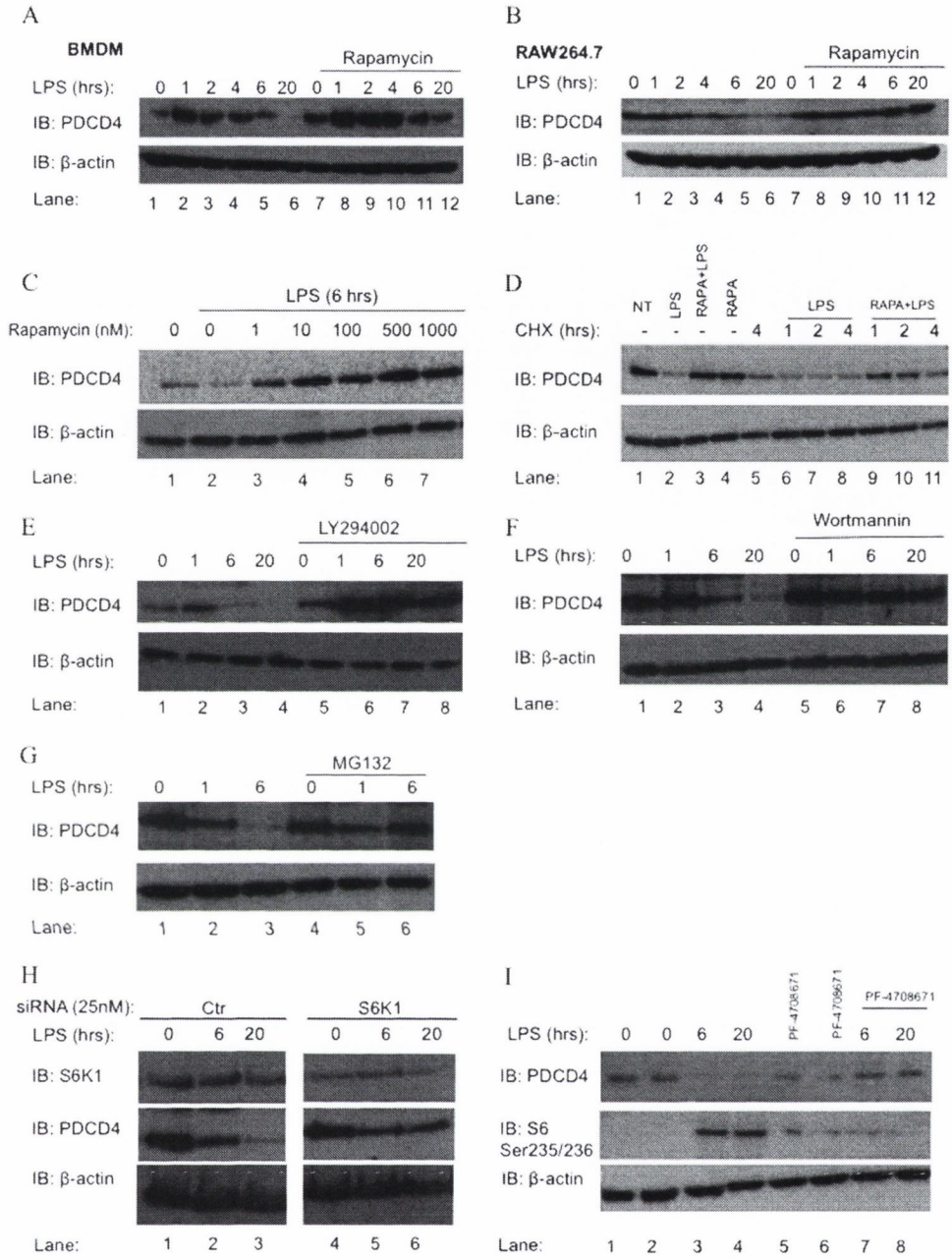


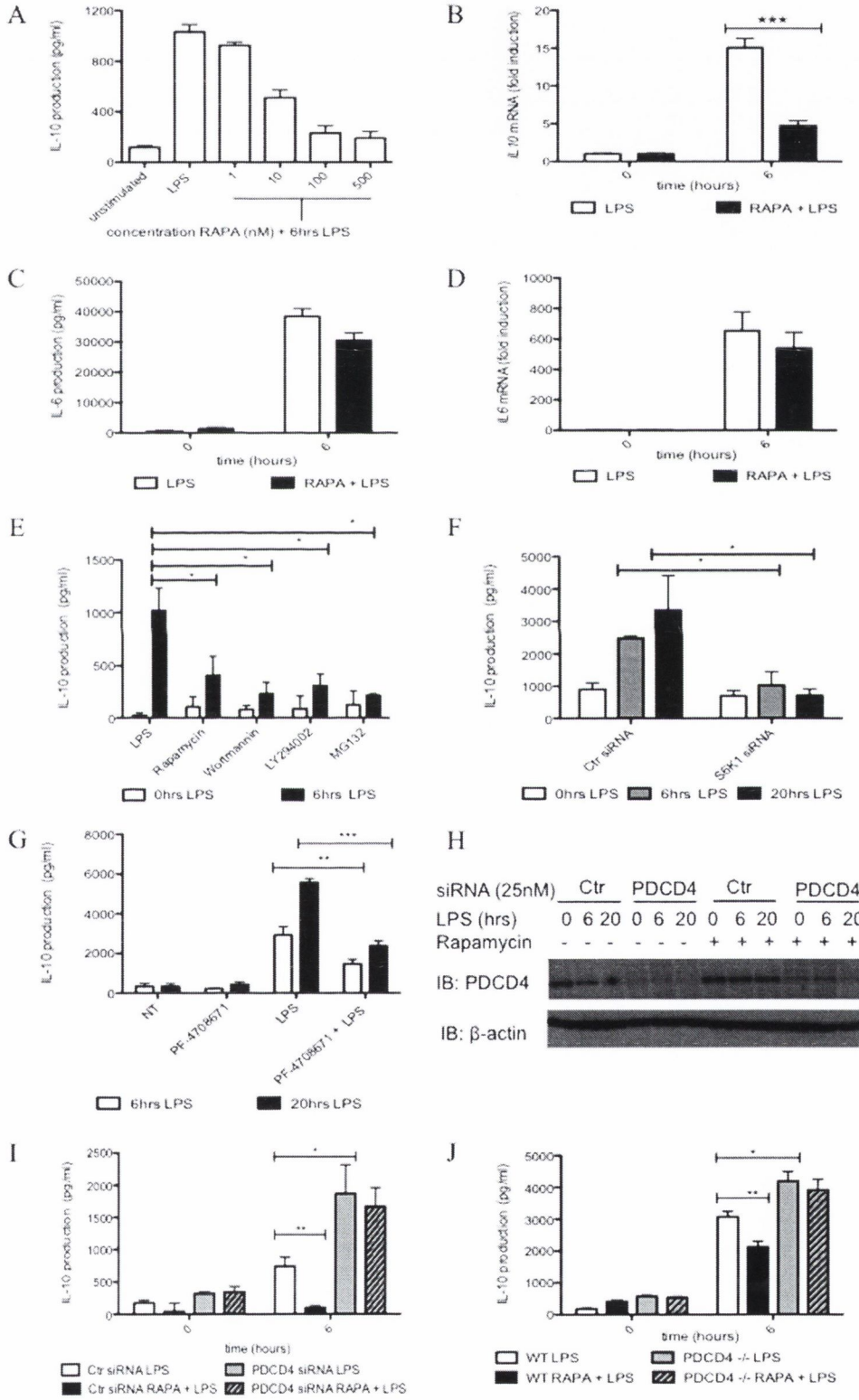
FIGURE 1. **LPS induces PDCD4 degradation requires mTOR signaling.** BMDM (*A–F*) or RAW264.7 (*B*) cells were serum-starved for 16 h, followed by pretreatment for 30 min with DMSO or 500 nM rapamycin (*RAPA*) followed by 100 ng/ml LPS for the indicated times. *C*, cells were pretreated with various concentrations of rapamycin for 30 min (indicated *above* each lane) *D*, cells were pretreated with for 30 min with DMSO or 500 nM rapamycin followed by for 20 h, followed by post treatment of cycloheximide for the indicated times. *E*, cells were pretreated with 10 μM LY294002 followed by 100 ng/ml LPS for the indicated times (*F*), or 5 μM wortmannin (*G*), or 5 μM MG132 and then stimulated with 100 ng/ml LPS for 6 h. Protein expression was measured by Western blot with antibodies specific for PDCD4 or β-actin. Data are representative of three separate experiments. *H*, RAW264.7 cells were transfected with siRNA encoding for S6K1 or control (*Ctr*) and serum-starved for 24 h. The cells were stimulated with (100 ng/ml) LPS for indicated times. *I*, cells were serum-starved for 16 h followed by pretreatment for 30 min with DMSO or 10 μM PF-4708671 followed by 100 ng/ml LPS for the indicated times. Protein expression of S6K1 (70 kDa), PDCD4 (52 kDa), or β-actin (45 kDa) were measured by Western blot with specific antibodies. Results are representative of three independent experiments. *IB*, immunoblot.

PDCD4 (5, 18). Knockdown of S6K1 using small interfering RNA (siRNA) resulted in a significant decrease in endogenous S6K1 expression (Fig. 1*H*, *top panel*, *lanes 4–6*). This led to an inhibition of LPS-induced degradation of PDCD4 protein (Fig. 1*H*, *middle panel*, compare *lanes 3* and *6*). Another method to implicate whether S6K1 is involved in this response was to pretreat RAW264.7 cells with S6K1 inhibitor PF-4708671. The cells pretreated with PF-478671 followed by 6 h of LPS stimulation, also led to an inhibition of LPS-induced PDCD4 protein degradation (Fig. 1*I*, compare *lanes 3* and *4* with *7* and *8*). These results show again that S6K1 is critical for PDCD4 degradation. It is therefore likely that the pathway stimulated by LPS here involves PI3K, mTORC1, and S6K1, leading to phosphorylation and degradation of PDCD4.

Rapamycin Inhibits the Induction of IL-10 by LPS through PDCD4—We next examined the effect of rapamycin on the induction of IL-10. As shown in Fig. 2*A*, pretreatment of RAW264.7 cells with rapamycin followed by 6-h stimulation

with LPS-inhibited IL-10 production with inhibition occurring from 1 nM and being optimal at 500 nM rapamycin (Fig. 2*A*). A similar decrease in *Il10* mRNA was observed at the 500 nM dose of rapamycin (Fig. 2*B*). This effect on IL-10 was specific as IL-6 (both at the protein and mRNA level) was not affected by pretreatment with rapamycin followed by a 6-h stimulation of LPS (Fig. 2, *C* and *D*, respectively). Pretreatment with LY294002, wortmannin, or MG132 also decreased induction of IL-10 in response to LPS (Fig. 2*E*), as did knockdown of S6K1 and PF-408671 S6K1 inhibitor (Fig. 2, *F* and *G*).

It has been shown previously that there is more IL-10 produced in response to LPS in cells lacking PDCD4 (6). We predicted that pretreatment with rapamycin in cells lacking PDCD4 would not affect IL-10 production if rapamycin medi-



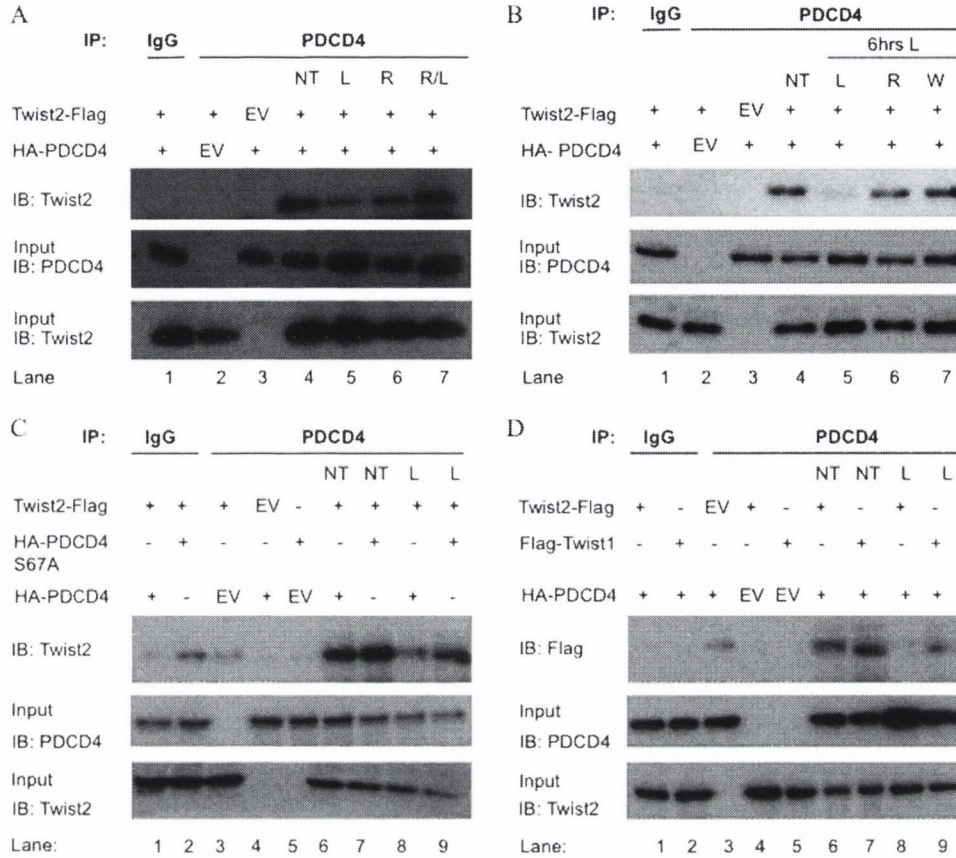


FIGURE 3. **PDCD4 interacts with Twist2.** *A* and *B*, HEK293/TLR4-MD2-CD14 cells were transfected with plasmids encoding Twist2-FLAG, HA-PDCD4, or empty vector (*EV*) as indicated. *A*, following 24-h post transfection and serum starvation, cells were left untreated or pretreated with DMSO (*L*) or 500 nM rapamycin (*R/L*) for 30 min followed with 6-h of LPS (100 ng/ml) stimulation. *B*, cells were pretreated with DMSO or 500 nM rapamycin (*R*) or 5 μM wortmannin (*W*) for 30 min, followed by 6 h LPS (100 ng/ml). *C* and *D*, HEK293/TLR4-MD2-CD14 cells were transfected with plasmids encoding Twist2-FLAG, HA-PDCD4, HA-PDCD4 S67A (*C*), FLAG-Twist1 (*D*), or empty vector (*EV*) as indicated. *C* and *D*, following 24 h post transfection and serum starvation, cells were left untreated stimulated 6 h with 100 ng/ml LPS. After treatment, cells were lysed, and 50 μl of whole cell lysate was kept for analysis. The remainder was immunoprecipitated (*IP*) with IgG or PDCD4 for 16 h at 4 °C. Whole cell lysates and immunoprecipitated samples were analyzed by Western blotting for Twist2-FLAG (18 kDa) or HA-PDCD4 (52 kDa) were measured with antibodies specific for FLAG and HA. Results are representative of three independent experiments.

ates its effect through PDCD4 stabilization. Therefore, we tested the effect of rapamycin on cells in which we knocked down PDCD4 (Fig. 2*H*). As can be seen in Fig. 2*I*, there was a 3-fold increase in IL-10 production in PDCD4 knockdown cells after LPS stimulation in comparison with the cells with control siRNA (Fig. 2*I*, 6-h LPS-treated samples, compare *gray* and *white bars*). Rapamycin had no effect on the induction of IL-10 by LPS in the cells in which PDCD4 was knocked down (Fig. 2*I*, 6-h LPS-treated samples, compare *gray hatched bar* with *gray bar*). In cells treated with control siRNA, rapamycin inhibited this IL-10 response (Fig. 2*I*, 6-h treated sample, compare *black bar* with *white bar*). Similar results were observed when PDCD4-deficient BMDM and wild-type BMDM were used as shown in Fig. 2*J*. IL-10 production in the PDCD4-deficient BMDM was increased compared with the wild-type BMDM (Fig. 2*J*, 6-h LPS treated samples, compare *gray bar* to *white bar*), but its induction was insensitive to rapamycin (Fig. 2*J*, 6-h LPS-treated samples, compare *gray hatched bar* with *gray bar*), unlike wild-type cells that showed inhibition by rapamycin (Fig. 2*J*, 6-h LPS-treated samples, compare *black bar* with *white bar*). These data confirm the importance of limiting PDCD4 via mTOR to induce IL-10 in response to LPS.

PDCD4 and Twist-2 Form a Complex—Next, we tested whether Twist2 and PDCD4 can interact. As shown in Fig. 3*A*,

FIGURE 2. **Rapamycin inhibits the induction of IL-10 by LPS through PDCD4.** *A*, RAW264.7 cells were serum-starved for 16 h followed by pretreatment for 30 min with DMSO or various concentrations of rapamycin for 30 min (as indicated) and stimulated 6 h with 100 ng/ml LPS. *B–D*, RAW264.7 cells were serum-starved for 16 h, followed by pretreatment with DMSO (LPS) or 500 nM rapamycin (*RAPA*+LPS) for 30 min followed by 6 h of 100 ng/ml LPS stimulation. *E*, RAW2647 cells were serum-starved for 16 h, followed by pretreatment with DMSO or 500 nM rapamycin, 10 μM LY294002, 5 μM wortmannin, or 5 μM MG132 for 30 min, followed by 6 h of LPS (100 ng/ml). *F*, RAW264.7 cells were transfected with siRNA encoding for S6K1 or control (*Ctr*) and serum-starved for 24 h, followed by treatment with 100 ng/ml LPS for indicated times. *G*, RAW264.7 cells were serum-starved for 16 h, followed by pretreatment with DMSO (LPS) or 10 μM PF-4708671 (RPF-4708671+LPS) for 30 min followed by 6 and 20 h of 100 ng/ml LPS stimulation. *H* and *I*, RAW264.7 cells were transfected with siRNA specific for PDCD4 or control (*Ctr*) and serum-starved for 24 h. The cells were pretreated with DMSO or (500 nM) rapamycin for 30 min followed by 6 h of LPS stimulation. *H*, protein expression were measured by Western blot with antibodies specific for PDCD4 (52 kDa) or β-actin (45 kDa). Data are representative of three separate experiments. *J*, BMDMs from wild-type (WT) and PDCD4 deficient ($PDCD4^{-/-}$) BMDMs were serum-starved for 16 h and pretreated with DMSO or 500 nM rapamycin, followed by 6 h LPS (100 ng/ml). After the indicated treatments, supernatants were analyzed by ELISA for IL-10 (*A*, *E*, *F*, *G*, *I*, and *J*) or IL-6 (*C*). RNA was extracted and the mouse IL-10 mRNA (*B*) and mouse IL-6 mRNA (*D*) were analyzed using TaqMan RT-PCR probes specific for murine IL-10 and IL-6. Data were normalized to GAPDH and 18S mRNA expression, and fold induction was calculated relative to untreated cells at 0 h. Data shown are representative of three separate experiments, with each point assayed in triplicate.*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *IB*, immunoblot.

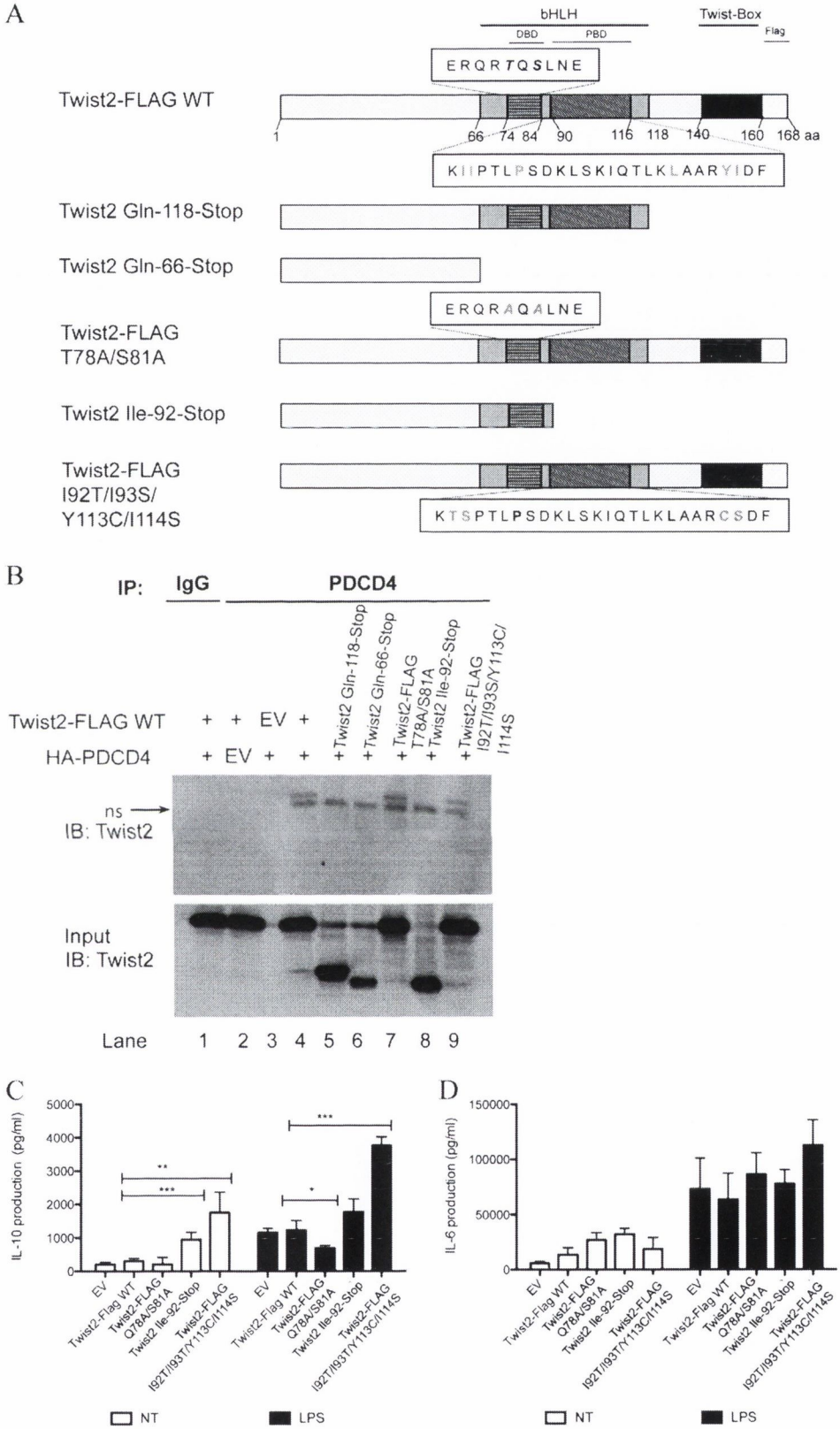
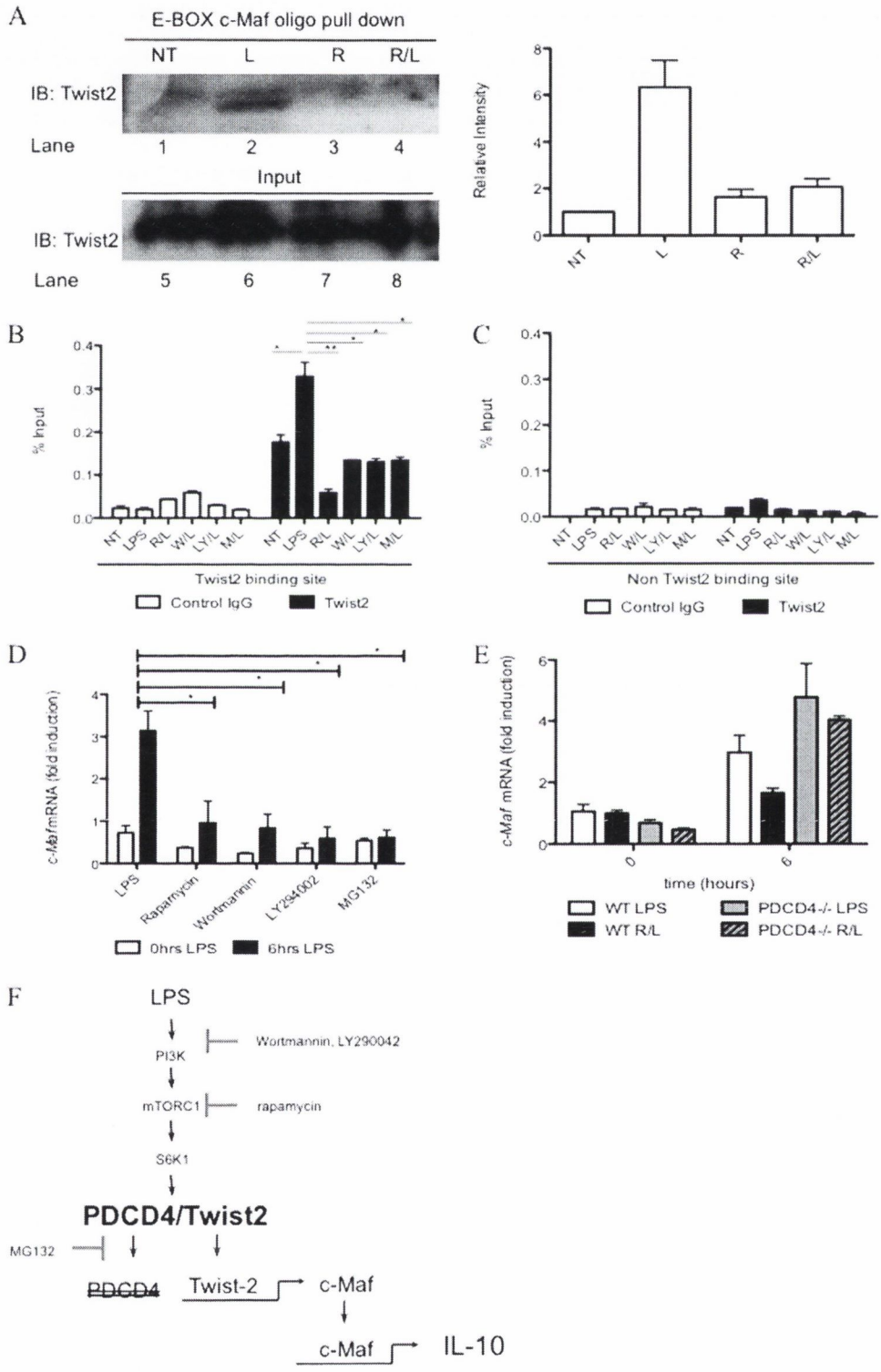


FIGURE 4. **Defining the domains responsible for the interaction of Twist2 with PDCD4.** *A*, schematic representation of Twist2-FLAG deletion mutants and Twist2-FLAG with mutations in the DNA binding domain (*DBD*) and protein binding domain (*PBD*) in the bHLH. *B*, HEK293T cells were transfected with equal amounts of various encoding plasmids Twist2-FLAG construct as described in *A* and HA-PDCD4 or empty vector (*EV*) plasmids as indicated. 24-h post transfection and serum starvation, cells were lysed, and 50 μl of whole cell lysate was kept for analysis. The remainder was immunoprecipitated (*IP*) with IgG or PDCD4 for 16 h at 4 °C. Whole lysates and immunoprecipitated samples were analyzed by Western blotting Twist2 (18 kDa). Results are representative of three independent experiments. *C* and *D*, RAW264.7 cells were transfected with equal amounts of various plasmids encoding Twist2-FLAG constructs and empty vector as described in *A* and HA-PDCD4. After 24 h, cells were left untreated or stimulated for 6 h with LPS (100 ng/ml). The supernatants were analyzed by ELISA for IL-10 (*C*) or IL-6 (*D*). Data shown are the mean ± S.D. from three separate experiments all carried out in triplicate *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

overexpressed HA-tagged PDCD4 interacted with overexpressed FLAG-tagged Twist2 (*top panel, lane 4*). Stimulation with LPS reduced this interaction (Fig. 3*A*, *top panel, lane 5*). Only a fraction of the overexpressed PDCD4 interacted with Twist2 because LPS did not cause a detectable decrease in the levels of total PDCD4 in the transfected cells (Fig. 3*A*, *middle panel, lane 5*). When cells were pretreated with rapamycin, the dissociation between PDCD4 and Twist2 following LPS stimulation was prevented (Fig. 3*A*, *lane 7*). Pretreatment of cells with wortmannin also inhibited the dissociation (Fig. 3*B*, compare *lane 7* with *lane 5*). We next tested whether deficiency of S6K-mediated phosphorylation of PDCD4 stabilized its interaction with Twist2. As shown in Fig. 3*C*, overexpressed HA-tagged PDCD4 interacted with overexpressed FLAG-tagged Twist2 (*top panel, lane 6*). Stimulation with LPS reduced this interaction (Fig. 3*C*, *top panel, lane 8*). The interaction can also be



determined in cells with overexpressed HA-tagged PDCD4 with a S6K phosphorylation site-deficient Ser-to-Ala mutant (Fig. 3*C*, *lane 7*) (5). However, after stimulation with LPS the decrease in the interaction between this mutant and the wild-type PDCD4 was attenuated (Fig. 3*C* compare *lane 9* with *lane 8*). A previous study has shown PDCD4 can interact with Twist1; therefore, we also tested Twist1 as a control (12). As shown in Fig. 3*D*, overexpressed HA-tagged PDCD4 interacted with overexpressed and overexpressed FLAG-tagged Twist1 (*top panel*, *lane 7*). Stimulation with LPS also reduced the interaction with Twist1 (Fig. 3*D*, *top panel*, *lane 9*). These results show that PDCD4 and Twist2 can form a complex, which can be disrupted by LPS in a rapamycin-sensitive manner.

Defining the Domains Responsible for the Interaction—Having confirmed an interaction between PDCD4 and Twist2, we next investigated which domains of Twist2 mediated the interaction. Five Twist2 constructs in addition to full-length Twist2 were generated and are depicted in Fig. 4*A*. Fig. 4*B* illustrates the results from the interaction analysis with the constructs. *Lane 4* (*top panel*) shows the interaction between PDCD4 and Twist2 as demonstrated previously in Fig. 3*A*. The higher percentage gel (18%) revealed a nonspecific band running faster than Twist2. Our results suggest that both the Twist Box and the bHLH domain are required for the interaction as removal of either domains abolished the interaction (Fig. 4*B*, *lanes 5*, *6*, and *8*). The mutations generated in the DNA binding domain (Fig. 4*B*, *lane 7*) did not affect the interaction between PDCD4 and Twist2; however, mutations in the protein binding domain appeared to reduce the interaction (Fig. 4*B*, *lane 9*). These results led us to investigate the importance of the different domains for IL-10 production. As shown in Fig. 4*C*, in RAW264.7 cells transfected with constructs lacking a functional Twist-Box and protein-binding domain (Twist2 Ile-92-stop) or mutated protein-binding domain (Twist2-FLAG I92T/I93S/T113C/I114S), there was an induction of IL-10 production (*white bars*). LPS was able to induce IL-10 in cells transfected with a plasmid encoding Twist2-FLAG WT (*black bar*, Twist2-FLAG WT). Transfection with a plasmid encoding Twist2 with the mutated DNA binding domain (*black bar*, Twist2-FLAG T78A/S81A) decreased its response, whereas transfection with a plasmid encoding the construct lacking the Twist-Box and protein binding domain (Twist2 Ile-92-stop) or mutated protein-binding domain (*Black bar*, Twist2-FLAG I92T/I93S/T113C/I114S) both showed a potentiated response. None of the constructs affected the induction of IL-6 by LPS (Fig. 4*D*, *black bars*). These results suggest that the Twist box and protein-binding domain in the bHLH domain are involved in the interaction with PDCD4, their absence allowing Twist2 to increase IL-10 production most likely due to an inability to be sequestered by PDCD4. The DNA-binding domain in Twist2 is needed for induction of IL-10.

Twist2 Binds to a Highly Conserved Site on the c-Maf Promoter after LPS Stimulation—Our data suggested that Twist2 might be released from PDCD4 to bind to the c-Maf promoter in response to LPS. We next examined the binding of Twist2 to the c-Maf promoter using a number of approaches. First, oligonucleotide pulldown assays were employed to investigate binding of Twist2 to the E-Box on the c-Maf promoter. As shown in Fig. 5*A*, *lane 2*, increased Twist2 binding to this specific and highly conserved site on the c-Maf promoter was induced by LPS. Pretreatment with 500 nM rapamycin inhibited this response (Fig. 5*A*, *lane 4*). To investigate this finding further, and in an endogenous context, ChIP at the Twist2 binding site in the c-Maf promoter was carried out. As shown in Fig. 5*B*, *black bars*, Twist2 bound to the c-Maf promoter basally, with binding being increased after 6 h LPS treatment. Pretreatment with rapamycin, wortmannin, LY294002, and MG132 inhibited the binding of Twist2, decreasing it below basal level. Twist2 did not bind to a non-Twist2 binding site in the c-Maf promoter (Fig. 5*C*, *black bars*) or the β-actin promoter (data not shown). These findings confirmed that LPS induces Twist2 binding to the Twist2 binding site at the E-Box on the c-Maf promoter. Rapamycin, wortmannin, LY294002, and MG132 all inhibited the induction of c-Maf mRNA by LPS (Fig. 5*D*). Induction of c-Maf mRNA by LPS was enhanced in PDCD4-deficient BMDM (Fig. 5*E*, 6-h LPS-treated samples, compare *gray bar* with *white bar*) but unlike in wild-type BMDM, this induction was not inhibited by rapamycin (Fig. 5*E*, 6-h LPS-treated samples, compare *gray hatched bar* with *gray bar*). These data indicate that PDCD4 targeting is more important for c-Maf induction by LPS.

DISCUSSION

Here, we demonstrate a previously undescribed aspect of IL-10 regulation by LPS. The stimulation of macrophages with LPS leads to the dissociation of PDCD4 from Twist2 with PDCD4 being degraded. Twist2 is thereby released and binds to

FIGURE 5. **Twist2 binds to a highly conserved site on the c-Maf promoter after LPS stimulation.** *A* and *B*, RAW264.7 cells were serum-starved for 16 h. Cells were then left untreated or pretreatment for 30 min with DMSO or 500 nM rapamycin and stimulated with 100 ng/ml LPS (*L*) for 6 h. Cells were also treated with 500 nM rapamycin alone for 6.5 h (*R*) (*A*, *left panel*). Cells were then lysed and an oligonucleotide (*oligo*) pulldown (*OPD*) assay was carried out with the E-Box Twist2-binding site oligonucleotide sequence on the c-Maf promoter. Samples were probed for Twist2 (18 kDa) by Western blot (*A*, *right panel*). Densitometry was carried are relative to non-treated controls. Bar graph represents the mean of two independent experiments. *B* and *C*, RAW264.7 cells were transfected with plasmids encoding Twist2-FLAG or HA-PDCD4. 24-h post transfection and serum starvation, cells were left untreated, or cells were pretreated for 30 min with DMSO or 500 nM rapamycin (*R/L*), 5 μM wortmannin (*W/L*), 10 μM LY294002 (*LY/L*), or 5 μM MG132 (*M/L*), and stimulated with 100 ng/ml LPS (*L*) for 6 h. After stimulation a ChIP assay was performed. Primers specific for the Twist2-binding site (~1141 base pairs upstream the c-Maf promoter of the genomic DNA) (*B*) and, additionally, the non-Twist2- binding site (~20 kb upstream the c-Maf promoter (*C*)) were designed, and binding events were measured as percent of input by real time PCR using antibodies against IgG (control antibody, *white bars*) or Twist2 (*black bars*). *D*, RAW264.7 cells were serum-starved for 16 h followed by pretreatment for 30 min with DMSO or 500 nM rapamycin, 5 μM wortmannin, 10 μM LY294002, or 5 μM MG132. *E*, BMDMs from wild-type (WT) and PDCD4-deficient ($PDCD4^{-/-}$) BMDMs were serum-starved for 16 h and pretreated with DMSO or 500 nM rapamycin, followed by 6 h LPS (100 ng/ml). *D* and *E*, after the indicated treatments, RNA was extracted, and the mouse c-Maf mRNA was analyzed using TaqMan RT-PCR probes specific for murine c-Maf. Data were normalized to GAPDH and 18S mRNA expression, and fold induction was calculated relative to untreated cells (*E*) or relative to untreated WT BMDM. Data shown are the mean ± S.D. from three separate experiments all carried out in triplicate. *, $p < 0.05$; **, $p < 0.01$. *F*, LPS activates PI3K/mTOR signaling inducing the expression of c-Maf and IL-10 by degradation of PDCD4. The degradation of PDCD4 allows Twist2 protein to bind on the c-Maf promoter, inducing c-Maf expression, which then induces IL-10 gene expression. The inhibitors shown maintain PDCD4-Twist2 complex inhibiting IL-10 induction.

the c-Maf promoter. The increase in c-Maf would lead to induction of IL-10. PI3K, mTOR, and S6K are all required for PDCD4 degradation, indicating another aspect of mTOR involvement in IL-10 induction (Fig. 5*F*). It has been shown previously that PDCD4 is a negative regulator of IL-10 production. PDCD4 is known to be degraded following LPS stimulation, resulting in increased IL-10 production (6). Our results are in agreement with these previous studies; however, we have also shown that pretreatment with rapamycin inhibits LPS-induced PDCD4 degradation. We have also shown that the increase of PDCD4 after rapamycin pretreatment was a result of protein stability, as has been shown in a previous study in MCF7 cells treated with conditioned medium and rapamycin pretreatment (17). It has also previously been shown that rapamycin, LY294002, or MG132 prevented the loss of PDCD4 protein after 8 and 24 h in macrophages exposed to conditioned medium (17). When the mTOR pathway is activated, mTOR can phosphorylate S6K1. S6K1 has previously been shown to be critical for the phosphorylation and proteasomal degradation of PDCD4 in T98G glioblastoma cells after serum activation and in HEK293 cells activated with TPA (5, 18). We have also shown that S6K1 is involved in PDCD4 degradation in response to LPS using RAW264.7 cells transfected with S6K1-specific siRNA and the S6K1 inhibitor PF-4708671. However, S6K1 depletion with siRNA appears to only partially block LPS-induced PDCD4 degradation. This is most likely due to the incomplete knockdown of S6K1 as demonstrated by Western blot in Fig. 1*H*. The residual S6K1 present could still lead to partial PDCD4 degradation. In total, our data add to our understanding of processes whereby LPS promotes PDCD4 degradation.

Our findings that PI3K inhibitors and rapamycin reduce LPS induction of IL-10 in macrophages are in agreement with previous studies (19, 20). It has previously been demonstrated that rapamycin has the ability to decrease IL-10 mRNA and protein in monocytes and dendritic cells treated with LPS (16, 20–22). However, a mechanism behind this was not completely understood.

PDCD4 has been described as an inhibitor of cap-dependent translation of mRNA, including IL-10 mRNA, through the interaction via MA3 domains with eukaryotic initiation factors eIF4a and eIF4G (8, 9). We have identified Twist2 as another binding partner of PDCD4. The interaction was shown to occur via the bHLH and Twist box. LPS caused a decrease in this interaction most likely through PDCD4 degradation. We also demonstrated that the DNA-binding domain in Twist2 was required for IL-10 induction.

Sharabi *et al.* (15) demonstrated that in Twist2-deficient mice, there was a decrease in IL-10 cytokine secretion and c-Maf mRNA after LPS stimulation (15). Furthermore, a study with c-Maf-deficient cells showed that c-Maf is an essential transcription factor for IL-10 gene expression in macrophages activated with LPS (23). Sharabi *et al.* (15) demonstrated that Twist2 could bind to the c-Maf promoter, suggesting that Twist2 is able to promote the expression of IL-10 via direct activation of c-Maf transcription (15). We confirmed these findings, demonstrating in addition that LPS increases Twist2 binding to the E-box of the c-Maf promoter in a PI3K and rapamycin-sensitive manner.

The mechanism for IL-10 production through the PDCD4-Twist2 complex as demonstrated in this study might be common to more TLR pathways and not only found in TLR4 pathway signaling, as it has been shown in innate immune cells that TLRs can recruit and activate PI3K (24–26). Activation of the PI3K pathway by TLR2 increases the production of IL-10, and inhibition of the PI3K pathway leads to a decrease of IL-10 production (19). This may imply that PDCD4/Twist2 could be involved.

Given the influence of IL-10 as a potent anti-inflammatory cytokine, these findings may have important therapeutic implications. IL-10 plays a critical role in preventing excessive inflammation and autoimmune diseases as it has been shown that IL-10 deficiency can lead to the development of inflammatory bowel disease and other auto-immune diseases (27). Therefore, the mechanisms that regulate IL-10 expression are critical to developing therapeutic strategies. In our study, we have shown that IL-10 expression can be regulated with the PDCD4-Twist2 complex through the PI3K-mTOR pathway, uncovering an important mechanism of IL-10 regulation.

Dysregulation of this process might lead to increased inflammation, or the specific activation of the pathways might have anti-inflammatory effects. Therefore, it will be important to define in further studies the role of the PDCD4-Twist2 complex in disease.

Taken together, the data indicate that rapamycin, by inhibiting mTORC1 activity, can prevent LPS-induced PDCD4 degradation. Rapamycin allows the PDCD4-Twist2 complex to remain stable, thereby preventing Twist2 from binding to the c-Maf promoter, limiting c-Maf expression. This in turn will limit IL-10 induction by LPS. Overall, we provide a novel insight into the important anti-inflammatory role of the mTOR/PDCD4/Twist2/c-Maf pathway in LPS-induced signaling, with this signaling process leading to IL-10 induction.

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