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Mal is not essential for TLR2 signalling and inhibits signalling by TLR3

Thesis submitted to the

University of Dublin

For the

Degree of Doctor of Philosophy

By

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Declaration

This thesis is submitted by the undersigned to the University of Dublin for the examination of Doctorate in Philosophy. The work herein is entirely my own with the exception of the following figure:

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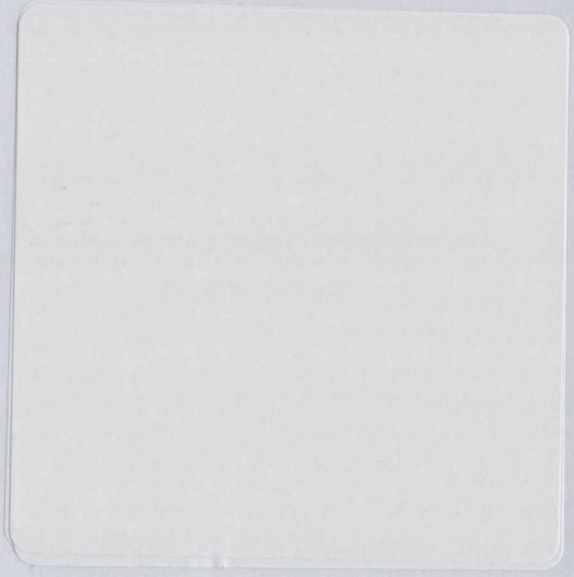

It is hard to believe that four years have passed and I am finally able to write this page! Many people have helped along the way but I must thank foremost Luke O'Neill my supervisor without whom this thesis would not have been possible. I am grateful for your support and encouragement along the way especially the motivational speeches when I was despairing. Huge thanks also go out to the members of the O'Neill lab (past and present) who made it a pleasure to go to the lab everyday and were always willing to lend a helping hand.

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Abbreviations

AP1	Activator protein 1
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine-5'-triphosphate
BMDM	Bone marrow derived macrophage
BSA	Bovine Serum Albumin
BTK	Bruton's tyrosine kinase
CARD	Caspase activation and recruitment domain
CFP	Cyan fluorescence protein
CLR	C-type lectin receptor
CTLD	C-type lectin-like domain
DAMP	Danger associated molecular pattern
DD	Death domain
DDX	Dead/H Box3
DMEM	Dulbecco's modified eagle medium
DUBA	De-ubiquitinating enzyme A
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetracetic acid
EV	Empty vector
FADD	FAS-associated death domain
FCS	Fetal Calf Serum
GPI	Glycosylphosphatidylinositol
HRP	Horseradish peroxidase

IBD	Inflammatory bowel disease
IFN	Interferon
IκB	Inhibitor of NFκB
IKK	IκB kinase
IL	Interleukin
IPTG	Isopropylthio-β-D-galactoside
IRAK	Interleukin 1 receptor associated kinase
IRF	Interferon response factor
JNK	c-jun N-terminal kinase
kDa	kilodalton
LB	Luria-Bertani broth
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTA	Lipoteichoic acid
Malp-2	Macrophage activating lipoprotein 2kDa
Mal	MyD88 adaptor like
MAP	Mitogen activated protein
MAVS	Mitochondrial antiviral signalling protein
MDA5	Melanoma differentiation-associated gene 5
MKK	MAP kinase kinase
MOI	Multiplicity of infection
MyD88	Myeloid differentiation factor 88

NAK	NFκB activating kinase
NAP1	NAK-associated protein 1
NEMO	NFκB essential modulator
NFκB	Nuclear factor kappa B
NLR	NOD-like receptor
NOD	Nucleotide Oligomerisation Domain
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PIP2	Phosphatidylinositol 4,5-bisphosphate
PMSF	Phenylmethylsuphonylflouride
PRR	Pattern recognition receptor
PVDF	Polyvinylidene difluoride
RA	Rheumatoid arthritis
RHIM	Rip 1 homotypic interaction motif
RIG-I	Retinoic acid-inducible gene-I
RIP	Receptor-interacting protein
RLR	RIG-like helicase receptor
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SARM	Sam and Arm containing protein
SDS	Sodium dodecyl sulphate

SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
TAB	TAK1 binding protein
TAG	TRAM adaptor with GOLD domain
TAK	Transforming growth factor β-activated protein kinase
TANK	TRAF family member-associated NFκB activator
TBK1	Tank binding kinase 1
TEMED	N, N, N, N,-tetramethylethylenediamine
TIR	Toll-IL1 receptor
TIRAP	TIR domain containing adaptor protein
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor associated factor
TRIF	TIR domain containing adaptor inducing IFN-β
TRAM	TRIF related adaptor molecule
UBC13	Ubiquitin-conjugating enzyme 13
UEV1A	Ubiquitin-conjugating enzyme E2 variant 1
VLR	Variable lymphocyte receptors
VIPER	Viral inhibitory peptide of TLR4
WCL	Whole cell lysate
YFP	Yellow fluorescence protein

Abstract

This thesis set out to systematically analyse the role of Mal in signalling by TLR2, TLR3 and TLR4 in murine macrophages and dendritic cells. It has revealed that Mal is not essential to TLR2 signal transduction, is required for TLR4 signalling and is inhibitory for signalling by TLR3. The absolute requirement for MyD88 in TLR2 and TLR4 signalling was also confirmed.

Initial interaction experiments demonstrated that MyD88 interacts with TLR1, TLR2, TLR4 and TLR6 and co-localises with TLR1, 2 and 6 within the cell upon stimulation with the appropriate ligand. MyD88 co-localises with TLR4 on the plasma membrane after stimulation with LPS and this co-localisation is disrupted by the translocation of TLR4 to an internal compartment of the cell. Mal was shown to interact with TLR1, TLR2 and TLR4 but not with TLR6.

There was a concentration-dependence for Mal seen in the immortalised macrophages treated with TLR1/2 ligand the Pam_3CSK4 or the TLR2/6 ligand Malp-2. There was little requirement for Mal seen at higher ligand concentrations and overall the role of Mal in TLR2 signalling was marginal. This was also seen when phosphorylation of p38 and JNK and degradation of IκB-α were examined with less signal transduction seen at lower ligand concentrations. Similarly, induction of cytokines by *S. typhimurium*, a TLR2 activator, showed little requirement for Mal especially at higher multiplicities of infection. MyD88 revealed its absolute requirement in TLR2 signalling with no IL6 production or activation of down stream signalling in response to the TLR2 ligands in the absence of MyD88.

Mal and MyD88 were required for IL6 induction in response to the TLR4 ligand LPS. Down stream signal transduction occurred normally in the absence of MyD88 and in a delayed manner in the absence of Mal through the activation of late NFκB and MAP kinase signalling by the TRIF-dependent pathway. Examination of the production of IL6 and TNF-α in primary macrophages and dendritic cells deficient in Mal also revealed a lack of requirement for Mal in TLR2 signalling and a total necessity for Mal in TLR4 signalling.

Mal and MyD88 deficiency boosted IL6 induction by the TLR3 ligand PolyIC in macrophages and dendritic cells. Enhanced IL6 and RANTES production was also seen in cells treated with the Mal inhibitor peptide VIPER in response to PolyIC stimulation. Phosphorylation of JNK, but not p38 or IκB-α degradation, was similarly potentiated in response to PolyIC, in Mal-deficient macrophages. IRAK2 was shown to form a trimer bridging Mal to TRAF3. Both TRAF3 and IRAK2 have seen shown to be involved in TLR3 signalling, therefore, the formation of a trimer containing these molecules and Mal could prevent them from functioning in response to TLR3 stimulation. As TLR3 and JNK activation are linked to apoptosis the inhibitory role of Mal through the trimer formation may be a mechanism to prevent cell death in response to TLR3 signal transduction.

This study, therefore, reveals that Mal is dispensable in TLR2 signalling with MyD88 probably coupling to the TLR2 receptor complex at sufficient levels to allow activation. TLR4 has total requirement for Mal with regards IL6 production. An inhibitory role for Mal in TLR3 signalling to JNK was also demonstrated possibly via its effect on the TRAF3/IRAK2 complex.

Chapter One

Introduction

1.1 Innate immunity

Innate immunity is the first line of defence against pathogen attack (Medzhitov, 2001). The molecules that make up the innate immune system are present in both plants and animals demonstrating its evolutionary importance (Janeway and Medzhitov, 2002). This implies that the innate immune system evolved before the split into plant and animal kingdoms and studies into the immune systems of plants and animals have revealed the genes involved in innate immunity are highly conserved (Hoffmann et al., 1999). Many multi-cellular organisms have an innate immune system but do not have adaptive immunity demonstrating its later evolution originating with the jawed vertebrates (Flajnik and Du Pasquier, 2004).

The cells involved in the innate immune response, such as macrophages and dendritic cells, recognise molecules common to pathogens but absent in the host. These molecules are recognised by pattern recognition receptors (PRRs) that are found on the plasma or endosomal membranes and in the cytosol of the immune cells (Kawai and Akira, 2008). The function of the PRRs is to alert the host to attack and induce an immediate immune response. They allow the activation of the complement cascade, opsonisation, phagocytosis and apoptosis (Brown, 2006). Where an adaptive immune response is available these immune cells are also involved in antigen presentation leading to T-cell differentiation (Tipping, 2006). Examples of PRRs utilised by the innate immune system are the Toll-like receptors (TLRs), the Nucleotide oligomerisation domain(NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) and the RIG-I like receptors (RLRs) (Becker and O'Neill, 2007; Bryant and Fitzgerald, 2009; Creagh and O'Neill, 2006).

1.2 Nod-like receptors

The Nod-like receptors or NLRs are a family of cytosolic proteins involved in innate immune signalling. 23 NLR proteins have been discovered to date in humans and 34 have been discovered in mice (Bryant and Fitzgerald, 2009). Their primary function is to recognise the presence of pathogens within the cells of the immune system and to regulate the production of interleukin 1-β (IL1-β) and IL18 (Martinon et al., 2009). They function to activate IL1-β and IL18 through the formation of multi-protein complexes termed inflammasomes. Once these complexes have formed due to the presence of a pathogen they allow the cleavage of pro-IL1-β and IL18 into their active forms. NLRP3 is a widely studied member of the NLR family that through its interactions with apoptosis-associated speck-like protein containing a CARD (ASC) and Caspase 1 recognises pathogens and many danger associated molecular patterns or DAMPs. These include adenosine-5'-triphosphate (ATP), monosodium urate crystals (MSU), amyloid-β and asbestos (Dostert et al., 2008; Halle et al., 2008; Kahlenberg et al., 2005; Shi et al., 2003).

1.3 C-type lectin receptors

The C-type lectin receptor superfamily or CLRs contain a large group of proteins linked by the presence of one or more C-type lectin-like domains (CTLDs). These domains are responsible for calcium-dependent carbohydrate binding by this family of proteins. The family has been split into 17 groups based on the amount and orientation of these CTLDs found in each protein (Zelensky and Gready, 2005). The transmembrane protein Dectin 1 is

a widely studied member of the CLR family that recognises β-glucans. It is of much interest due to its location on myeloid cells such as monocytes and the fact that it can recognise the presence of fungal β-glucans and activate innate immune signalling (Huysamen and Brown, 2009). It also recognises mycobacteria and endogenous ligands. The engagement of β-glucans by Dectin 1 leads to dendritic cell maturation and the production of cytokines and this activity is aided by TLR signalling pathway activation (Reid et al., 2009).

1.4 RIG-I like receptors

The RIG-I like receptors or RLRs are RNA helicase proteins responsible for the binding of dsRNA in the cytoplasm of cells. Retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are members of the RIG-I like receptor family (Nakhaei et al., 2009). They both contain N-terminal Caspase activation and recruitment domains (CARD), a central ATPase domain and helicase domains.

The RIG-I like receptors exist as monomers in the cytosol of cells and homodimerisation due to ligand binding is required to unmask their CARDs allowing for recruitment of mitochondrial antiviral signalling protein (MAVS, also known as IPS 1) which acts as their adaptor protein (Bowie and Fitzgerald, 2007; Kawai and Akira, 2009). These receptors are non-redundant for one another and as such recognise distinct dsRNA. RIG-I is responsible for the binding of short sequences of dsRNA such as those found in influenza virus, whereas MDA-5 binds picornaviruses and PolyIC (Kato et al., 2006).

1.5 Toll-like receptors

The discovery of the protein Toll in the fruit fly in the early 1980s was the first breakthrough in the discovery of the Toll-like receptors (TLRs). Toll was initially thought to only be required for dorso-ventral pattern development in the fruit fly (Hashimoto et al., 1988), however, studies into Toll mutant flies found them to be highly susceptible to infection by fungi and gram-positive bacteria (Lemaitre et al., 1996). When this second function for Toll was further examined it was discovered that Toll was involved in fly immunity and was activated by an upstream PRR. The activation of Toll resulted in a protease cascade activating the transcription factor Dorsal, a fly version of Nuclear factor kappa B (NFκB) (O'Neill, 2004).

Toll is a type-1 transmembrane protein with leucine rich repeats (LRRs) on its extracellular surface and a cytoplasmic domain with homology to the type-1 interleukin 1 (IL1) receptor. For that reason this area is known as the Toll/IL1 receptor (TIR) domain (Baker et al., 1997).

Investigations into the defence mechanisms of plants around the same time led to the discovery of N-protein, a protein involved in the immune response of the tobacco plant against tobacco mosaic virus (Whitham et al., 1994). This protein also shared homology with Toll and the IL1 receptor cytoplasmic domain.

This homologous domain involved in plant and animal innate immunity was used to identify genes in the human genome with similar sequences and ultimately led to the discovery of the 10 human and 13 mouse TLRs characterised to date (Kawai and Akira, 2007).

1.6 Toll-like receptor ligands

As a result of the early investigations into TIR domain containing proteins the landmark discovery of the TLRs in the mouse and human genomes was made. The TLRs are type-1 transmembrane proteins with molecular weight of 90-115 kilodalton (kDa) (Miggin and O'Neill, 2006). They consist of the TIR domain at their C-terminus and 16-28 copies of the LRRs (20-30 amino acid residues with conserved LxxLxLxxN motifs) at their N-terminus (Jin and Lee, 2008).

The first TLR to be characterised was TLR4 which recognises the complex of MD2 and lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria (Medzhitov et al., 1997). This led to the identification of the other TLRs which can be split into two groups based on their cellular localisation.

The TLRs found on the plasma membrane are TLR1, 2, 6, 4, 5 and 10. TLR2 forms complexes with TLR1 or TLR6 to bind tri- and di-acylated lipopeptides respectively (Omueti et al., 2005). TLR10 is a part of the TLR2 subfamily of TLRs but to date its ligand and method of activating the TLR2 signal cascade remains unclear. It appears that TLR10 can interact with TLR2 to recognise tri-acylated lipopeptides and that the heterodimer of TLR10 and TLR2 recruits myeloid differentiation factor 88 (MyD88) to signal. However, this ligand binding and MyD88 recruitment did not lead to the activation of NFκB or interferons (Guan et al., 2010). TLR5 recognises bacterial flagellin (Hayashi et al., 2001).

The TLRs found on the endosomal membrane are TLR3, 7, 8 and 9. They are all involved in the recognition of nucleotides with TLR3 binding dsRNA, TLR7/8 recognising ssRNA and anti-viral molecules such as imiquimod and TLR9 binding poly-unmethylated

cytosine and guanine rich regions (CpG) in DNA (Alexopoulou et al., 2001; Hemmi et al., 2002; Hemmi et al., 2000).

The binding of ligands to the TLRs results in the initiation of signalling in the cytosol of the cell. It is thought that dimerisation and ligand binding leads to conformational changes in the TIR domains of the TLRs allowing the adaptor proteins to be recruited. This was seen recently when the structure of TLR9 was examined upon stimulation with CpG. A non-active dimer of TLR9 was seen in cells that was unable to recruit MyD88, however, upon the addition of CpG the orientation of the TIR domains of the TLR9 dimer was altered and hence allowed for MyD88 recruitment (Latz et al., 2007).

1.7 TIR domain containing adaptor proteins

To date four activating and one inhibitory TIR domain containing adaptor proteins have been identified: MyD88, MyD88 adaptor-like protein (Mal), TIR domain-containing protein inducing interferon-β (TRIF), TRIF related adaptor molecule (TRAM) and Sam and Arm containing protein (SARM) (Kenny and O'Neill, 2008). The adaptors of interest in this project are MyD88 and Mal.

1.7.1 MyD88

MyD88 was the first adaptor discovered and is so-called due to its role in the terminal differentiation of $M1D^{+}$ myeloid precursors in response to IL6. MyD88 is induced in these cells upon IL6 treatment. The 88 refers to the gene number in the list of induced

genes (Lord et al., 1990). With regards to innate immunity MyD88 was first shown to be involved in signalling by the IL1 receptor (IL1R1) and then in signalling by various TLRs (Medzhitov et al., 1998; Wesche et al., 1997). The generation of MyD88-deficient mice verified these discoveries (Kawai et al., 1999; Takeuchi et al., 2000b). Mice lacking MyD88 were unable to produce cytokines in response to all TLR ligands tested with the exception of TLR3 which utilises the TRIF signalling pathway only. Hence, MyD88 has been implicated in signal activation by all TLRs bar TLR3. A recent study has revealed an inhibitory role for MyD88 in TLR3 signalling in the corneal epithelium cells of mice. This inhibition was shown to be specific to the phosphorylation of the mitogen activated protein (MAP)-kinase protein c-jun N-terminal kinase or JNK (Johnson et al., 2008).

There are several regions of MyD88 that are crucial for its ability to signal. At its C-terminus is the TIR domain, common to all the adaptor proteins, which allows it to directly interact with its TLR of interest and with the other adaptors through TIR-TIR interactions. At its N-terminus there is a death domain (DD), a second protein-protein interaction domain, which allows MyD88 to recruit the IL1 receptor associated kinases (IRAKs) to the signalling complex through a DD-DD interaction (O'Neill and Bowie, 2007).

1.7.2 Mal

The second adaptor protein to be discovered was Mal (also known as TIR domain containing adaptor protein or TIRAP). Mal was discovered by high-throughput sequencing of a human dendritic cell expressed sequence tag (EST), complementary DNA library. This

revealed a gene with a region highly similar to the TIR domains of MyD88, TLR2 and TLR4 (Fitzgerald et al., 2001). Upon further investigation the gene was revealed as Mal, the second adaptor molecule which is required for TLR signalling. At the same time a second group also identified Mal using bioinformatical studies into genes with potential TIR domain sequences (Horng et al., 2001).

Mice lacking the entire gene coding for Mal or with a truncated version of Mal were generated to examine its role in TLR signalling. Both investigations revealed a requirement for Mal in TLR2 and TLR4 signalling only and it is postulated that Mal acts to bridge these TLRs to MyD88 and hence aid in the activation of the MyD88-dependent signalling pathway (Horng et al., 2002; Yamamoto et al., 2002a).

Several regions of Mal have been characterised as being important for its signalling ability. At its N-terminus Mal has a phosphatidylinositol-4,5-bisphosphate (PIP2)-binding motif which recruits it to the plasma membrane in micro domains known to be rich in TLR4 (Kagan and Medzhitov, 2006). Phosphorylation of Mal at several tyrosine's (86 and 187) by Bruton's tyrosine kinase (BTK) is also required for its activation (Gray et al., 2006; Jefferies et al., 2003). This phosphorylated Mal is then targeted by suppressor of cytokine signalling 1 (SOCS1) for degradation (Mansell et al., 2006). IRAK1 and IRAK4 also phosphorylate Mal leading to its ubiquitination and degradation by the proteasome (Dunne et al., 2010a).

At its C-terminus Mal has a putative tumour necrosis factor receptor-associated factor 6 (TRAF6) binding domain allowing Mal to directly interact with TRAF6 (Mansell et al., 2004). A Caspase1 cleavage site at position 198 (aspartic acid) in the C-terminus of

Mal has also been discovered and this cleavage is required for the activation of Mal (Miggin et al., 2007).

A single nucleotide polymorphism (SNP) in the gene encoding Mal for an amino acid at position 180 was discovered through population genetic studies which converted a serine residue (S) to a leucine residue (L). Individuals heterozygous for this SNP are protected against several infectious diseases including Malaria and Systemic lupus erythematosus (SLE) (Castiblanco et al., 2008; Khor et al., 2007). Individuals homozygous for serine at this position are hyper-responsive to infection and individuals homozygous for leucine are unable to mount an immune response requiring Mal.

A second SNP in the gene encoding Mal at position 96 in which an aspartic acid (D) is converted to an asparagine (N). The conversion of aspartic acid to asparagine resulted in decreased NFκB activation and reduced IL8 production. Membrane trafficking and MyD88 recruitment were impaired in the presence of Mal 96N and this was suggested to be due to altered post-translational changes in Mal 96N (George et al., 2010).

1.7.3 TRIF

The next adaptor discovered was TRIF. Due to the ability of cells derived from MyD88- and Mal-deficient mice to produce cytokines in response to TLR3 and TLR4 stimulation the presence of a second signalling pathway was investigated. Through database studies using the known TIR domain sequences of MyD88 and Mal the third TIR domain containing adaptor TRIF was discovered (Yamamoto et al., 2002b). TRIF was also independently discovered in a yeast-two hybrid screen with TLR3 (Oshiumi et al., 2003a).

The generation of TRIF-deficient mice revealed TRIF was required for the activation of the interferon regulatory factor (IRF) pathway leading to interferon (IFN) production in response to PolyIC, the TLR3 ligand, and LPS, the TLR4 ligand (Yamamoto et al., 2003a).

Several domains in TRIF are vital for its ability to signal. It contains a TRAF6 binding domain near its N-terminus and a TIR domain and a receptor-interacting protein (RIP) homotypic interaction motif (RHIM) are located at the C-terminus (Han et al., 2004; Meylan et al., 2004; Sato et al., 2003).

1.7.4 TRAM

TRAM was discovered through bioinformatical analysis searching for genes containing TIR domain sequences (Fitzgerald et al., 2003). Investigations into TRAM using over-expression studies, dominant-negative mutants and interaction studies with TRIF showed that TRAM functions exclusively on the TLR4 pathway (Oshiumi et al., 2003b). The generation of TRAM-deficient mice confirmed that TRAM acts to bridge TRIF to TLR4 (Yamamoto et al., 2003b). There was no role for TRAM in TLR3 signalling demonstrating TRIF is the sole adaptor required for TLR3 signal activation.

Research into TRAM has revealed two conformational changes that are required for its activity. The N-terminus of TRAM must undergo myristoylation to anchor TRAM at the membrane and allow signalling (Rowe et al., 2006). TRAM must also be phosphorylated on serine 16 by protein kinase C ε (PKCε) to be activated (McGettrick et al., 2006). A splice variant of TRAM, termed TRAM adaptor with GOLD domain (TAG), has recently

been discovered which negatively regulates MyD88-independent signal transduction in response to TLR4 stimulation (Palsson-McDermott et al., 2009).

1.7.5 SARM

The final adaptor discovered was SARM. It was first revealed as a homolog of a sterile α-motifs (SAMs) and HEAT/armadillo repeats containing protein found in *Drosophila melanogaster* (Mink et al., 2001). Investigations into the role of SARM in innate immunity were only carried out when a TIR domain was identified, after in-depth investigations into its homologs in *C. elegans* (Couillault et al., 2004).

SARM was then shown to be inhibitory to TRIF signalling due to its ability to directly bind TRIF. Over-expression of SARM resulted in abolishment of TRIF-dependent, but not MyD88-dependent, NFκB and IRF7 activation. This inhibition required the SAM and TIR domains of SARM but not its HEAT/Armadillo repeats (Carty et al., 2006).

A second study into SARM has since revealed it may have a separate role in neuro-protection as neurons from SARM-deficient mice were more resistant to apoptosis induced by oxygen and glucose deprivation (OGD) than neurons from wild type mice. There appeared to be no role for SARM in TLR signalling as when macrophages from SARM-deficient mice were tested with various TLR ligands all responses were normal (Kim et al., 2007c). The two different roles for SARM therefore require further investigation. The important signalling domains of the five TIR domain containing adaptor proteins are outlined in Figure 1.1.

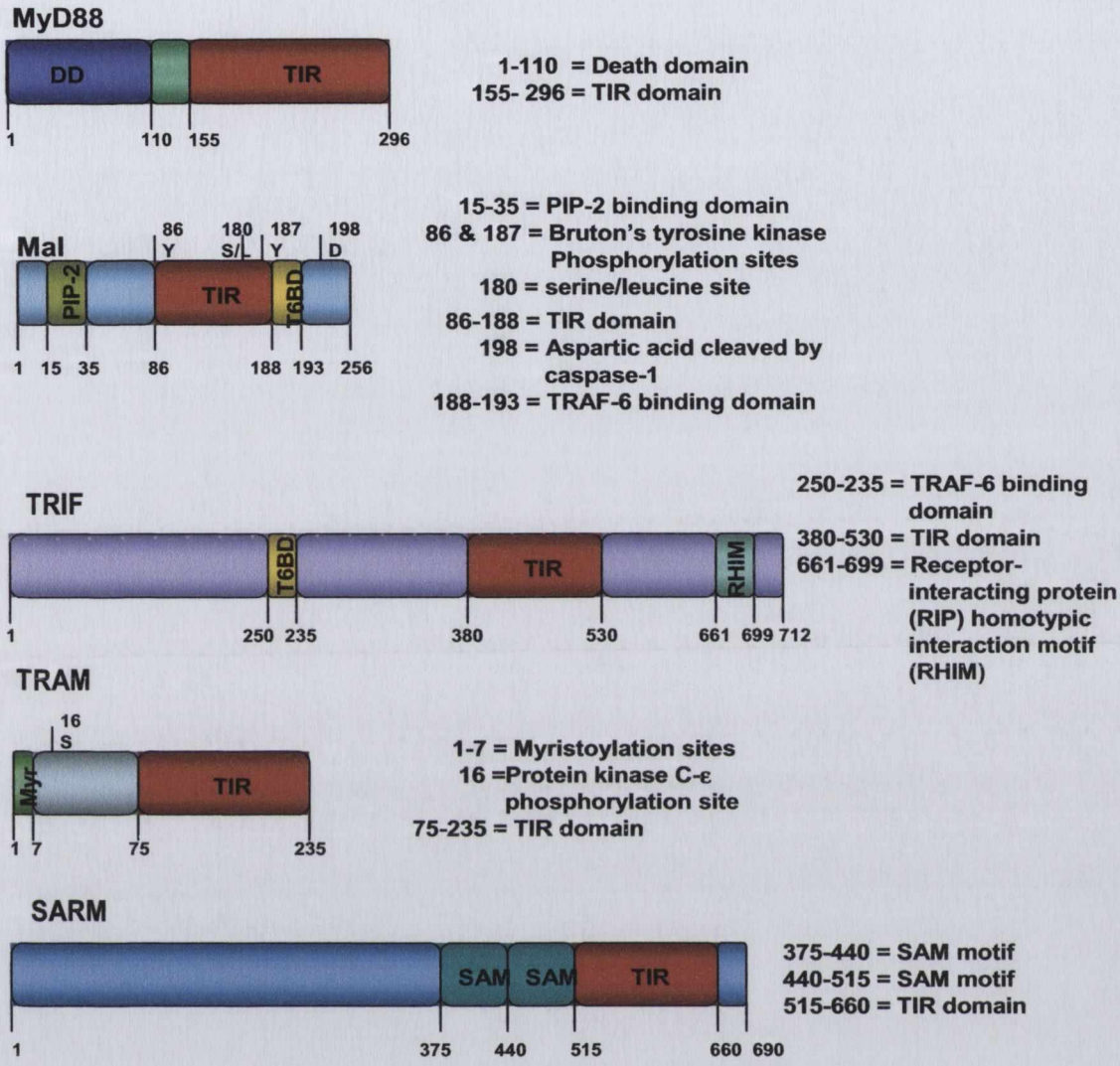

Figure 1.1. **The TIR domain containing adaptor proteins**

The regions important for the activity of the five TIR domain containing proteins are shown above. MyD88 has a C-terminal death domain (DD) and the N-terminal TIR domain. Mal has a PIP2 binding domain, two BTK phosphorylation sites, the serine to leucine SNP, a TIR domain, a Caspase1 cleavage site and a putative TRAF6 binding domain. TRIF contains a putative TRAF6 binding domain, a TIR domain and a RHIM region. TRAM contains a myristoylation site, a PKCε phosphorylation site at serine 16 and the TIR domain. SARM consists of two Sam motifs and the TIR domain.

1.8 Signalling pathways initiated by the TLRs

Once the adaptor proteins bind the activated TLRs via TIR-TIR interactions they utilise their other functional domains to activate the two common signalling pathways induced by TLR activation – the MyD88-dependent and the TRIF-dependent signalling pathways.

1.8.1 The MyD88-dependent signalling pathway

When the TLRs have been activated through the binding of a ligand they undergo conformational changes to allow the recruitment of MyD88 to their TIR domains (except in TLR3 signalling in which TRIF is recruited). This in turn leads to the recruitment of IL1-receptor associated kinase 4 (IRAK4) which upon binding to MyD88 recruits IRAK1 and IRAK2. The IRAKs are then activated by either autophosphorylation or by phosphorylating each other. The recruitment of the IRAKs to MyD88 via a death domain homotypic interaction results in the translocation of the MyD88 signalling complex from the TLR at the membrane to the cytosol. The phosphorylation of the IRAKs leads to the recruitment of tumour necrosis factor (TNF)-receptor associated factor 6 (TRAF6). The ubiquitinating factors UEV1A (ubiquitin-conjugating enzyme E2 variant 1) and UBC13 (ubiquitin-conjugating enzyme 13) are recruited to TRAF6 in order to ubiquitinate it. The K63-linked poly-ubiquitination of TRAF6 is crucial for the continuation of the MyD88-dependent signalling cascade. Once TRAF6 has been poly-ubiquitinated it recruits transforming growth factor β-activated kinase (TAK1) and TAK binding proteins 1, 2 and 3 (TAB1, 2

and 3) to its ubiquitin chains through a direct interaction with TAB2. TAK1 autophosphorylation occurs with the aid of its adaptor proteins TAB1, TAB2 and TAB3. The phosphorylation of TAK1 allows it to phosphorylate the NFκB essential modulator (NEMO) complex (a trimer of IκB kinase (IKK)γ, IKKα and IKKβ). IKKγ is also targeted for ubiquitination by the TRAF6 and TAK1 complex. The phosphorylation of IKKα and IKKβ allow them in turn to phosphorylate inhibitor of NFκB-α (IκB-α) and target it for ubiquitination and proteasomal degradation. The degradation of IκB-α allows NFκB translocation to the nucleus where it can initiate the synthesis of pro-inflammatory cytokines (Kawai and Akira, 2006; Krishnan et al., 2007).

The activation of TAK1 through autophosphorylation also results in it phosphorylating MAP kinase kinases such as MKK3, 4, 6 and 7. These MAP kinase kinases then in turn phosphorylate their target kinases which include p38 which is phosphorylated by MKK3 and 6 and JNK which is activated by MKK4 and 7. The phosphorylation of these kinases allows them to continue the phosphorylation cascade and activate transcription factors such as activator protein 1 (AP1) (O'Neill, 2006).

The end products of MyD88 recruitment are the activation of NFκB, AP1 and p38 and JNK MAP kinases. These transcription factors and MAP kinases ensure the cell produces an adequate cytokine response to pathogen attack by producing pro-inflammatory cytokines such as IL6 and TNF-α (O'Neill, 2003). The MyD88-dependent pathway is outlined in Figure 1.2.

The MyD88-dependent pathway can also lead to the activation of the type 1 interferons. This is thought to only occur when the endosomal TLRs that use MyD88 to signal, such as TLR7, TLR8 and TLR9 are activated. A complex containing MyD88,

IRAK1, IRAK4, TRAF6 and IRF7 has been seen where IRAK1 phosphorylates IRF7 and IFNα is produced. MyD88 is also involved in IRF1 and IRF5 activation leading to IFNβ production (O'Neill and Bowie, 2007). The MyD88-dependent signalling pathway leading to interferon production is outlined in Figure 1.3.

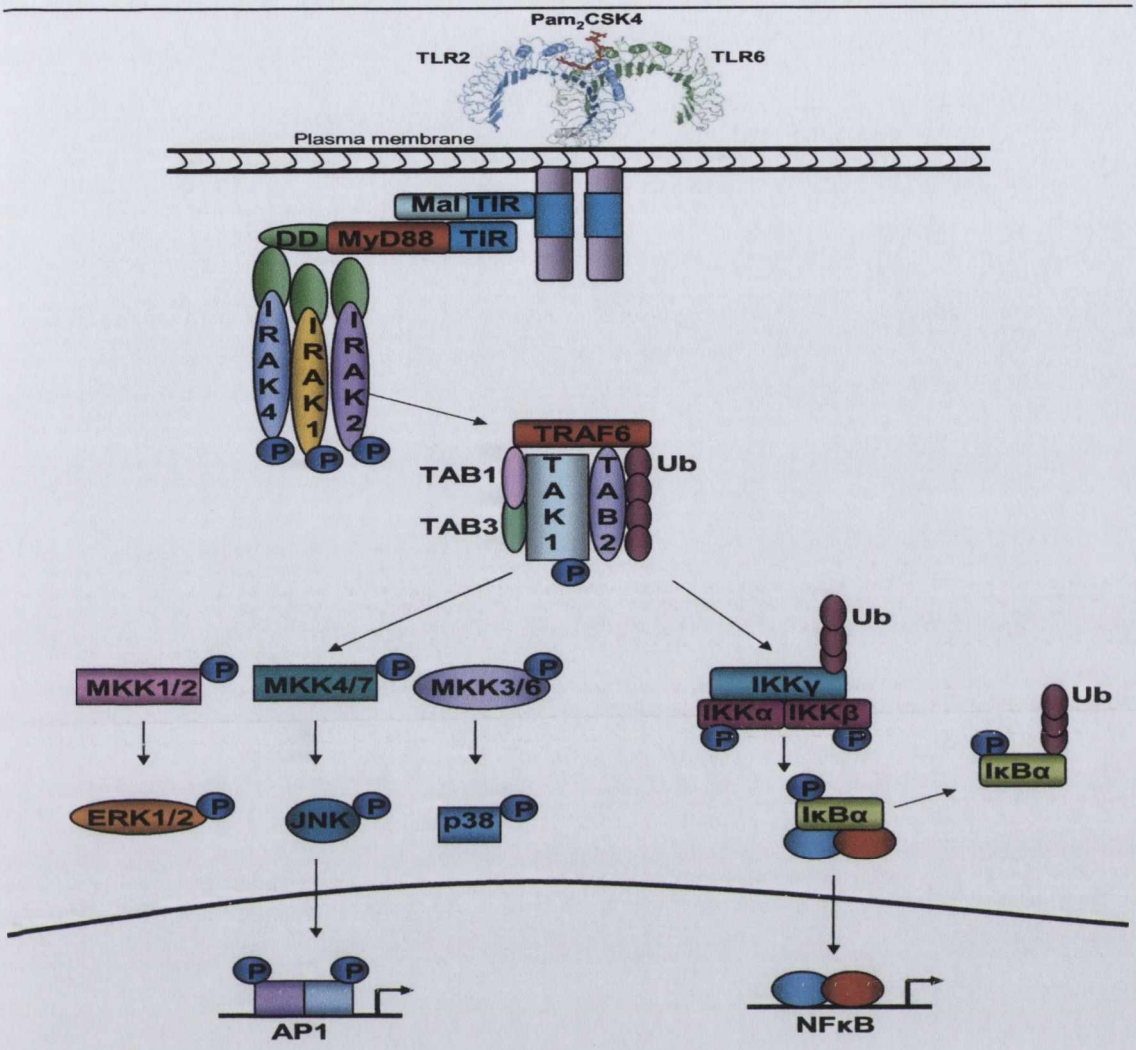

Figure 1.2. **The MyD88-dependent signalling pathway**

Upon ligand recognition the TLRs interact with MyD88 via a TIR homotypic interaction. IRAK1, 2 and 4 are recruited to MyD88 via a death domain interaction and become phosphorylated. They in turn recruit TRAF6 which becomes K63-linked poly-ubiquitinated. TAB2 is recruited to TRAF6 and it recruits TAK1, TAB1, and TAB3. Phosphorylation of TAK1 leads to the phosphorylation of MKK1, 2, 3, 4, 6 and 7. These MAP2 kinases then phosphorylate their targets such as ERK1/2 JNK and p38 resulting in translocation of AP1 into the nucleus. TAK1 also targets IKKγ, IKKα and IKKβ for ubiquitination or phosphorylation. The phosphorylation of IKKα and IKKβ allow then to target IκBα for degradation through phosphorylation and ubiquitination allowing NFκB to translocate to the nucleus.

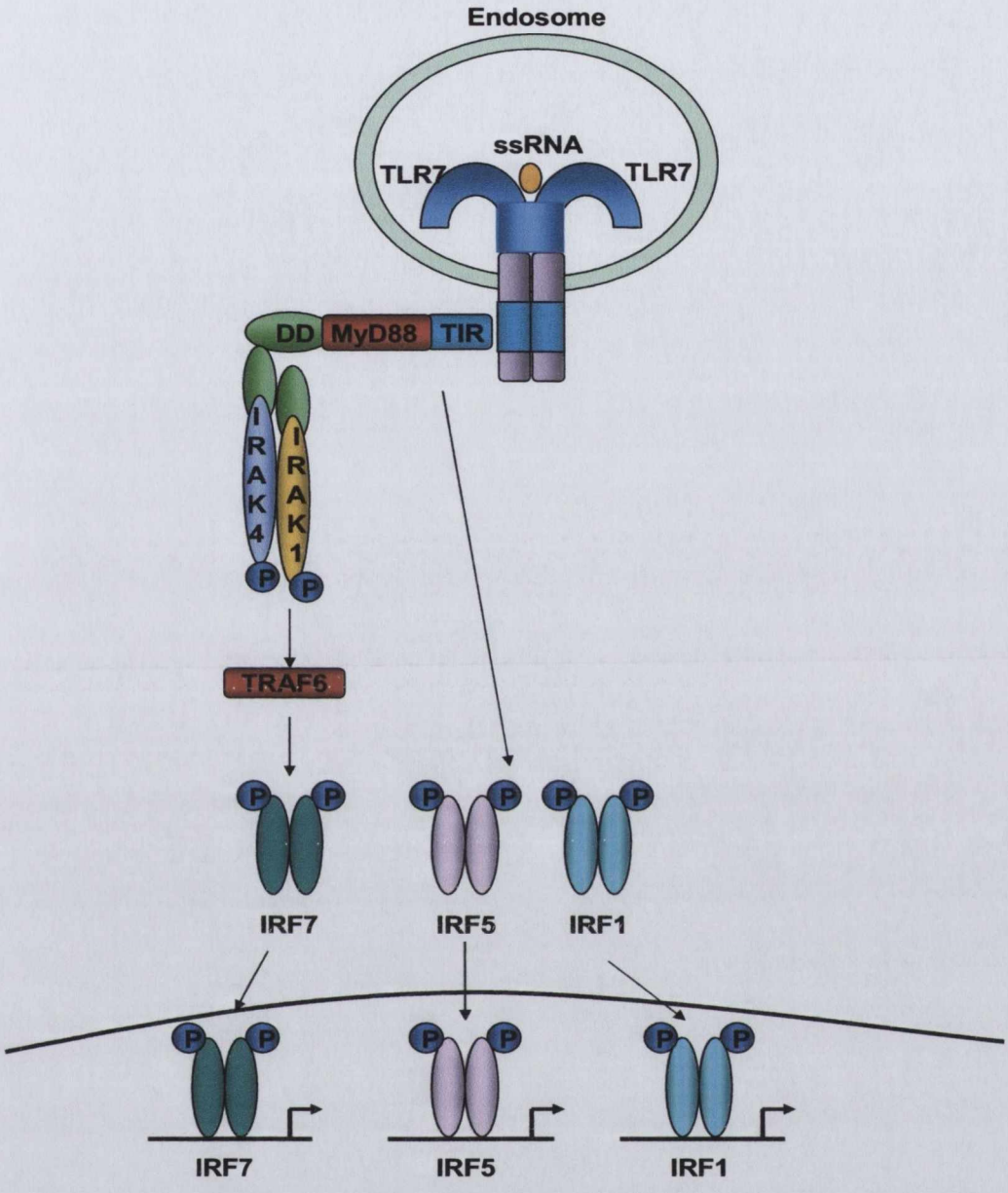

Figure 1.3. **The MyD88-dependent signalling pathway also leads to interferon production**

Upon ligand recognition the endosomal TLRs interact with MyD88 via a TIR homotypic interaction. IRAK4 and IRAK1 are recruited to MyD88 via a death domain interaction and become phosphorylated. They in turn recruit TRAF6 which becomes K63-linked poly-ubiquitinated. This allows the phosphorylation of IRF7 and induction of IFNα. The interaction between the endosomal TLRs and MyD88 also leads to phosphorylation of IRF5 and IRF1 resulting in INFβ production.

1.8.2 The TRIF-dependent signalling pathway

TRIF can utilise its ability to bind directly to TRAF6 via its TRAF6 binding domain to activate the MyD88-dependent signalling pathway as outlined above. TRAF family member associated NFκB activator (TANK) binding kinase 1 (TBK1) and IKKε are used by TRIF to activate the IRF3 and IRF7 pathways by phosphorylation with the help of NFκB activating kinase (NAK)-associated protein 1 (NAP1) and TRAF3 both of which interact with TRIF.

At its C-terminus TRIF has the TIR domain and a receptor-interacting protein (RIP) homotypic interaction motif (RHIM) domain. The RHIM domain functions to bind RIP1 and 3 which activate and inhibit TLR3 signalling to NFκB respectively (Kawai and Akira, 2006). The final pathway activated by TRIF leads to apoptosis and is thought to involve RIP1, FAS-associated death domain (FADD) and Caspase8 and is MyD88-independent (Kaiser and Offermann, 2005). The TRIF-dependent signalling pathway is outlined in Figure 1.4.

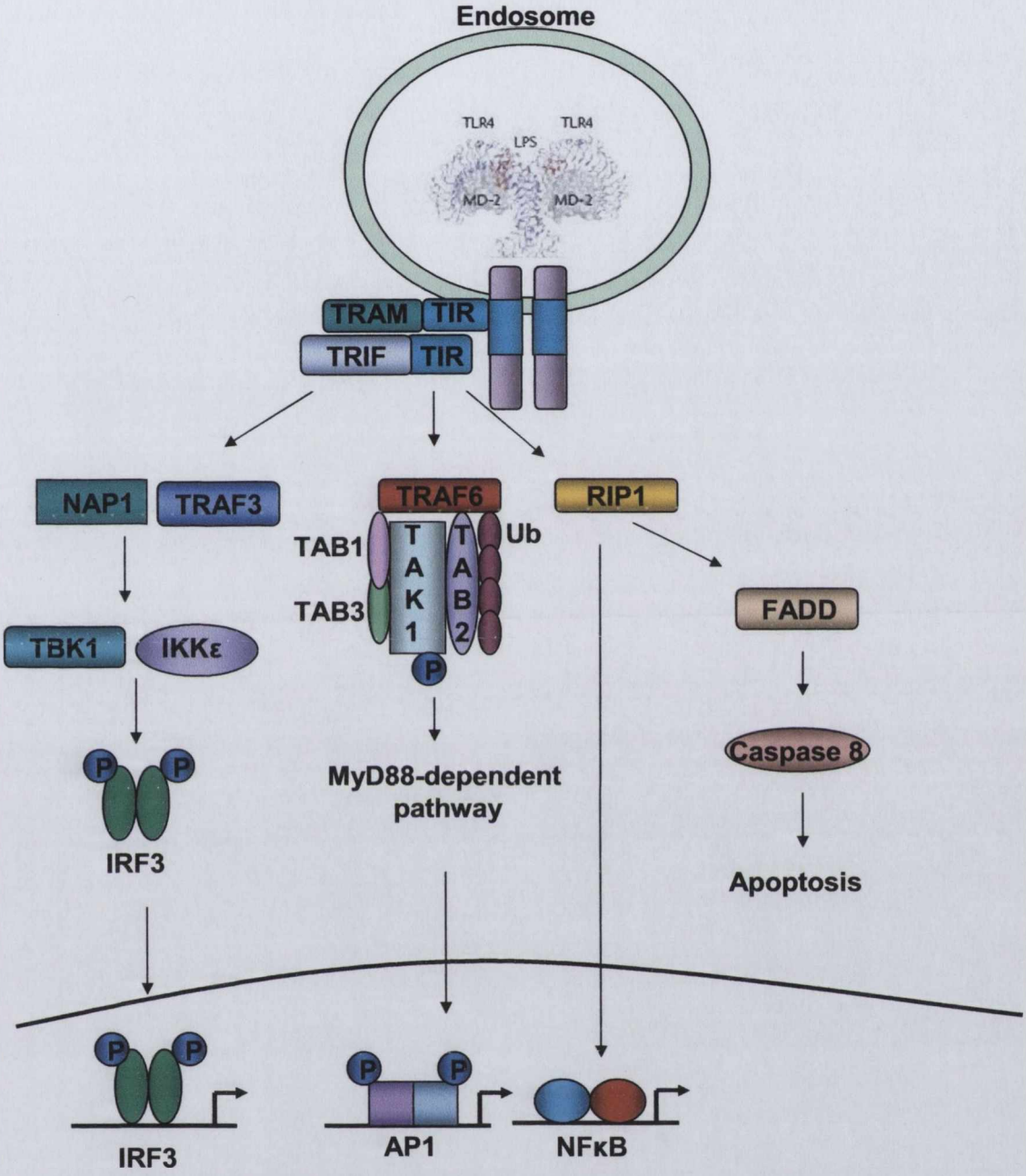

Figure 1.4. **The TRIF-dependent signalling pathway**

Upon ligand recognition TLR4 interacts with TRAM via a TIR homotypic interaction. TRAM recruits TRIF to the TLR complex and signal transduction is initiated. TRIF can recruit TRAF6 to activate the MyD88-dependent pathway. The interaction of TRIF and RIP1 also activates NFκB. TRIF can also recruit NAP1 and TRAF3 to allow phosphorylation of IRF3 through TBK1 and IKKε. TRIF can also recruit FADD through its interaction with RIP1 resulting in apoptosis due to the activation of Caspase8.

1.8.3 Regulation of the MyD88- and TRIF-dependent signalling pathways

Regulation of the MyD88- and TRIF-dependent signal cascades is crucial to ensure that there is no over-activation of inflammation as this can be detrimental to the host. As such there have been many negative regulators of TLR signalling discovered to date. One method to down regulate TLR signalling is to target crucial proteins for degradation. Triad3A is an E3 ubiquitin protein ligase that targets TLR3, 4, 5 and 9 but not TLR2 for degradation (Chuang and Ulevitch, 2004). Another example of ubiquitination as a method of control was outlined above when Mal is targeted for poly-ubiquitination by SOCS1 (Mansell et al., 2006).

Ubiquitination not only serves to target proteins for degradation in TLR signalling as several proteins require K63-linked poly-ubiquitination to become activated as outlined above for TRAF6. Hence, de-ubiquitinating enzymes serve as good negative regulators of TLR signalling. An example of one such protein is A20 which removes K63-linked ubiquitin from RIP1 and replaces it with K48-linked ubiquitin chains thus targeting RIP1 for degradation (Wertz et al., 2004). A20 also targets TRAF6 for de-ubiquitination (Boone et al., 2004). A second example of a de-ubiquitinating enzyme is de-ubiquitinating enzyme A (DUBA). DUBA targets TRAF3 and removes its crucial K63-linked poly-ubiquitin chains rendering TRAF3 inactive (Kayagaki et al., 2007).

A recently discovered group of RNA termed micro RNA are also crucial to the negative regulation of TLR signalling. Examples of this include miR-146 which targets IRAK1 and TRAF6 (Taganov et al., 2006) and miR-21 which negatively regulates TLR4 by targeting PDCD4 and promoting IL10 production (Sheedy et al., 2010). Thus, the micro

RNAs represent ideal candidates for further investigations into negative regulation of TLR signalling.

A splice variant of MyD88 known as MyD88s that does not contain the intermediate domain of MyD88 also functions as a negative regulator of TLR signalling by blocking the interactions between MyD88 and IRAK4. It is induced in response to LPS stimulation and as such activates a negative feedback loop in TLR4 signalling (Burns et al., 2003). SARM and TAG also negatively regulate TLR signalling as outlined above.

There are also accessory proteins involved in TLR signalling that aid in mounting a successful TLR response in the presence of a pathogen. The pellinos are a family of E3 ubiquitin ligases that interact with TRAF6 and TAK1 to aid in signal transduction. They are also the proposed ubiquitin ligases required for the K63-linked poly-ubiquitination of IRAK1 (Moynagh, 2009). The ubiquitination of IRAK1 has been linked with the activation of NFκB (Windheim et al., 2008).

DEAD/H Box 3 or DDX3 is a RNA helicase protein shown to be involved in the TBK1/IKKε-dependent up-regulation of the *IFNb* promoter in response to PolyIC or viral treatment of cells. It therefore ensures that sufficient levels of IFNβ are produced in the TLR signalling cascade. It was discovered as a protein inhibited by the K7 protein of vaccinia virus (Schroder et al., 2008). It is targeted for inhibition by many viruses allowing them to evade the immune system which demonstrates its crucial role in successful TLR signal transduction.

1.9 TLR2, TLR3 and TLR4

The main focus of this project is the role MyD88 and Mal play in TLR2, TLR3 and TLR4 signalling. These TLRs have unique as well as shared features.

1.9.1 TLR2

It is known that TLR2 is vital to the recognition of many molecules including peptidoglycan (PGN), glycosylphosphatidylinositol (GPI) anchors, lipoteichoic acid (LTA) and lipopeptides (Lien et al., 1999). Upon ligand binding, TLR2 uses Mal to bridge to MyD88 to activate the MyD88-dependent signalling pathway.

1.9.1.1 Lipopeptides

Bacterial lipopeptides are found on the cell walls of many micro-organisms. They are anchored to the cell wall by conserved N-terminal lipid chains. These lipids contain di-0-acylated S-(2,3-dihydroxpropyl)-cysteinyl residues at the N-terminus which are coupled to polypeptides. These S-(2,3-dihydroxpropyl)-cysteine residues can also be acylated with a third amine-linked fatty acid. In order to do this a diacylglycerol group is added to the sulfhydryl group of the cysteine, the cleavage sequence is removed and the acyl group is added to the free N-terminal group of the cysteine. The di-acylated glycerol groups are attached to the cysteine via a thioester bond and the tri-acylated glycerol is attached via an amide bond (Chambaud et al., 1999). The most common forms of lipid chains found in

these lipopeptides are palmitoyl groups. Many bacteria do not possess the enzyme necessary to convert the lipopeptide from di-acyl to tri-acyl and hence both forms exist. Therefore there are two types of lipopeptide that TLR2 must be able to recognise and bind.

In order to bind the wide range of lipopeptides found on pathogens TLR2 dimerises with either TLR1 or TLR6 to recognise tri- and di-acylated lipopeptides respectively. This discovery was made through the generation of TLR1-, TLR2- and TLR6-deficient mice (Takeuchi et al., 2001; Takeuchi et al., 2002).

1.9.1.2 The TLR1/2 signalling complex

The TLR1/2 complex binds tri-acylated lipopeptides such as the synthetic ligand Pam_3CSK4 (Omueti et al., 2005). This synthetic ligand retains most of the ability to stimulate the TLR1/2 complex when compared to full-length lipopeptides. Recently the crystal structure of the interface between TLR1, TLR2 and Pam_3CSK4 has been solved (Jin et al., 2007) and is shown in Figure 1.5. In order to achieve this, the extracellular domains of human TLR1 and TLR2 were fused to the hagfish variable lymphocyte receptors (VLRs). The VLRs were chosen as they contained conserved LxxLxLxxN motifs and therefore allowed minimal structural incompatibility.

The extracellular domains of TLR1 and TLR2 are horseshoe shaped and can be split into three distinct regions – the N-, central and the C-terminal subdomains as shown in Figure 1.5A. The binding of Pam_3CSK4 to the TLR1/2 heterodimer results in a change in their conformation by inducing the formation of an "m" shaped heterodimer. Two of the lipid chains on Pam_3CSK4 are embedded in a pocket at the border of the central and C-

terminal domains of TLR2 and the amide-linked lipid chain is found in the same region of TLR1 as shown in Figure 1.5B. Both pockets are lined with hydrophobic residues from LRRs 9-12 but the TLR1 pocket is only a quarter the size of the TLR2 pocket. The binding of Pam_3CSK4 to TLR2 takes up 90% of the space in the TLR2 internal pocket suggesting room is left to allow for the binding of different ligands by TLR2.

TLR1 and TLR2 also interact with each other near the ligand binding regions therefore ensuring more stabilisation of the heterodimer. The regions involved in the interaction between TLR1 and TLR2 are hydrophobic and hence aid in the interaction between the two proteins. It has been previously shown that TLRs dimerise in the absence of ligand as was seen for TLR1/2, TLR2/6 and TLR9 (Latz et al., 2007; Triantafilou et al., 2006). This suggests that the binding of ligand is solely required to aid intracellular activation by interactions between the TIR domain of the TLRs and the TIR domain containing proteins.

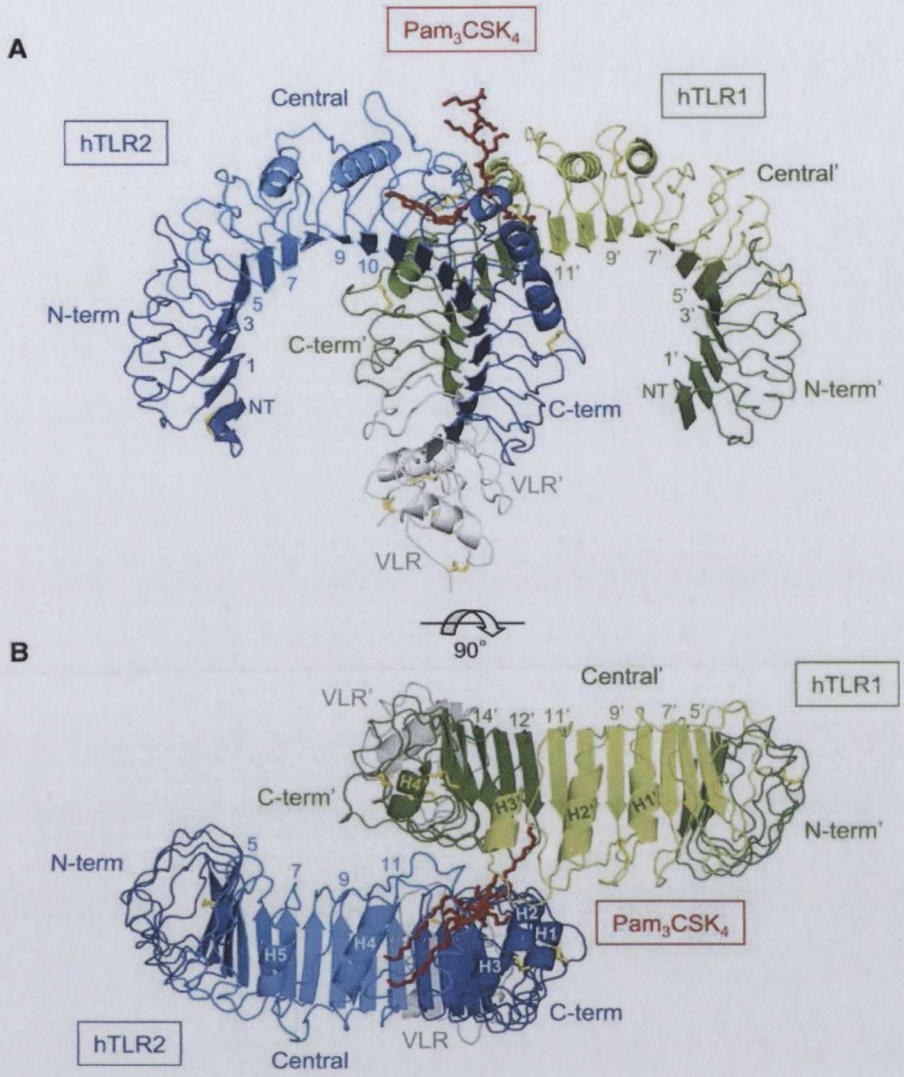

Figure 1.5. **Crystal structure of the TLR1/2 signalling complex**

The horseshoe shaped extracellular domains of TLR1 and TLR2 are shown with both containing three distinct regions; the N-terminal and C-terminal (in dark green on TLR1 and dark blue on TLR2) and the central region (light green for TLR1 and light blue for TLR2) (A). The ligand Pam_3CSK4 is shown in red and its interaction with both TLR1 and TLR2 occurs at the interface between the central and C-terminal regions. One lipid chain of Pam_3CSK4 is located in a pocket in TLR1 and two lipid chains in a pocket in TLR2 (B). Taken from (Jin et al., 2007).

1.9.1.3 The TLR2/6 signalling complex

The TLR2/6 complex binds di-acylated lipopeptides such as macrophage-activating lipopeptide 2kD (Malp-2) from *Mycoplasma fermentans* (Omueti et al., 2005). It also interacts with the synthetic di-acylated ligand Pam_2CSK4. The crystal structure of this interface has also recently been solved. The analysis was carried out similarly to the TLR1/2 study with the fusion of the extracellular domains of mouse TLR2 and TLR6 to the hagfish variable lymphocyte receptor B (VLRB) (Kang et al., 2009). As was seen with the crystal structure of TLR1 and TLR2 there was the formation of a horseshoe shape for TLR2 and TLR6 with each TLR containing 20 leucine rich repeats (LRRs). Again three distinct regions of the extracellular domains were identified with both TLRs containing N-terminal and C-terminal domains separated by a central domain as shown in Figure 1.6A and B. The interaction of TLR2 and TLR6 with Pam_2CSK4 resulted in the formation of the "m" shaped structure seen for TLR1/2 signalling and is again thought to alter the conformation of the intracellular domains to allow signal transduction.

During the examination of these regions it was discovered that the N-terminal of both TLRs was similar to that seen for the TLR1/TLR2 complex. The ligand binding pocket of TLR2 was also very similar to that seen in the TLR1/TLR2 complex with variations seen mainly due to species differences therefore suggesting that the interaction of TLR2 with the lipopeptides is the main driving force of TLR1/2 and TLR2/6 signalling. However, the central and C-terminal domains of TLR6 and interactions between TLR2 and TLR6 were very different that those seen for the TLR1/2 complex.

TLR6, which shares 56% amino acid identity with TLR1 and is overall very structurally similar to TLR1, showed a marked difference in its folding pattern at the ligand binding site and the region involved in its interaction with TLR2. The presence of two phenylalanines at positions 343 and 365 in LRR11 of TLR6 were found to block the pocket opening of TLR6 therefore preventing it from binding acyl chains of lipopeptides. Mutation of these phenylalanines to a methionine and a leucine respectively resulted in the ability of the TLR2-TLR6 complex to respond to tri-acylated lipopeptides. TLR6 also contains a greater amount of hydrophobic residues in the region important for its interaction with TLR2 resulting in an 80% increase in the dimerisation interface. When TLR1 and TLR2 bind Pam_3CSK4 it is the presence of three acyl groups that allows the conformational changes between TLR1 and TLR2 and gives the heterodimer stability. As TLR6 has limited contact with Pam_2CSK4 it must form a larger interaction surface with TLR2 to ensure the heterodimer is stable and can initiate intracellular signal transduction.

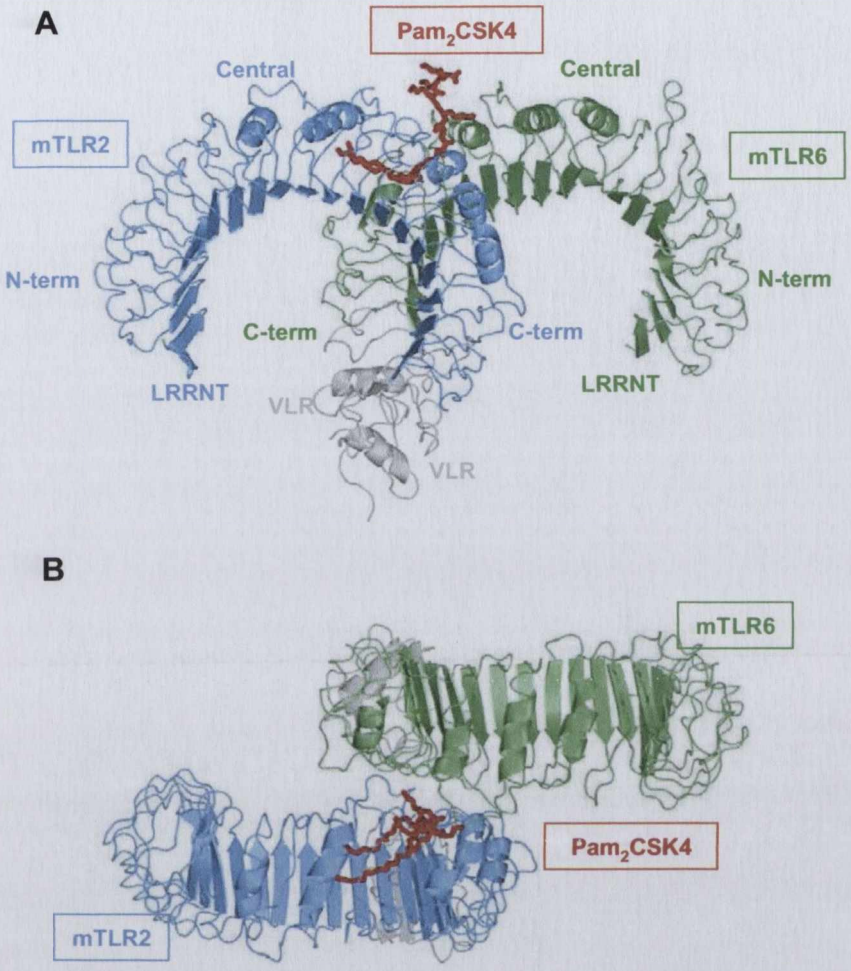

Figure 1.6. **Crystal structure of the TLR2/6 signalling complex**

The horseshoe shaped extracellular domains of TLR2 and TLR6 are shown with both containing three distinct regions; the N-terminal, C-terminal and the central region (blue for TLR2 and green for TLR6) (A). The ligand Pam_2CSK4 is shown in red and its interaction with both TLR2 and TLR6 occurs at the interface between the central and C-terminal regions. Both lipid chains of Pam_2CSK4 are located in a pocket in TLR2 with very little interaction seen between TLR6 and Pam_2CSK4 (B). A greater surface area of TLR2 and TLR6 interact in comparison to that seen for TLR1 and TLR2. Taken from (Kang et al., 2009).

1.9.1.4 The role of MyD88 and Mal in TLR2 signalling

As outlined above TLR2 requires MyD88 to signal. This was confirmed though the generation of MyD88-deficient mice and treating cells derived from them with TLR1/2 and TLR2/6 ligands. A total dependence on MyD88 for a TLR2 response has been demonstrated (Takeuchi et al., 2000a; Underhill et al., 1999b).

The generation of Mal-deficient mice led to the discovery that it was also required for TLR2 signalling. In these studies the effects of TLR2 ligand stimulation on Mal-deficient cells were only examined at single concentration and it was concluded that Mal was absolutely required for TLR2 and TLR4 signalling to NFκB (Horng et al., 2002).

Modelling studies have postulated that the TIR domains of MyD88, TLR2 and TLR4 are largely electro-positive whereas the TIR domain of Mal is largely electro-negative (Dunne et al., 2003). A second study revealed that TLR4 localises to areas on the plasma membrane rich in phosphatidylinositol 4,5-bisphosphate (PIP2) and that Mal contains a PIP2 binding domain that allows it to bring MyD88 to the plasma membrane (Kagan and Medzhitov, 2006). From these studies a bridging role for Mal on the MyD88-dependent signalling pathway was theorised with Mal recruiting MyD88 to TLR2 and TLR4.

Once this bridging role for Mal had been established the focus turned more to the functional regions important for signalling and the polymorphisms associated with disease as outlined in section 1.7.2.

1.9.2 TLR3

TLR3 is one of the four TLRs found on the endosomal membrane and is involved in the recognition of viral pathogens. Through the sequential transfection of TLR1 through to TLR6 into 293T cells and stimulation with PolyIC, a synthetic dsRNA molecule, TLR3 was identified as the receptor for dsRNA. The generation of TLR3-deficient mice further confirmed this as they were incapable of mounting an immune response upon PolyIC stimulation resulting in decreased NFκB activation and type I IFN production (Alexopoulou et al., 2001).

1.9.2.1 TLR3 signalling

Polyriboinosinic polyribocytidylic acid (PolyIC) is a synthetic dsRNA ligand and the main ligand used to examine TLR3 signal transduction. TLR3 is also activated in response to viral stimulation and this has been shown for Influenza A Virus and West Nile virus (Le Goffic et al., 2007; Wang et al., 2004). Viral detection by the innate immune system, however, seems to utilize both TLR3 and the RLRs to coordinate the appropriate response (Schroder and Bowie, 2005).

1.9.2.2 The TLR3 signalling complex

TLR3 homodimerises to bind dsRNA and the crystal structure of the TLR3/dsRNA interface has been elucidated (Botos et al., 2009; Liu et al., 2008). The TLR3 monomers

are the usual horseshoe shape seen for TLR1, TLR2 and TLR6 and contain 23 LRRs. The dimerised TLR3 can only bind dsRNA that is between 40 and 50 base pairs in length and this can only occur in an acidic environment (pH 6.5 and below) indicating that the location of TLR3 is in the endosome.

The dsRNA binds at two sites on the convex surface of TLR3, there is an N-terminal binding site involving the leucine rich repeat N-terminal (LRRNT) and LRRs1-3 and a C-terminal binding site involving LRRs 19-21. The presence of positively charged residues in these areas aids the binding of the sugar-phosphate backbones of the dsRNA. The fact that TLR3 does not bind directly to any individual bases but the sugar-phosphate backbone of the RNA reveals the mechanism by which TLR3 can non-specifically recognise dsRNA from many viruses. The crystal structure is shown in Figure 1.7A and an aerial view is shown in Figure 1.7B.

The TLR3 homodimers only interact at a small portion of their LRR C-terminals (LRRCT). The bound dsRNA does not change the overall shape of the TLR3 proteins again suggesting that ligand binding is central to allowing TIR-TIR interactions with the adaptor proteins rather than altering the N-terminal domains of the activated TLRs. Unlike the TLR1/2 interaction with Pam_3CSK4 and the TLR2/6 interaction with Pam_2CSK4 which utilise the presence of hydrophobic pockets, the bonds between TLR3 and dsRNA are mainly on the surface of TLR3 with hydrogen and ionic bonding playing a central role.

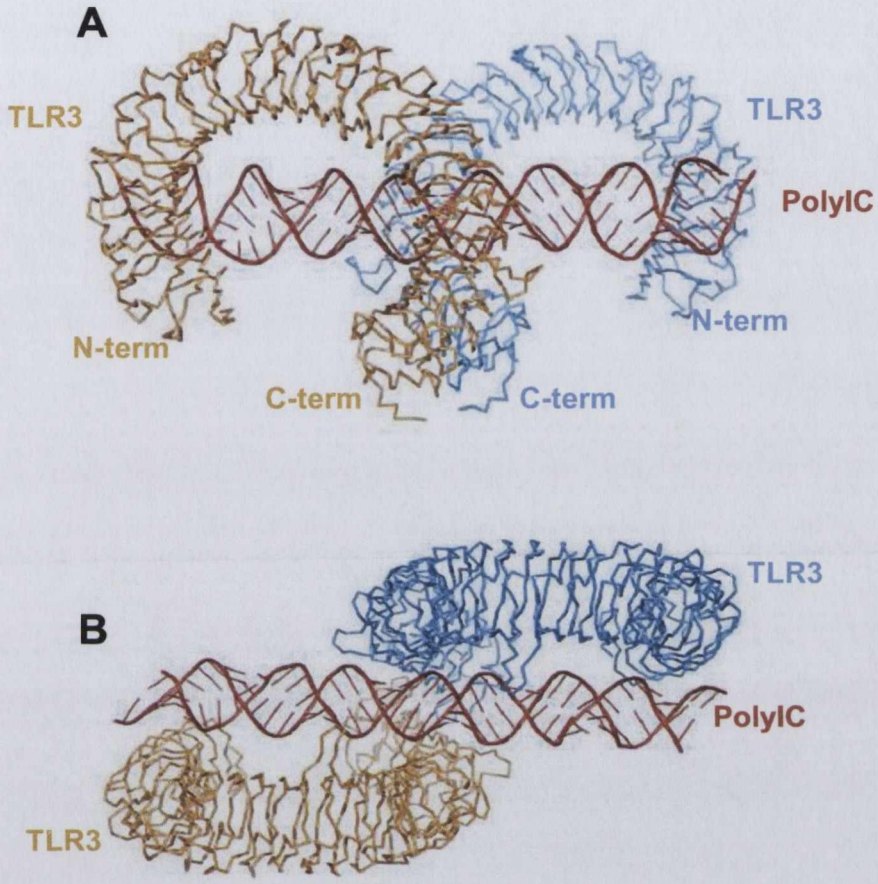

Figure 1.7. **The crystal structure of the TLR3 signalling complex**

The two TLR3 molecules (shown in orange and blue) dimerise upon binding of PolyIC (shown in red) and this allows the conformational changes in their TIR domains to allow the recruitment of TRIF. At the C-terminal of the TLR3 molecules there is a region of interaction between the two. The TLR3 molecules interact with PolyIC at their N- and C-terminals (A and B). Adapted from (Botos et al., 2009)

1.9.2.3 The role of MyD88 and Mal in TLR3 signalling

TLR3 is the only TLR discovered to date that does not use MyD88 to signal and as such is the sole TLR that utilises TRIF directly (Oshiumi et al., 2003a). This was verified through the generation of TRIF-deficient mice (Yamamoto et al., 2003a). In this study it was revealed that both TLR3 and TLR4 also use TRIF to signal but it has since been shown that TLR4 requires the presence of TRAM to bridge to TRIF (Fitzgerald et al., 2003; Oshiumi et al., 2003b).

A recent study demonstrated an inhibitory role for MyD88 in TLR3 signalling. In cells lacking MyD88 there was enhanced production of RANTES and phosphorylation of c-jun N-terminal kinase (JNK) in response to TLR3-induced PolyIC stimulation (Johnson et al., 2008). To date no evidence linking Mal to the inhibition of TLR3 signalling has been shown.

1.9.3 TLR4

TLR4 was the first mammalian TLR discovered due to it similarity to the *toll* gene in *Drosophila melanogaster*. It is the receptor responsible for binding lipopolysaccharide (LPS) found on gram-negative bacteria (Takeuchi et al., 1999a). It signals at the plasma membrane and from within endosomes. It is the only TLR to date that utilises all four activating adaptor proteins.

1.9.3.1 TLR4 signalling

TLR4 uses both the MyD88- and TRIF-dependent signalling pathways in response to LPS as it can interact with both Mal and TRAM, the bridging adaptors for MyD88 and TRIF (Fitzgerald et al., 2001; Oshiumi et al., 2003b). A complex mechanism exists using several proteins to deliver LPS to TLR4 that is initiated by the binding of LPS to lipopolysaccharide binding protein (LBP). LBP then transfers the LPS onto CD14. CD14 is found in two forms; a GPI-anchored form and a soluble form. It was initially thought to be the receptor for LPS but it has no transmembrane and cytoplasmic domain and as such functions to transfer the LPS to MD2. MD2 is vital to allow LPS induced TLR4 signal transduction and is found in the TLR4-LPS complex (Fitzgerald et al., 2004).

1.9.3.2 The TLR4 signalling complex

The crystal structure of the TLR4, LPS and MD2 complex has recently been solved (Park et al., 2009). TLR4 was shown to be horseshoe shaped as has been seen for other TLRs. Prior to LPS binding TLR4 and MD2 do interact but the presence of LPS is required to allow dimerisation of TLR4 and in the complex there are two TLR4 and two MD2 molecules bound to two LPS molecules. The binding of LPS results in the formation of the "m" shaped multi-protein complex seen for TLR1/2, TLR2/6 and TLR3 signalling. The TLR4 molecules can again be divided into three regions; the N- and C-terminal and the central domains. Thus, it seems all the TLRs examined adopt a similar structure and that this structure is not greatly altered by the binding of a ligand.

MD2 contains a hydrophobic pocket that allows it to interact with 5 of the 6 lipid chains found in LPS. The binding of LPS to MD2 leads to a conformational change in MD2 which further stabilises its interaction with TLR4 through hydrophilic interactions. The phenylalanine (F) 126 loop found in MD2 is crucial to allow dimerisation of the TLR4 and MD2 complexes through hydrophilic interactions. Mutation of this residue in MD2 results in no dimerisation of the TLR4-MD2 monomers. The crystal structure of TLR4, MD2 and the TLR4 antagonist Eritoran has also been solved. In this complex the F126 loop of MD2 was exposed and in this structure no dimerisation occurred and therefore no downstream signal activation was seen. This may explain the mechanism behind the antagonistic nature of Eritoran (Kim et al., 2007a).

The sixth lipid chain of LPS is found on the surface of MD2 and it is required to directly bind the LPS to TLR4. This binding occurs through hydrophobic interactions between the lipid chain and phenylalanines on the surface of TLR4. Hydrophilic interactions also occur between TLR4 and LPS. The two TLR4 molecules also homodimerise at the C-terminal regions between LRRs 15 and 17 and this interaction is vital for down stream signal activation. The crystal structure of the TLR4-MD2 and LPS dimer is shown in Figure 1.8A and an overhead view is shown in Figure 1.8B.

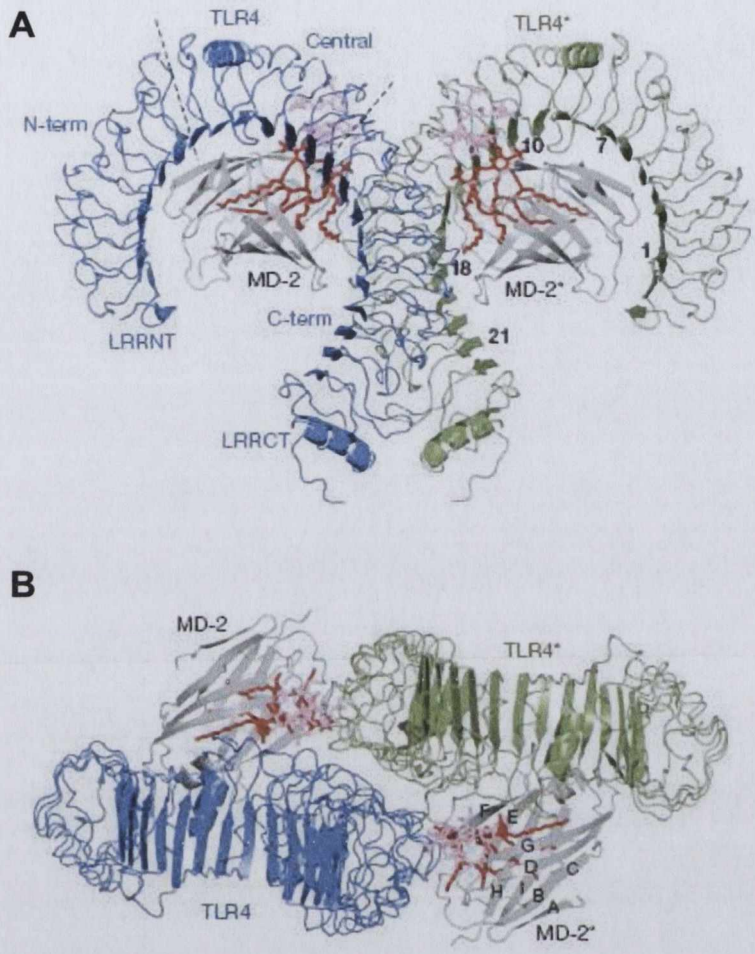

Figure 1.8. **The crystal structure of the TLR4 signalling complex**

TLR4 and MD2 can interact but upon LPS binding they dimerise with a second set of TLR4 and MD2. The TLR4 molecules are shown in blue and green, the MD2 is grey and the LPS is red (A and B). LPS interacts with both MD2 and TLR4. The binding of LPS allows for interaction between the two TLR4 molecules at the C-terminal LRRs. Taken from (Park et al., 2009).

1.9.3.3 The role of MyD88 and Mal in TLR4 signalling

MyD88 is crucial to TLR4 signalling to NFκB and uses Mal to bridge to TLR4 as Mal contains a PIP2-binding domain to bring MyD88 to the plasma membrane. MyD88- or Mal-deficient mice are completely incapable of producing cytokines upon LPS stimulation confirming the role of both in TLR4 signalling (Fitzgerald et al., 2001; Takeuchi et al., 2000b).

1.10 Project aims and objectives

The role of MyD88 and Mal in TLR1/2, TLR2/6, TLR3 and TLR4 signalling has not been thoroughly examined. There is much known about the ligands that activate each pathway but the exact interactions between these TLRs and MyD88 and Mal has not been clarified. It is suggested that MyD88 can interact with TLR1 and TLR6 and that Mal only interacts with TLR2 and TLR4 but this has not been confirmed experimentally. The potential inhibitory role of Mal in TLR3 signalling and the mechanism behind this and the inhibitory role of MyD88 have also not been investigated.

This project, therefore, attempted to clarify the roles played by MyD88 and Mal in TLR2, TLR3 and TLR4 signalling.

1.10.1 Aims

The aims of this project were to:

1. Investigate if MyD88 can directly interact with TLR1, TLR2, TLR4 and TLR6
2. Investigate if Mal can directly interact with TLR1, TLR2, TLR4 and TLR6
3. Confirm that MyD88 is crucial for all TLR2 and TLR4 signal transduction
4. Clarify the role of Mal in the TLR2 and TLR4 signalling pathways
5. Determine if Mal is an inhibitor of TLR3 signalling in a manner similar to that of MyD88
6. Decipher the mechanism of MyD88 and/or Mal inhibition of TLR3 signalling

Overall this project aims to shed new light on the role of Mal in TLR2, TLR3 and TLR4 signalling.

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Ligands

Pam_3CSK4 was purchased from Calbiochem (San Diego, CA, USA). LPS and Malp-2 were obtained from Alexis (Braunschwerg, Germany). PolyIC was purchased from Amersham (GE Healthcare UK LTD, Calfont St. Giles, Bucks), Sigma (Poole, Dorset, UK) Invivogen (Toulouse, France) and Imgenex (San Diego, CA, USA). R848 and murine CpGB was purchased from Invivogen (Toulouse, France).

2.1.2 Antibodies

The phospho-p38 and JNK Map kinase and the IκB antibodies were purchased from Cell Signaling technologies (Beverly, MA, USA). Ha antibody was obtained from Covance (Princeton, New Jersey). Myc and GFP antibodies were from Santa Cruz Biotechnology, Inc (Santa Cruz, California). Flag and β-actin antibodies were purchased from Sigma (Poole, Dorset, UK). Human TLR1 and TLR6 antibodies were purchased from Abcam (Cambridge, UK). Human TLR2 antibody was from Imgenex (San Diego, CA, USA). The IL6, TNF-α and RANTES Duoset ELISA kits were purchased from RnD Systems (Minneapolis, MN, USA). Maxi and mini prep endotoxin free plasmid purification kits were purchased from Promega (Madison, Wisconsin, USA).

2.1.3 Polymerase chain reaction reagents

All reagents used for the polymerase chain reaction (PCR), restriction digestion and ligation assays were purchased from New England Biolabs LTD (Ipswich, MA, USA). Primers were designed in house and made by Eurofins MWG Operon (Ebersberg, Germany). All sequencing was also carried out by Eurofins MWG Operon.

2.1.4 Cell culture reagents

Dulbecco's modified eagle medium (DMEM), Roswell Park Memorial Institute (RPMI) and sterile phosphate buffered saline (PBS) were from Gibco (Bioscences, Dun Laoghaire, Dublin). Fetal calf serum (FCS) was purchased from Biosera (East Sussex, UK). Penicillin/streptomycin and trypsin were from Sigma (Poole Dorset, UK). Genejuice was from Novagen (Merck, Damstadt, Germany).

2.1.5 General reagents

Glutathione-sepharose 4B beads were obtained from Amersham (GE healthcare UK LTD, Calfont St. Giles, Bucks, UK). Protein A/G-plus agarose beads were from Santa-cruz. Enhanced Chemiluminescence reagent was purchased from cell signaling technologies (Beverly, MA, USA). Pre-stained molecular weight marker was from New England Biolabs LTD (Ipswich, MA, USA). Polyacrylamide was from National diagnostics LTD. 32-P labelled ATP was from Perkin Elmer (Ballymount Dublin). The

Mal inhibitor peptide and control peptide were from Calbiochem (Gibbstown, NJ). The Mal inhibitor peptide VIPER and the control peptide were a gift from Tatyana Lysakova (Andrew Bowie Lab TCD). General laboratory chemicals were from Sigma (Poole, Dorset, UK).

2.2 Expression vectors

Myc-MyD88 was a gift from Marta Muzio (Mario Negri Institute, Milan, Italy), Ha-Mal and GST-TLR4 were generated in house by Dr. Aisling Dunne. Flag tagged TLR1, 2, 3, 4 and 6, yellow fluorescence protein (YFP)-TLR1, 2, 4 and 6 and cyan fluorescence protein (CFP)-Mal and MyD88 were kind gifts from Dr. Kate Fitzgerald (University of Massachusetts Medical School, Worchester, MA, USA). GFP-Caspase1, pGBKT7- EV, TLR1-TIR-Myc, TLR2-TIR-Myc, TLR4-TIR-Myc and TLR6-TIR-Myc and pACT2-EV, Mal-Ha and MyD88-Ha were a gift from Sinead Miggin (Institute of Immunology, National University of Ireland, Maynooth, Co. Kildare, Ireland). All constructs used were human.

2.3 Bacterial transformation and plasmid purification

DH5-α E.coli cells were thawed on ice prior to transformation with the plasmids of interest. 2 ng DNA and 50 µl bacteria were incubated on ice for 5 min. The mixtures were then heat shocked at 42^0C for 2 min and chilled on ice for a further 2 min. 500 µl Luria-Bertani broth (LB) with no antibiotic was then added and the bacteria were incubated at

37^0C for 1 h at 200 revolutions per minute (rpm). The bacteria were centrifuged at 13 Krpm for 1 min, resuspended in 100 µl LB broth and plated onto agar plates containing the required antibiotic. The plates were incubated overnight at 37^0C allowing for colony growth. A single colony was grown in 3 ml LB plus antibiotic for 6-8 h at 37^0C with 200 rpm and transferred to 100 ml LB overnight with 200 rpm to allow bacterial growth. The following day the bacteria were centrifuged and the plasmid was purified using the maxi prep Promega endotoxin free plasmid purification kit, according to the manufacturer's instructions.

2.4 Cell lines

Immortalised bone marrow derived macrophages (BMDM) from wild type, MyD88-, Mal- and TRIF-deficient mice were a gift from Douglas Golenbock (University of Massachusetts Medical School, Worchester, MA, USA) and generated as previously described (Blasi et al., 1990; Hornung et al., 2008; Roberson and Walker, 1988). All of the immortalised cells were tested in comparison to primary cells for the following: 1) expression of surface antigens: HLA class II molecules, F4/80, CD11b and CD11c. 2) Response to a panel of TLR ligands including lipopeptides, PolyIC, LPS, resiquimod and CpG ODN. If the BMDMs were not nearly identical to non-immortalised cells from the same animal in these assays, they were discarded. There have been a number of papers reporting the use of these cells, including: Verstak et al., (Verstak et al., 2009), Nagpal et al., (Nagpal et al., 2009) and Lysakova-Devine et al.,(Lysakova-Devine et al., 2010).

The primary bone marrow derived macrophages and dendritic cells from wild type and Mal-deficient mice were generated in house. The primary bone marrow derived macrophages from wild type, TLR1-, TLR2- and TLR6-deficient mice were a gift from Kate Fitzgerald. THP-1, U373 and human embryonic kidney (HEK)-293T cells were purchased from ECACC (Salisbury, UK).

2.5 Cell culture

Cell lines were stored in liquid nitrogen at a concentration of $1x10^7$cells/ml in 50% FCS, 40% DMEM and 10% DMSO in plastic cryo vials. To thaw the cells they were removed from the liquid nitrogen and immediately resuspended in 6 ml warm DMEM. The cells were centrifuged at 1200 rpm for 3 min and the pellet was resuspended in 5 ml DMEM. The cells were grown in a small flask until confluent and then expanded.

The immortalised bone marrow derived macrophages, U373s and HEK-293T cells were cultured in DMEM supplemented with 10% (v/v) FCS and 1% (v/v) penicillin-streptavidin. The primary macrophages were cultured in DMEM supplemented with 10% (v/v) FCS, 1% (v/v) penicillin-streptavidin and 20% (v/v) of supernatant taken from L929 cells (a murine M-CSF-producing cell line). The primary dendritic cells were cultured in RPMI supplemented with 10% (v/v) FCS, 1% (v/v) penicillin-streptavidin and 40 ng/ml GM-CSF. Cells were grown at 37^0C with 5% CO_2 and cultured when flasks reached 80-90% confluency. Cells were washed in 5 ml 1xPBS and incubated with 2 ml trypsin at 37^0C for 5 min to lift them off the flask. Cells were then collected in 10 ml DMEM and centrifuged

at 1200 rpm for 3 min. The cell pellet was resuspended in 1 ml DMEM and 10 µl was used to count the cells using the following method:

40 µl DMEM, 10 µl trypan blue and 10 µl cells were mixed. 10 µl of the mixture was pipetted into a hemacytometer and 5 boxes of the central grid were counted. This allowed the following calculation which gave the number of cells present:

Average of 5 boxes x 25(total no. of boxes in grid) x 6(dilution factor) x 10,000=no.cells/ml

The amount of cells required was calculated and cells were plated for an experiment.

The suspension cell line, THP-1, were grown in RPMI media supplemented with 10% (v/v) FCS and 1% (v/v) pen-strep at 37^0C with 5% CO_2 until media was cloudy. Cells were cultured by centrifuging the media to pellet the suspension cells. The pellets were pooled in 1xPBS and centrifuged again to pellet. The cells were then counted and set-up as for the adherent cell lines.

2.6 Transient transfection of HEK-293T cells

HEK-293T cells were set-up at concentrations between $1x10^5$/ml and $3x10^5$/ml and transfected using Genejuice, a liposomal based transfection reagent, for the live cell imaging, co-immunoprecipitations and GST-pulldowns using the following method:

3 µl Genejuice per 1 µg of DNA and 800 µl serum-free DMEM were mixed and incubated at room temperature for 5 min. 1-20 µg of the DNA of interest was added to the Genejuice/SF-DMEM mix and incubated at room temperature for 15 min. The volume of media on the cells was reduced and the DNA/Genejuice mix was added to the cells drop-wise. The cells were incubated for 24-48 h at 37^0C with 5% CO_2.

2.7 SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

Samples were resolved on Sodium Dodecyl sulphate (SDS) polyacrylamide gel using a constant current of 30 mA per gel. Samples were first run through a stacking gel (1 ml 30% bisacrylamide mix, 0.75 ml 1M Tris pH 6.8, 4.1 ml ddH_2O, 60 µl 10% (w/v) SDS, 60 µl 10% (w/v) ammonium persulphate and 6 µl TEMED) to condense the protein. The proteins then passed through the 10% resolving gel (5 ml 30% bisacrylamide mix, 3.8 ml Tris pH8.8, 5.9 ml ddH_2O, 150 µl 10% (w/v) SDS, 150 µl 10% (w/v) APS and 6 µl TEMED) and separated according to their size. The proteins were run in a bio-rad gel box filled with 1x SDS-running buffer (10x = Tris 25 mM, glycine 192 mM, 0.1% (w/v) SDS). A pre-stained molecular marker was run along side the proteins as molecular weight standards.

2.8 Western blotting

After the samples were electrophoresed on an SDS-gel they were transferred onto a polyvinylidene difloride (PVDF) membrane and treated with antibodies to allow detection of the protein of interest by enhanced chemiluminescence (ECL).

2.8.1 Transfer method

The resolved proteins were transferred onto PVDF using a wet transfer system with all components soaked first in transfer buffer (25 mM Tris-HCl pH8, 0.2 M glycine, 20% (v/v) methanol). The gel was immersed in 1x transfer buffer and placed in a layer of filter

paper and sponge. The gel was covered in PVDF and a second layer of filter paper and a sponge were placed on top of the PVDF. The assembly was placed in a cassette and a constant current of 150 mA was applied for 2 h or 30 mA overnight.

2.8.2 Blocking the membrane

Once the proteins from the gel had transferred onto the PVDF it was removed from the cassette and incubated at room temperature for 1 h in blocking solution. The blocking solution used was either 5% (w/v) Marvel (non-fat dried milk, 5 g per 100 ml of Tris buffered saline plus Tween-20 [TBST]) or 5% (w/v) Bovine Serum Albumin (BSA) depending on the antibody requirements. The membrane was blocked with gentle shaking.

2.8.3 Incubation with antibody

The membrane was transferred into a 50 ml tube containing 5 ml 5% Marvel or BSA plus 1:1000 to 1:5000 dilution of the appropriate primary antibody. The membrane was incubated with the primary antibody by rolling the tube at room temperature for 2 h or overnight at 4^0C. The membrane was then washed three times in 1x TBST for 5 min.

The membrane was then placed in a 50 ml tube containing 5 ml 5% marvel plus 1:1000 to 1:5000 dilution of the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. The membrane was rolled for 45 min to 1 h at room temperature, washed five times for 5 min and developed by enhanced chemiluminescence (ECL) according to the manufacturers' instructions.

2.8.4 Stripping and re-probing

The same membrane could be used to examine several proteins if the membrane was stripped of the first set of antibodies and re-probed with a second antibody of interest. The membrane was washed in 1xTBST for 5 min after ECL to remove any chemiluminescent agent. The membrane was stripped for 15 min by placing it in 15 ml 1x re-blot plus solution (Chemicon International) with gentle shaking. The membrane was then washed for 1 min in 1xTBST, blocked, incubated with primary and secondary antibodies and developed as outlined in section 2.8.3.

2.9 Yeast two-hybrid assay

The yeast strain *S. cerevisiae* AH109 was used to study interactions as it requires the presence of amino acids in the agar to grow. When transformations were carried out the yeast grew on plates that lacked one or more of the essential nutrients as the plasmid introduced allowed the yeast to produce the amino acid required. In the presence of the pGBKT7 plasmids, which contained the promoter binding domain (BD) of the transcriptional activator, the yeast could grow on plates lacking tryptophan. In the presence of the pACT2 plasmids, which contained the activating domain (AD) of the transcriptional activator, the yeast grew on plates lacking leucine. If the BD and AD of the proteins coded for by the plasmids that were transformed into the yeast interacted the yeast would grow on plates lacking tryptophan, leucine and histidine.

2.9.1 Growth of AH109 cells

A small volume of the glycerol stock of the AH109 cells was streaked onto a YPDA agar plate (difco peptone 20 g, yeast extract 10 g, yeast agar 20 g, ddH2O to 1 L, autoclaved and added 50 ml 40% (w/v) glucose) and were incubated at 30^0C for 2-3 days. Several colonies were added to 1 ml YPDA media, vortexed to mix and grown for 18 h in 50 ml YPDA at 30^0C at 250 rpm.

2.9.2 Transformation of AH109 cells

10-20 ml of the overnight culture was transferred into 300 ml YPDA media to give an OD_{600nm} of 0.2-0.3. The 300 ml culture was incubated at 30^0C for 3 h at 250 rpm to give a final OD_{600nm} of 0.5 ± 0.1. The culture was centrifuged for 5 min at 1,000 rpm, the supernatant was decanted, the pellets were resuspended in a total volume of 50 ml ddH_2O, pooled and centrifuged for 5 min at 1,000 rpm. The pellet was resuspended in 1.5 ml 1x TE/LiAc.

2 µl pGBKT7-TLR1, TLR2, TLR4, TLR6 and empty vector (EV) were mixed with 10 µl of herring testes carrier DNA (pre-boiled for 10 min) and 100 µl of the yeast and vortexed to mix. 600 µl of 1xPEG/LiAc was added to each sample and the yeast was incubated at 30^0C for 30 min at 200 rpm.

After the incubation 70 µl DMSO was added to each sample, the cells were mixed by gentle inversion and heat shocked at 42^0C for 15 min. The cells were then chilled on ice for 2 min, centrifuged at 14 Krpm for 5 sec and the pellet was resuspended in 400 µl 1x TE.

200 µl of the resuspended cells were plated on SD-T plates (yeast nitrogen base without amino acids 6.7 g, agar 20 g, drop-out supplement -Ade, -try, -his, -leu 0.6 g, 10x leucine, histidine and adenine 100 ml, to 1 L with ddH_2O, autoclaved and 50 ml 40% (w/v) glucose and 15 ml 0.2% (w/v) adenine added) and grown at 30^0C for 1 week.

The yeast was then retransformed with the prey proteins pACT2-MyD88, Mal and EV as outlined above. The transformed yeast was grown on SD-TL plates to ensure both plasmids were transformed.

2.9.3 Protein-protein interaction assay

The yeast were also grown on SD-TLH (-tryptophan, leucine and histidine) plates for 1 week at 30^0C. Only yeast containing proteins that could interact would grow. A SD-TLH plate was divided in three and colonies taken from the original SD-TLH plates were streaked as follows:

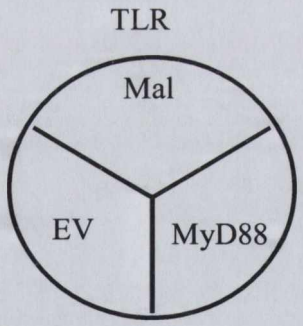

Figure 2.1. **Layout of yeast two-hybrid plate**. Each type of transformed yeast was streaked onto a third of a SD-TLH plate to identify the protein-protein interactions between TLR-1, 2, 4 and 6 and MyD88, Mal and EV.

After each transformation 1 ml of the yeast were lysed in 5x sample loading buffer and analysed by SDS-PAGE with Western blotting to ensure the presence of the bait proteins (Myc-tagged) and the prey proteins (Ha-tagged).

2.10 Live cell imaging using confocal microscopy

HEK-293T cells were set-up at $1x10^5$cells/ml in 35 mm glass bottom dishes and incubated for 24 h at 37^0C with 5% CO_2. The cells were then transiently transfected with 1 µg YFP-TLR1, TLR2, TLR4 or TLR6 and CFP-MyD88 or Mal. 24 h post transfection the cells were given 1 ml fresh media and incubated for a further 24 h at 37^0C with 5% CO_2. The cells were then examined with an Olympus Fluoview FV1000 Imaging system. The excitation light for imaging was provided by the Green Helium-Neon and the Red Helium-Neon lasers. The cells were then stimulated for 5-30 min with either 50 nM Pam_3CSK4 (TLR1 and TLR2), 100 ng/ml LPS (TLR4) or 50 nM Malp-2 (TLR6) and examined as outlined above.

2.11 Co-immunoprecipitation assay

HEK-293T cells were set-up at $3x10^5$ cells/ml in 10 cm dishes and incubated for 24 h at 37^0C with 5% CO_2. The cells were transiently transfected with the plasmids of interest as outlined in section 2.6 and incubated for a further 24-48 h at 37^0C with 5% CO_2. To ensure the plasmids were expressed as protein the cells were lysed in 300 µl 5x sample

loading buffer, sonicated and analysed by SDS-PAGE with Western blotting using the relevant antibody as outlined in sections 2.7 and 2.8

For the co-immunoprecipitation assays the cells were transiently transfected with plasmid as outlined in section 2.6. After 24 h the cells were washed twice in 1xPBS, lysed in 700 µl high stringency lysis buffer (Hepes pH7.5 50 mM, NaCl 150 mM, EDTA 2 mM, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, and 1 µg/ml leupeptin) transferred into microfuge tubes and rolled at 4^0C for 1 h to ensure lysis occurred. The samples were then centrifuged at 13 Krpm for 10 min to remove debris and the supernatants were pre-cleared in 20 µl protein A/G-plus agarose bead slurry for 1 h at 4^0C to remove any protein that bound directly to the beads and not the antibody.

The samples were centrifuged at 2,400 rpm to pellet the A/G beads and the lysates were transferred into fresh microfuge tubes. 60 µl of each sample was removed at this point (whole cell lysate, WCL), 20 µl 5x sample loading buffer was added and the WCLs were boiled for 5 min. 2 µg of the relevant antibody and 50 µl of A/G beads slurry were added and the samples were rolled at 4^0C for 3 h to allow the antibodies to bind their proteins of interest.

The samples were centrifuged at 2,400 rpm for 3 min to pellet the beads, the beads were washed three times in high stringency lysis buffer, 60 µl 5x sample loading buffer was added and the samples were boiled for 5 min. The samples were analysed by SDS-PAGE and Western blotting as outlined in sections 2.7 and 2.8.

2.12 GST-pulldown assay

The interactions seen in the yeast two-hybrid assay and live cell imaging were also tested by GST-pulldown. This involved the use of GST-MyD88 and GST-Mal fusion proteins and the generation of GST-TIR-TLR1, TLR2 and TLR6 fusion proteins.

2.12.1 Cloning of TLR1, 2, 3 and 6 TIR domains into pGex-4T2 vector

The TIR domains of TLR1, TLR2, TLR3 and TLR6 were sub-cloned from the flag or YFP TLR plasmids used in the co-immunoprecipitation and live cell imaging assays. Primers for each TIR domain were designed with the sequence for the restriction enzymes *EcoR* I and *Sal* I at each end:

TLR1-TIR forward primer

EcoR I

TAT AGA ATT CGC AGG GCC AGG AAC ATA

TLR1-TIR reverse primer

Sal I

A TTG TCG ACG CTA TTT CTT TGC TTG CTC

TLR2-TIR forward primer

EcoR I

TAT AGA ATT CCC AGG AAA GCT CCC AGC

TLR2-TIR reverse primer

Sal I

A TTG TCG ACG CTA GGA CTT TAT CGC AGC

TLR3-TIR forward primer

EcoR I

TAT AGA ATT CCC GCA GCA TAT ATA

TLR3-TIR reverse primer

Sal I

A TTG TCG ACG TTA ATG TAC AGA GTT

TLR6-TIR forward primer

EcoR I

TAT AGA ATT CCC TTA GAA GAA CTC CAA AGA

TLR6-TIR reverse primer

Sal I

A TTG TCG ACG TTA AGA TTT CAC ATC ATT

The TIR domains were amplified out of the flag-TLR1, YFP-TLR2, flag-TLR3 and flag-TLR6 plasmids using the following PCR programme:

94^0C – 3min
94^0C – 45 sec
58^0C – 1 min
72^0C – 1 min
} 35 cycles
72^0C – 5 min
4^0C - ∞

And the following recipe:

Master Mix	X1	X3
dNTPs [10mM]	1µl	3µl
10xthermoPol Buffer	5µl	15µl
$MgCl_2$ [25mM]	5µl	15µl
F. primer [10mM]	0.5µl	1.5µl
R. primer[10mM]	0.5µl	1.5µl
Taq polymerase	1µl	3µl
DNA @ 200ng/ml	2.5µl	7.5µl
ddH_2O	Up to 50µl	Up to 150µl

Table 2.1 **Reagents for polymerase chain reaction (PCR)**

The reagents were mixed and added to the DNA of interest allowing the TIR domains of interest to be amplified out of their vectors by PCR.

ddH_2O was used as the control for each primer set. After the PCR 10 µl of DNA loading buffer (50% (v/v) glycerol, 50% (v/v) ddH_2O and 10 mg/ml orange-G) was added to each sample and the entire sample was run on a 1% agarose/TAE gel (1 g agarose, 100 ml 1xTAE, 1 µg/ml ethidium bromide) and run at 100 V in a DNA gel rig filled with 1xTAE (50x = Tris 242 g, glacial acetic acid 57.1 ml, 0.5 M EDTA pH8 100 ml, up to 1 L with ddH_2O). A UV light box was used to take a picture and to cut the amplified DNA out of the agarose gel. The DNA was isolated from the agarose using a Qiagen gel extraction kit according to the manufacturer's instructions. 20 µl DNA was eluted with ddH_2O and the concentration was determined using a Nanodrop spectrophotometer.

The PCR products and the pGex-4T2 empty vector were restriction digested with the *EcoR* I and *Sal* I restriction enzymes:

Master mix	X1	X2 (vector)	X4 (insert)
EcoRI buffer	2µl	4µl	8µl
BSA	0.2µl	0.4µl	0.8µl
EcoR I	0.5µl	1µl	2µl
Sa l I	0.5µl	1µl	2µl
DNA	–	1/3 of insert	Up to 20µl
ddH_2O	Up to 20µl	Up to 20µl	–

Table 2.2. **Reagents for restriction digestion assay**

The reagents were mixed, added to the PCR products and the samples were incubated at 37^0C for 3 h.

The samples were incubated at 37^{0}C for 3 h and the products were isolated with the Qiagen gel extraction kit to remove any restriction enzymes. The samples were then ligated into the cut pGex-4T2 vector using the following recipe:

T4 ligase	1µl
10x T4 ligase Buffer	2µl
Vector	5µl
Insert	5µl
ddH_2O	7µl

The samples were incubated for 1 h at room temperature and transformed into DH5-α bacterial cells as outlined in section 2.3. The plates were incubated overnight at 37^{0}C.

Eight colonies of the pGex-4T2 TLR1-TIR, TLR2-TIR, TLR3-TIR and TLR6-TIR bacteria were grown overnight in 5 ml LB broth plus 1 µg/ml ampicillin, centrifuged at 13 Krpm for 5 min and the DNA was isolated from the cells using the Promega endotoxin free mini-prep kit as per the manufacturers' instructions. The DNA isolated from the colonies was restriction digested as before with the *EcoR* I and *Sal* I restriction enzymes for 3 h at 37^{0}C. 5 µl DNA loading buffer was added to the samples and they were analysed on a 1% agarose gel at 100 V with a standard DNA ladder along side to verify the band size. An image was taken using a UV light box. A product of 500 bp was expected.

Four samples of each plasmid that digested as anticipated were sent for sequencing to confirm there were no mutations introduced into the TIR domains during the PCR process. Sequence alignments were carried out using the ClustalW algorithm (Thompson et

al., 1994). One sample of each plasmid was seen to have no mutations and these samples were used to make the GST-TLR-TIR domain fusion proteins.

2.12.2 GST-TLR1, TLR2, TLR3, TLR4 and TLR6-TIR fusion protein preparation

The pGex-4T2 empty vector, TLR1-TIR, TLR2-TIR, TLR3-TIR, TLR4-TIR and TLR6-TIR vectors were transformed into the *E.* coli Rosetta gami2 strain (as outlined in section 2.3) which is commonly used to express mammalian proteins in bacterial cells as it expresses tRNAs common in mammalian systems but rare in bacteria. The bacteria were incubated for 18 h in 30 ml LB broth containing 1 μg/ml ampicillin, 34 μg/ml chloramphenicol and 12.5 μg/ml tetracycline at 37^0C and 250 rpm. The cultures were then diluted 1:100 in 500ml fresh LB broth and incubated at 37^0C and 250 rpm until the OD_{600nm} was 0.5 ± 0.1.

To induce the transcription and translation of the GST-fusion proteins in the bacteria 100 μM isopropylthio-β-D-galactoside (IPTG) was added and the cultures were incubated for 24 h at 18^0C at 250 rpm. The bacteria were then centrifuged at 6,000 rpm for 10 min.

The bacterial pellets were resuspended in 40 ml NETN lysis buffer (Tris-HCl pH8 20 mM, NaCl 100 mM, EDTA 1 mM, 1% (v/v) Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mg/ml lysozyme). The bacteria were incubated on ice for 15 min and sonicated to ensure adequate lysis. The samples were centrifuged at 14 Krpm for 50 min at 4^0C to pellet any debris. The lysates were incubated with 600 μl glutathione-sepharose 4B beads for 18 h at 4^0C on a roller to couple the fusion proteins to the beads.

The samples were then centrifuged at 2,500 rpm for 10 min at 4^0C to pellet the beads and washed 5 times in 1xNETN. The beads were resuspended in a total volume of 1 ml with 1xPBS (plus 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mg/ml lysozyme) and stored at 4^0C. 5, 10 and 20 μl of each GST-fusion protein prep was mixed with 5x sample loading buffer and analysed by SDS-PAGE. The gel was stained with coomassie brilliant blue solution (50% (v/v) methanol, 10% (v/v) glacial acetic acid, 2.5 g coomassie brilliant blue, up to 1 L with ddH_2O) for 1 h with gentle shaking and destained overnight in destain solution (50% (v/v) methanol, 10% (v/v) glacial acetic acid, up to 1 L with ddH_2O). The amount of each fusion protein used in the GST-pulldown assays was determined based on the level of expression of each at the different volumes analysed.

2.12.3 GST-MyD88 and GST-Mal fusion protein preparation

The pGex-4T2 empty vector, MyD88 and Mal containing vectors were transformed into the *E.* coli BL21 strain (as outlined in section 2.3) which is commonly used to express mammalian proteins in bacterial cells. The bacteria were incubated for 18 h in 30 ml LB broth containing 1 μg/ml ampicillin at 37^0C and 300 rpm. The cultures were then diluted 1:100 in 500 ml fresh LB broth and incubated at 37^0C for 2-3 h (until the OD_{600nm} was 0.5 ± 0.1) at 300 rpm.

To induce the transcription and translation of the GST-fusion proteins in the bacteria 100 μM IPTG was added and the cultures were incubated for another 3 h at 37^0C

at 300 rpm. The bacteria were then centrifuged at 6,000 rpm for 10 min and pellets were frozen overnight at -80^{0}C.

The following day the bacterial pellets were resuspended in 40 ml NETN lysis buffer and the fusion proteins were isolated as outlined above. Several volumes of each GST-fusion protein prep were mixed with 5x sample loading buffer and analysed by SDS-PAGE along side a GST-fusion protein prep of known concentration of 1 µg/ml. The gel was stained with coomassie brilliant blue solution for 1 h with gentle shaking and destained overnight in destain solution. The amount of each fusion protein used in the GST-pulldown assays was determined based on the level of expression of each in comparison to the 1µg/ml protein.

2.12.4 GST-pulldown assay with MyD88 and Mal

HEK-293T cells were set-up at 3x10^{5}cells/ml in 10 cm dishes and incubated overnight at 37^{0}C with 5% CO_2. The cells were transiently transfected with 3 µg Myc-MyD88, Ha-Mal or EV for 24 h. The following day the cells were washed twice in 1xPBS and lysed in 700 µl low stringency lysis buffer (Hepes pH7.5 50 mM, NaCl 100 mM, 10% (v/v) glycerol, 0.5% (v/v) Nonidet P-40, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, and 1 µg/ml leupeptin) by rolling at 4^{0}C for 1 h. The cell debris was removed by centrifuging the samples at 13 Krpm for 10 min. The supernatants were next incubated for 1 h with 20 µl glutathione-sepharose 4B beads at 4^{0}C as a pre-clear step to remove any non-specific binding of proteins to the beads. The samples were centrifuged at 2,400 rpm for 5 min to pellet the beads, 60 µl of the

supernatants were removed for the WCLs and the samples were incubated with GST, GST-MyD88 or GST-Mal for 3 h at 4^0C on a roller.

The beads were pelleted by centrifuging the samples at 2,400 rpm for 5 min and were then washed three times in low stringency lysis buffer. 60 µl of 5x sample loading buffer was added to the beads and they were boiled for 5 min. The samples were then analysed by SDS-PAGE and Western blotting as outlined in sections 2.7 and 2.8. The membranes were blocked in 5% marvel for one hour, incubated for 2 h in a 1:1000 dilution of the anti-Myc or anti-Ha primary antibodies in 5% marvel and incubated in a 1:1000 dilution of the HRP-conjugated anti-mouse secondary antibody. The membranes were developed by ECL.

2.12.5 GST-pulldown assay with endogenous TLR1, TLR2 and TLR6

THP-1 cells were set-up at $2x10^5$ cells/ml in a total volume of 250 ml and incubated at 37^0C with 5% CO_2 overnight. The cells were then counted and $1x10^7$cells/ml per point were transferred into microfuge tubes and centrifuged at 1,200 rpm for 5 min. The cell pellets were lysed in 700 µl 1% CHAPs lysis buffer (Tris HCl pH 7.5 30 mM, NaCl 150 mM, 10% CHAPS, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, and 1 µg/ml leupeptin) and rolled at 4^0C for 1 h to ensure adequate lysis of the cells. The samples were centrifuged at 13 Krpm for 10 min to remove any cell debris. The GST-pulldown assay was then carried out as outlined in section 2.12.4.

The samples were then analysed by SDS-PAGE and Western blotting as outlined in sections 2.7 and 2.8. The membranes were blocked in 5% marvel for 1 h, incubated

overnight at 4^0C in a 1:1000 dilution of the human-TLR1, TLR2 or TLR6 primary antibodies in 5% marvel and incubated in a 1:1000 dilution of the HRP-conjugated anti-rabbit secondary antibody. The membranes were developed by ECL.

2.12.6 GST-pulldown with over-expressed TLR2 and TLR6

HEK-293T cells were set-up at $3x10^5$ cells/ml in 10 cm dishes and incubated at 37^0C with 5% CO_2 overnight. The cells were then transiently transfected with 3 µg flag-TLR2, flag-TLR6 or EV, as outlined in section 2.6, and incubated at 37^0C with 5% CO_2 for 24-48 h. The cells were washed twice in 1xPBS and lysed in 700 µl high stringency lysis buffer by rolling at 4^0C for 1 h and the GST-pulldown assay was carried out as outlined in section 2.12.4. The samples were analysed by SDS-PAGE and Western blotting using the flag antibody.

2.12.7 GST-pulldown with membrane fractionation

HEK-293T cells were set-up at $3x10^5$cells/ml in 10 cm dishes and incubated at 37^0C with 5% CO_2 overnight. The cells were transiently transfected with 3 µg flag-TLR2, TLR6 or EV for 24 h. The cells were washed twice in 1x PBS and lysed in 300 µl membrane lysis buffer (Tris HCl pH 7.5, $MgCl_2$ 10 mM, EDTA 1 mM, sucrose 250 µM and 0.1 mM phenylmethylsulfonyl fluoride). Each sample was douce homogenised 35 times, 50 µl was removed for the WCLs and the samples were spun in an ultracentrifuge at 50 Krpm for 50 min at 4^0C. The supernatant was transferred to fresh microfuge tubes and

5x sample loading buffer was added, the pellet was resuspended in 75 µl 5x sample loading buffer and sonicated. The samples were then boiled for 5 min. The membrane fraction, cytosolic fraction and WCLs were analysed by SDS-PAGE and Western blotting using the flag antibody.

To carry out the GST-pulldown assay on the concentrated flag-TLR2 and flag-TLR6 samples isolated from the membrane fractionation step the experiment was initiated as outlined above, however, the membrane fraction (pellet) was lysed in 75 µl high stringency lysis buffer, sonicated and the volume was increased to 700 µl with lysis buffer. The assay was then carried out as described in section 2.12.4.

2.12.8 GST-pulldown with TLR-TIR domain fusion proteins

HEK-293T cells were set-up at $3x10^5$ cells/ml in 10 cm dishes and incubated at 37^0C with 5% CO_2 overnight. The cells were transiently transfected with 3 µg Myc-MyD88, Myc-IRAK2, Ha-Mal, Ha-TRIF, GFP-Caspase1 or EV and incubated for 24 h at 37^0C with 5% CO_2. The cells were treated as outlined in section 2.12.4 and incubated with the GST-TLR1, TLR2, TLR3, TLR4 and TLR6 TIR domain containing fusion proteins for 3 h at 4^0C. The beads were washed and the samples were analysed by SDS-PAGE and Western blotting with the Myc, Ha and GFP antibodies.

For the TLR1-TIR fusion protein optimisation the cells were set-up as above, transfected for 24 h with 3 µg Myc-MyD88, EV, Mal or Caspase1 and the pulldown was carried out using GST-TLR1-TIR only at increasing amounts of 5, 7, 10, 12, 15 and 20 µl.

2.13 ELISA (enzyme linked immunosorbent assay)

Duoset ELISA kits from RnD Biosystems were used to analyse the levels of murine IL6 and TNF-α produced by the macrophages and dendritic cells in response to TLR stimulation. Duoset ELISA kits from RnD Biosystems were also used to analyse the levels of human IL6 and RANTES produced by the U373s.

2.13.1 Cell preparation and stimulation

The immortalised macrophages, primary macrophages, dendritic cells and the U373s were set-up at $2x10^5$ cells/ml in 96 well plates (200 µl/well) and incubated overnight at 37^0C with 5% CO_2. The media was replaced before stimulation and the volume was brought down to 100 µl. The cells were stimulated with various concentrations of Pam_3CSK4, Malp-2, LPS, PolyIC, R848 and mCpGB. After 18 h stimulations the supernatants were collected and analysed by ELISA for the production of IL6, TNF-α or RANTES.

2.13.2 Treatment with Mal inhibitor peptide

The wild type immortalised macrophages were set up at $2x10^5$ cells/ml in 96 well plates and incubated overnight at 37°C. Fresh DMEM was added to the cells and the cells were pre-treated with 1 µg/ml inhibitor peptide or Mal inhibitor peptide (Calbiochem) for 1

h before stimulation with PolyIC or LPS. After 18 h stimulations the supernatants were collected and analysed by ELISA for IL6 production.

2.13.3 Treatment with the Mal inhibitor peptide VIPER

The human astrocytoma cell line U373 was used to assess the activity of the Mal inhibitor peptide VIPER. The cells were set-up at $2x10^5$ cells/ml and incubated overnight at 37°C. Fresh DMEM was added the following day and the cells were pre-treated with 5 μM VIPER or control peptide for 1 h before stimulation with Pam_3CSK4, PolyIC and LPS. After 18 h stimulations the supernatants were collected and analysed by ELISA for IL6 and RANTES production.

2.13.4 Treatment of ligands with Polymyxin B

A 250 μg/ml stock of Sigma PolyIC and Amersham PolyIC, a 100 ng/ml stock of LPS and a 10 μg/ml stock of R848 were incubated overnight at 4°C with 100 μg/ml Polymyxin B. 10 μl of each ligand was added to the wild type and Mal-deficient macrophages and the cells were incubated for 18 h at 37°C. This gave a final concentration of 25 μg/ml PolyIC, 10 ng/ml LPS and 1 μg/ml R848 and a final concentration of 10 μg/ml Polymyxin B on the cells. After the 18 h stimulations the supernatants were tested for the production of IL6.

2.13.5 ELISA plate preparation

The IL6, TNF-α or RANTES capture antibody was diluted in 1xPBS (pH 7.2-7.4) and 50 µl was applied to each well of a flat bottomed high binding 96 well plate. The plate was sealed and incubated overnight at room temperature. Each well of the plate was then rinsed with wash buffer (0.05% (v/v) Tween-20 in PBS, pH 7.2-7.4) three times. The plate was blotted on tissue paper to ensure the removal of any bubbles and 150 µl of reagent diluent (1% (v/v) BSA in PBS, pH7.2-7.4) was added to each well. The plate was sealed and incubated at room temperature for a minimum of 1 h. The wash step was repeated prior to addition of the standards and samples.

2.13.6 ELISA procedure

The IL6, TNF-α or RANTES standards were prepared, as per the manufacturers' instructions, in reagent diluent. The samples were diluted 1:2 to 1:8 in reagent diluent depending on the ligand used to stimulate the cells. A total of 50 µl of sample or standard per well was added to the prepared plate. The plate was sealed and incubated at room temperature for 2 h or at 4^0C overnight. The wash step was repeated to remove the samples and standards and 50 µl per well of detection antibody (prepared in reagent diluent) was added to the plate. The plate was sealed and incubated at room temperature for 2 h. The plate was washed three times and 50 µl per well of streptavadin-HRP (prepared in reagent diluent) was added to the plate. The plate was incubated in the dark for 20 min, the wash step was repeated and 50 µl per well of a 50:50 mix of the substrate solution was added.

After 10-20 min incubation the reaction was stopped by the addition of 25 µl 1 M H_2SO_4. The optical density of each well was determined immediately using a microplate reader with the wavelength set to 450 nm. The standard curve was calculated by plotting the mean absorbance of the standards against their concentration. A best fit line was drawn through the points and the concentrations of the samples were calculated based on the R^2 value given by the graph.

2.13.7 Statistical analysis

Data were expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. For comparison between two groups, Student's *t* test was used. A *p* value of <0.05 was considered significant.

2.14 Induction of downstream signalling molecules upon TLR activation

The immortalised macrophages were tested for their ability to induce phosphorylation of p38 and JNK and the degradation of IκB-α.

2.14.1 Cell preparation and stimulation

The wild type, MyD88-, Mal- and TRIF-deficient immortalised macrophages were set-up at $4x10^5$cells/ml in 6 wells of a 12 well plate and incubated overnight at 37^0C with 5% CO_2. The following day the cells were given fresh media (600 µl) and stimulated with

200 nM and 50 nM Pam_3CSK4, 200 nM and 50 nM Malp-2 and 100 ng/ml LPS for 45, 30, 15, 10, 5 and 0 min, or with 25 μg/ml PolyIC for 90, 60, 45, 30, 15 and 0 min. The supernatants were discarded and the cells were washed in 250 μl 1xPBS. The cells were transferred into microfuge tubes and centrifuged at 1,200 rpm for 5 min. The supernatants were discarded and the cell pellets were lysed in 100 μl low stringency lysis buffer on ice for 15 min.

2.14.2 Bradford assay

In order to determine the protein content of the samples a Bradford assay was next carried out. 2.5 μl of ddH_2O and 2.5 μl of each sample were added to a flat bottomed 96 well plate in duplicate. 250 μl Coomassie Bradford reagent was added to each well, after vigorous mixing. The optical density of each well was read at 595 nm in a microplate reader. The standard curve was calculated by plotting the mean absorbance of the standards against their concentration. A best fit line was drawn through the points and the concentrations of the samples were calculated based on the R^2 value given by the graph. The samples were normalised to the same concentration by the addition of 5x sample loading buffer (Tris HCl pH 6.8 125 mM, 2% (w/v) SDS, 10% (v/v) glycerol, 200 ng/ml bromophenol blue and 50 mM dithiothreitol) and ddH_2O.

2.14.3 Sample analysis

The samples were run on 10% SDS-gels and transferred onto PVDF as outlined in sections 2.7 and 2.8. The samples tested for phosphorylation of p38 and for IκB-α degradation were blocked in 5% BSA, incubated in 1:1000 dilution of anti phospho-p38 or IκB-α primary antibody in 5% BSA overnight at 4^0C and in a 1:1000 dilution of HRP-conjugated anti-rabbit secondary antibody for 1 h at room temperature. The samples tested for the phosphorylation of JNK were washed once for 5 min in 1xTBS, blocked in 5% marvel for 1 h, incubated overnight in 1:1000 dilution of anti phospho-JNK primary antibody in 5% BSA at 4^0C and in a 1:1000 dilution of HRP-conjugated anti-rabbit secondary antibody for 1 h at room temperature. The membrane was then developed by ECL. The samples were also tested for the presence of β-actin as the loading control. The membranes were blocked for 1 h in 5% marvel, incubated at room temperature for 1 h in a 1:10,000 dilution of β-actin primary antibody, washed three times for 5 min in 1xTBST and incubated for 45 min in HRP-conjugated anti-mouse secondary antibody before being developed by ECL.

2.15 JNK2 K/R kinase assay

The wild type and Mal-deficient immortalised bone marrow derived macrophages were stimulated with various TLR ligands. Antibodies against MKK4 and MKK7 (a gift from J. Saklatvala, Imperial college, London, UK) were incubated with the cell lysates to immunoprecipitate the total amount of MKK4 or MKK7 in the lysates. The IP samples

were then tested for their ability to phosphorylate a kinase dead form of JNK2 (K/R) (a gift from M. Kracht, University of Giessen, Giessen, Germany) using ^{32}P labelled ATP.

2.15.1 Preparation of JNK2 K/R protein

The pGex-JNK2 K/R (kinase dead form) was transformed into BL21 cells as outlined in section 2.3. The bacteria were then grown and pelleted as outlined in section 2.12.3.

The GST-JNK2 K/R beads were then washed four times in Buffer A (50 mM Tris, 0.1 mM EDTA, 0.5% (w/v) sodium deoxycholate, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) and washed twice in buffer A plus 0.27 M sucrose. The beads were then incubated with 30 mM glutathione for 15 min at room temperature with regular mixing. This allowed the JNK2 K/R to be cleaved from the glutathione beads.

A Bradford assay was then carried out to determine the concentration of the JNK2 K/R and 1, 2, 5 and 10 μg was analysed by SDS-PAGE with coomassie staining. 1, 2, 5 and 10 μg BSA were used as controls. The JNK2 K/R was stored at -80°C.

2.15.2 Stimulation and MKK4/7 immunoprecipitation

The immortalised bone marrow derived macrophages from wild type and Mal-deficient mice were set-up in 12 well plates at $6x10^5$cells/ml and incubated overnight at 37°C. The following day the cells were given fresh media and stimulated with 100 nM

Pam_3CSK4, 100 ng/ml LPS and 1 μg/ml R848 for 15 and 30 min or with 25 μg/ml PolyIC and 3 μg/ml mCpGB for 45 and 90 min. The wild type cells were also stimulated with 50 ng/ml IL1-α for 10 and 30 min.

The supernatants were then removed; the cells were lifted in 500 μl ice cold 1xPBS and centrifuged at 1,200 rpm at 4°C for 4 min. The cell pellets were lysed in 300 μl low stringency lysis buffer (Hepes pH 7.5 50 mM, NaCl 100 mM, EDTA 1 mM, 10% (v/v) glycerol, 0.5% (v/v) Nonidet P-40, 0.5% (v/v) sodium deoxycholate, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) on a roller at 4°C for 45 min.

A Bradford assay was carried out to normalise the protein concentrations. The lysates were then pre-cleared in 20 μl A/G bead slurry at 4°C for 45 min on a roller. The lysates were centrifuged at 2,400 rpm for 4 min to pellet the beads and transferred to a fresh microfuge tube. The lysates were incubated overnight at 4°C on a roller in 40 μl protein A/G-plus agarose beads with 3 μl anti-MKK4 or MKK7 or 1 μg anti-rabbit IgG control.

2.15.3 JNK2 Kinase assay

The following day the beads were washed three times in 800 μl low stringency lysis buffer and three times in 800 μl kinase buffer (Tris HCl pH 7.4 150 mM, $MgCl_2$ 30 mM, 0.5% (v/v) sodium deoxycholate, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). The beads were

incubated in 20 µl reaction media (^{32}P ATP 5 µCu, ATP 20 nM, JNK2 K/R 2 µg and kinase buffer) at 37°C and 1,000 rpm for 30 min.

8 µl 5x sample loading buffer was added to the samples before boiling for 5 min. The samples were centrifuged at 14 Krpm for 1 min and analysed by SDS-PAGE as described in section 2.7. The samples were then transferred onto PVDF as outlined in section 2.8 and placed in a cassette with 2-3 films. The cassette containing the membrane was stored at -80°C until developed. The membranes were then tested for the presence of JNK2 and MKK4 or MKK7 by Western blotting.

Chapter Three

Mal is not essential for TLR2 signalling

Chapter 3

3.1 Introduction

As mentioned in chapter 1, TLR2 and TLR4 are transmembrane receptors found on the plasma membrane. TLR2 is the receptor utilized for the recognition of lipoproteins found on the surface of gram-positive bacteria (Aliprantis et al., 1999). It also binds peptidoglycan and zymosan from yeast cell walls (Sandor et al., 2003). TLR2 utilises TLR1 and TLR6 to aid in the recognition of the vast amount of lipoproteins found on pathogens. TLR1 heterodimerises with TLR2 to initiate signalling in the presence of tri-acylated lipopeptides and TLR6 interacts with TLR2 to bind di-acylated lipopeptides (Ozinsky et al., 2000; Takeuchi et al., 2001; Takeuchi et al., 2002). TLR4 is the receptor responsible for the interaction with lipopolysaccharide (LPS), a component of gram-negative bacterial cell outer membranes (Chow et al., 1999).

Once the TLRs have bound their ligand and dimerised the next step in signal transduction requires the TLRs to interact with the adaptor proteins. In the case of TLR2 and TLR4 signalling Mal and MyD88 are recruited to the TIR domain of the activated TLRs to initiate the MyD88-dependent signalling pathway. TLR4 can also recruit TRAM and TRIF to initiate the TRIF-dependent signalling pathway. The adaptor proteins bind the TLRs via a TIR-TIR homotypic interaction. Internal signal pathways are then activated resulting in the modulation of gene expression and the production of pro-inflammatory cytokines and chemokines (Miggin and O'Neill, 2006).

Mal and MyD88 are TIR-domain containing adaptor proteins shown to be required for TLR2 and TLR4 signalling. Mal is thought to be a bridging adaptor linking these TLRs

to MyD88 and thus initiating the MyD88-dependent signalling pathway. Many features of Mal have been revealed, including the identification of several phosphorylation sites, a Caspase1 cleavage site and a PEST domain allowing it to be degraded by SOCS1 (Gray et al., 2006; Mansell et al., 2006; Miggin et al., 2007). Phosphorylation of Mal by IRAK1 and IRAK4 also results in it being ubiquitinated and hence degraded (Dunne et al., 2010a). A single nucleotide polymorphism has also been reported in the gene encoding Mal, which converts a serine at position 180 to a leucine (Khor et al., 2007). A heterozygous state for this polymorphism has been shown to be protective against several diseases including Malaria and Systemic lupus erythematosus (SLE) (Castiblanco et al., 2008).

Previous work in the area of TLR2 and TLR4 signalling revealed an absolute requirement for Mal and MyD88 (Horng et al., 2002; Yamamoto et al., 2002a). However, an in depth examination of the dependencies of these signalling molecules in relation to both adaptor proteins has never been investigated. Also the precise involvement of Mal and MyD88 in these heterodimeric complexes has not been investigated to date.

With these questions in mind I set out to identify where in the TLR1/2, TLR2/6 and TLR4 signalling complexes Mal and MyD88 bound to initiate signal transduction. I also sought to thoroughly explore the role of Mal and MyD88 in the activation of signalling molecules and the production of cytokines downstream of the TLR1/2, TLR2/6 and TLR4 complexes.

3.2 Results: Interaction experiments

The first aim of the project was to determine where in the TLR1/2, TLR2/6 and TLR4 signalling complexes Mal and MyD88 bound. I therefore carried out yeast two-hybrid assays, live cell imaging via confocal microscopy, co-immunoprecipitation assays and GST-pulldown assays to clarify these interactions.

3.2.1 A yeast two-hybrid assay reveals that MyD88 interacts with TLR1, TLR2, TLR4 and TLR6, and that Mal interacts with TLR2 and TLR4 only

A yeast two-hybrid assay was first carried out to identify the interacting partners of TLR2 and TLR4 signalling. The AH109 strain of yeast were transformed with the TIR domains of human TLR1, TLR2, TLR4 and TLR6 and grown for 3 days on agar plates lacking tryptophan to ensure only transformed yeast grew. The yeast were re-transformed with full length MyD88, Mal or empty vector and grown on agar plates lacking tryptophan, histidine and leucine.

As shown in Figure 3.1A yeast containing TLR1 grew in the presence of MyD88 (section 2) indicating TLR1 and MyD88 interacted. TLR1 did not interact with Mal (section 1). Similarly, as shown in Figure 3.1B, TLR6 interacted with MyD88 (section 2) but not Mal (section 1). TLR2 and TLR4 interacted with both MyD88 (section 2) and Mal (section 1) as shown in Figures 3.1C and 3.1D respectively. None of the TLRs interacted non-specifically with the empty vector construct (section 3, Figure 3.1A-D).

To ensure the TLRs and adaptor proteins were expressed at similar levels a sample of the yeast was lysed at each step. Figure 3.1E shows the Myc-tagged TLRs were similarly expressed. Figure 3.1F demonstrates that Ha-tagged MyD88 and Mal were also similarly expressed.

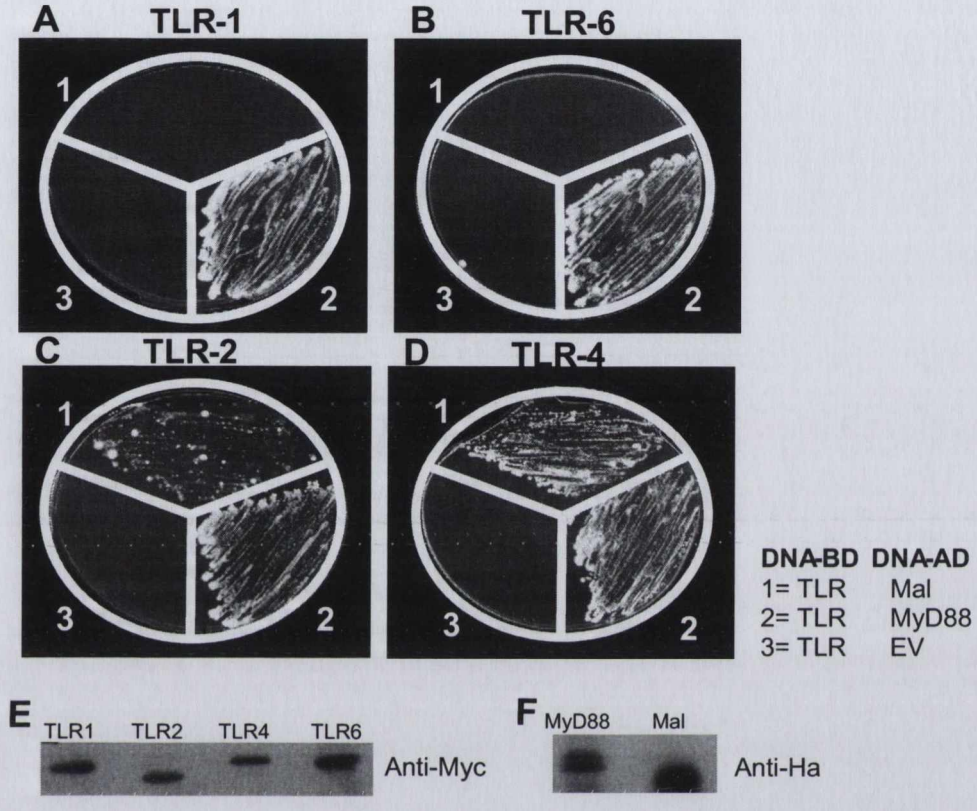

Figure 3.1. **MyD88 interacts with TLR1, TLR2, TLR4 and TLR6, whilst Mal interacts with TLR2 and TLR4 only**

The AH109 strain of yeast was transformed with the TIR domains of TLR1, 2, 4 and 6 and grown at 30^0C for three days on agar plates lacking tryptophan. The yeast were re-transformed with Mal, MyD88 or empty vector and grown on plates lacking tryptophan, histidine and leucine at 30^0C for one week (A-D). After both transformations yeast extracts were tested for the presence of the TLR-Myc tagged (E) and Ha-tagged adaptor (F) proteins by Western blot. These data are representative of three experiments.

3.2.2 Live cell imaging reveals that MyD88 only co-localises with TLR1, TLR2, TLR4 and TLR6 after stimulation

To confirm the interactions shown in the yeast two-hybrid assay HEK-293T cells were transiently transfected with YFP-tagged TLR1, TLR2, TLR4 or TLR6 and CFP-tagged MyD88. The live cells were examined using a confocal microscope, stimulated with the relevant TLR ligands and re-examined.

As shown in Figure 3.2A CFP-MyD88 (red) resided inside the cells, YFP-TLR1 (green) was found on the membrane and in the cytosol and the two did not co-localise (overlay panel). As revealed in Figure 3.2B upon stimulation with 50 nM Pam_3CSK4 for 30 min the MyD88 and TLR1 co-localised within the cell as indicated by the arrows (yellow overlay panel).

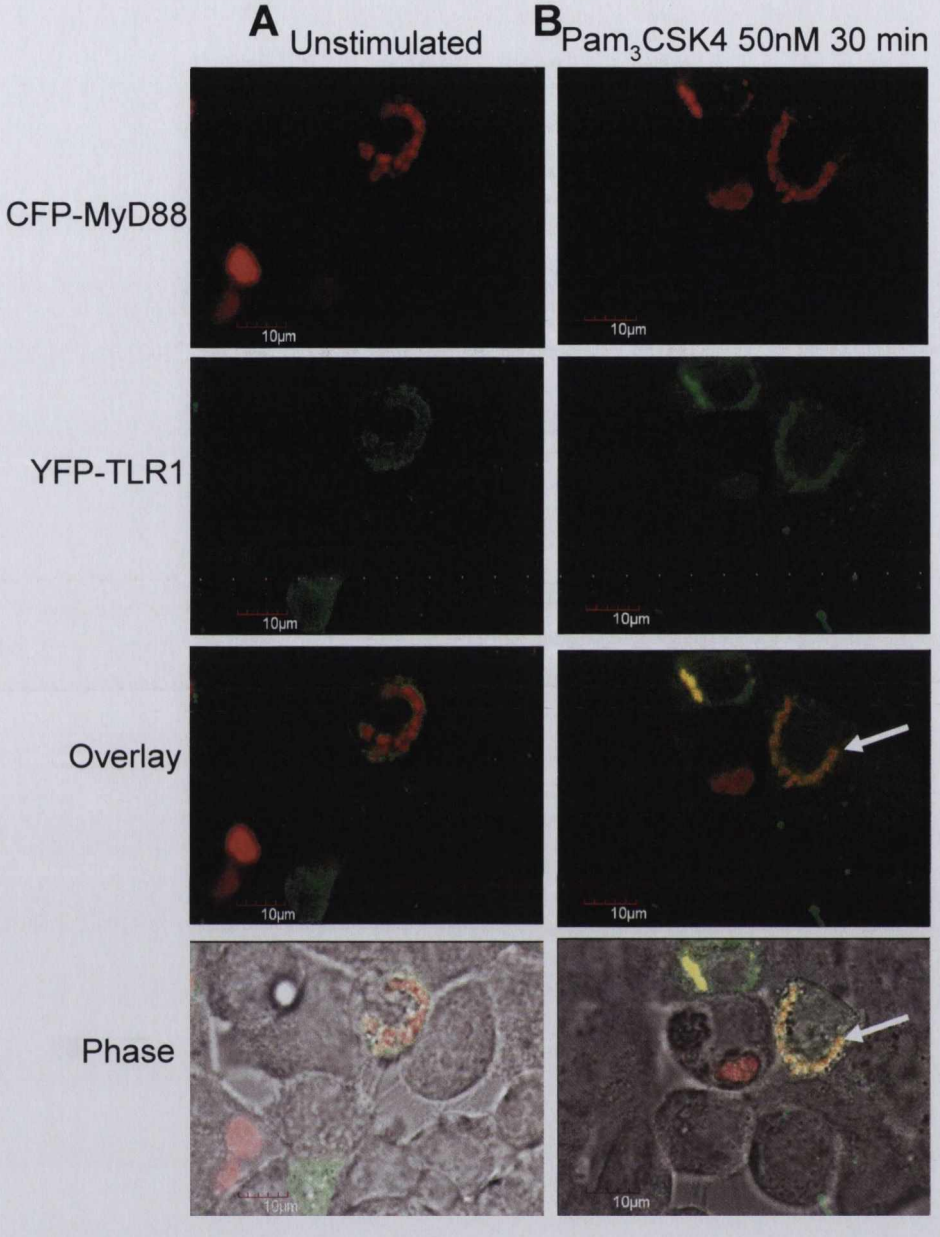

Figure 3.2. **MyD88 and TLR1 co-localise upon stimulation with 50 nM Pam_3CSK4**

HEK-293T cells were transiently transfected with 3 µg YFP-TLR1 and CFP-MyD88. 48 h post transfection the cells were examined using the Olympus Fluoview FV1000 Imaging system (A).The cells were then stimulated with 50 nM Pam_3CSK4 for 30 min and re-examined by confocal microscopy (B). An average of 20% of cells examined expressed both proteins. The cells shown are representative of three independent experiments.

I next examined the ability of TLR2 and MyD88 to co-localise. As shown Figure 3.3A MyD88 (red) was again in found in the cytosol of the cell and TLR2 (green) was mainly at the plasma membrane. There was no area of co-localisation (overlay). 30 min post stimulation with 50 nM Pam_3CSK4 the cells were re-examined and as shown in Figure 3.3B MyD88 and TLR2 co-localised inside the cells as indicated by the arrows (yellow overlay panel).

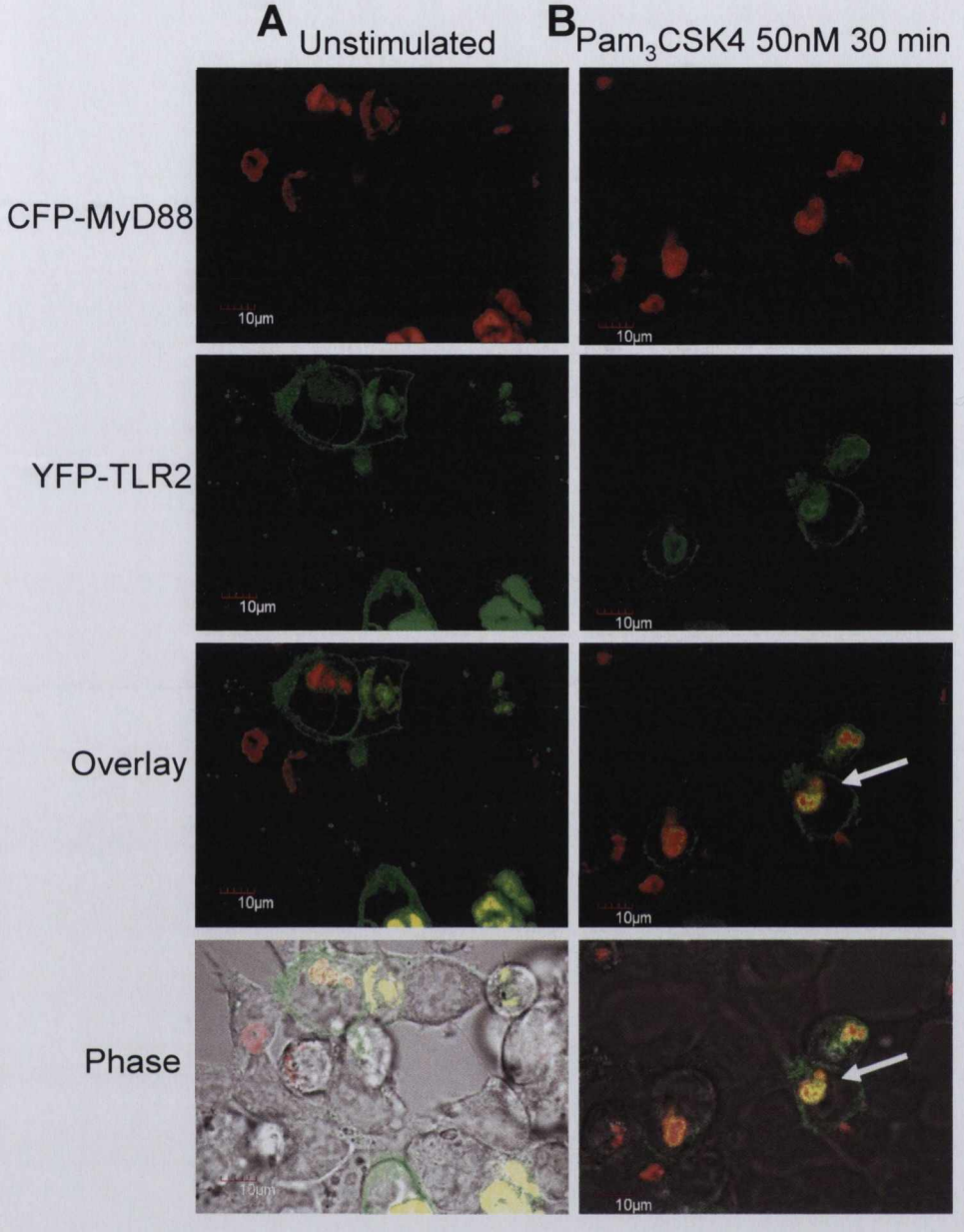

Figure 3.3. **MyD88 and TLR2 co-localise upon stimulation with 50 nM Pam_3CSK4**

HEK-293T cells were transiently transfected with 3 µg YFP-TLR2 and CFP-MyD88. 48 h post transfection the cells were examined using the Olympus Fluoview FV1000 Imaging system (A).The cells were then stimulated with 50 nM Pam_3CSK4 for 30 min and re-examined by confocal microscopy (B). An average of 20% of cells examined expressed both proteins. The cells shown are representative of three independent experiments.

The HEK-293T cells were also transfected with YFP-TLR4 and CFP-MyD88. Figure 3.4A shows CFP-MyD88 (red) localised inside the cell and TLR4 on the membrane and in the cytosol (green) with no co-localisation (overlay). The cells were then stimulated with 100 ng/ml LPS for 5 min and as shown in Figure 3.4B this resulted in the co-localisation of TLR4 with MyD88 on the plasma membrane as indicated by the arrows (yellow colour in overlay panel). When the cells were re-examined 20 min post LPS stimulation this co-localisation could no longer be found as shown in Figure 3.4C (overlay panel). The TLR4 had internalised at this point and this disrupted its interaction with MyD88.

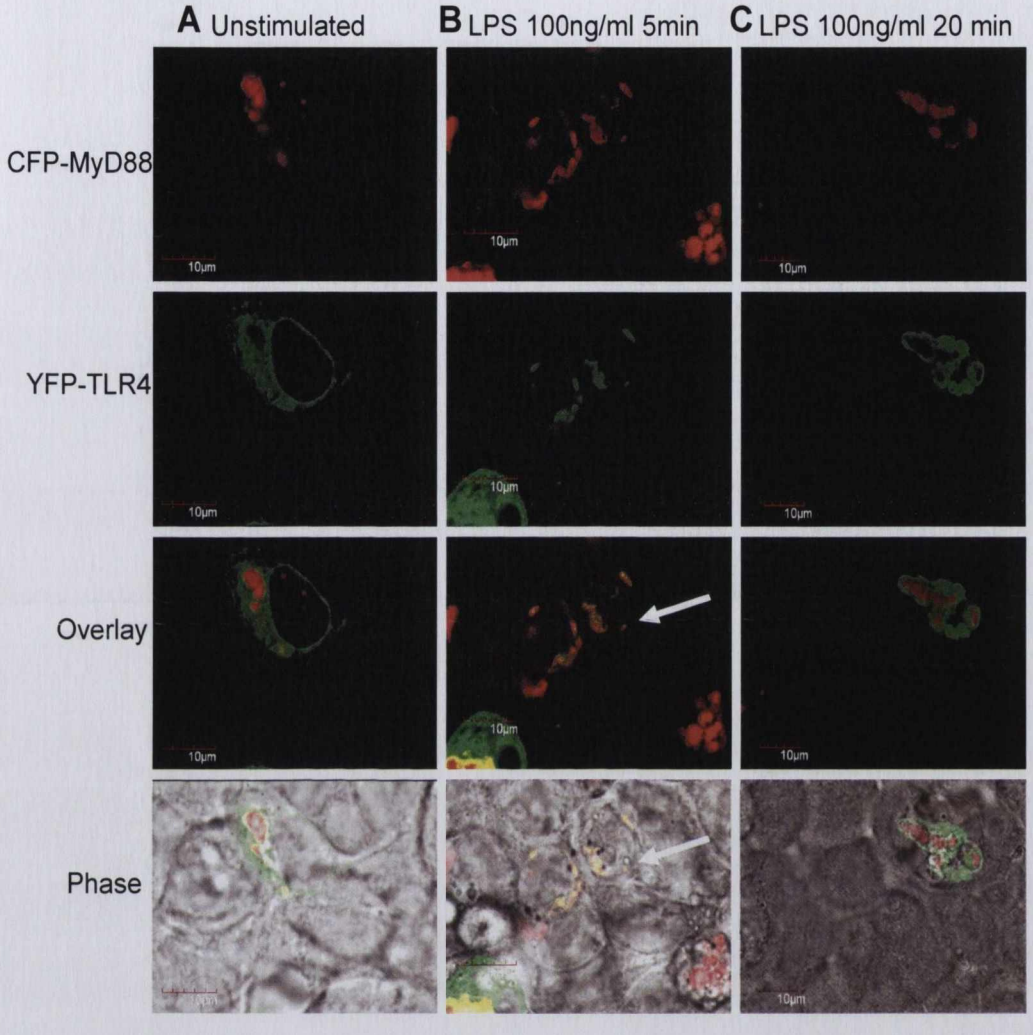

Figure 3.4. **MyD88 and TLR4 co-localise upon stimulation with 100 ng/ml LPS**

HEK-293T cells were transiently transfected with 3 µg YFP-TLR4 and CFP-MyD88. 48 h post transfection the cells were examined using the Olympus Fluoview FV1000 Imaging system (A).The cells were then stimulated with 100 ng/ml LPS for 5 (B) and 20 (C) min and re-examined by confocal microscopy. An average of 20% of cells examined expressed both proteins. The cells shown are representative of three independent experiments.

Finally YFP-TLR6 and CFP-MyD88 were transfected into the HEK-293T cells and examined by confocal microscopy. As shown in Figure 3.5A MyD88 (red) was again localised within the cell and TLR6 (green) was mainly at the membrane. The two did not co-localise (overlay). Figure 3.5B demonstrates that 30 min stimulation with 50 nM Malp-2 allowed MyD88 and TLR6 to co-localise within the cell as indicated by the arrows (yellow colour in overlay panel).

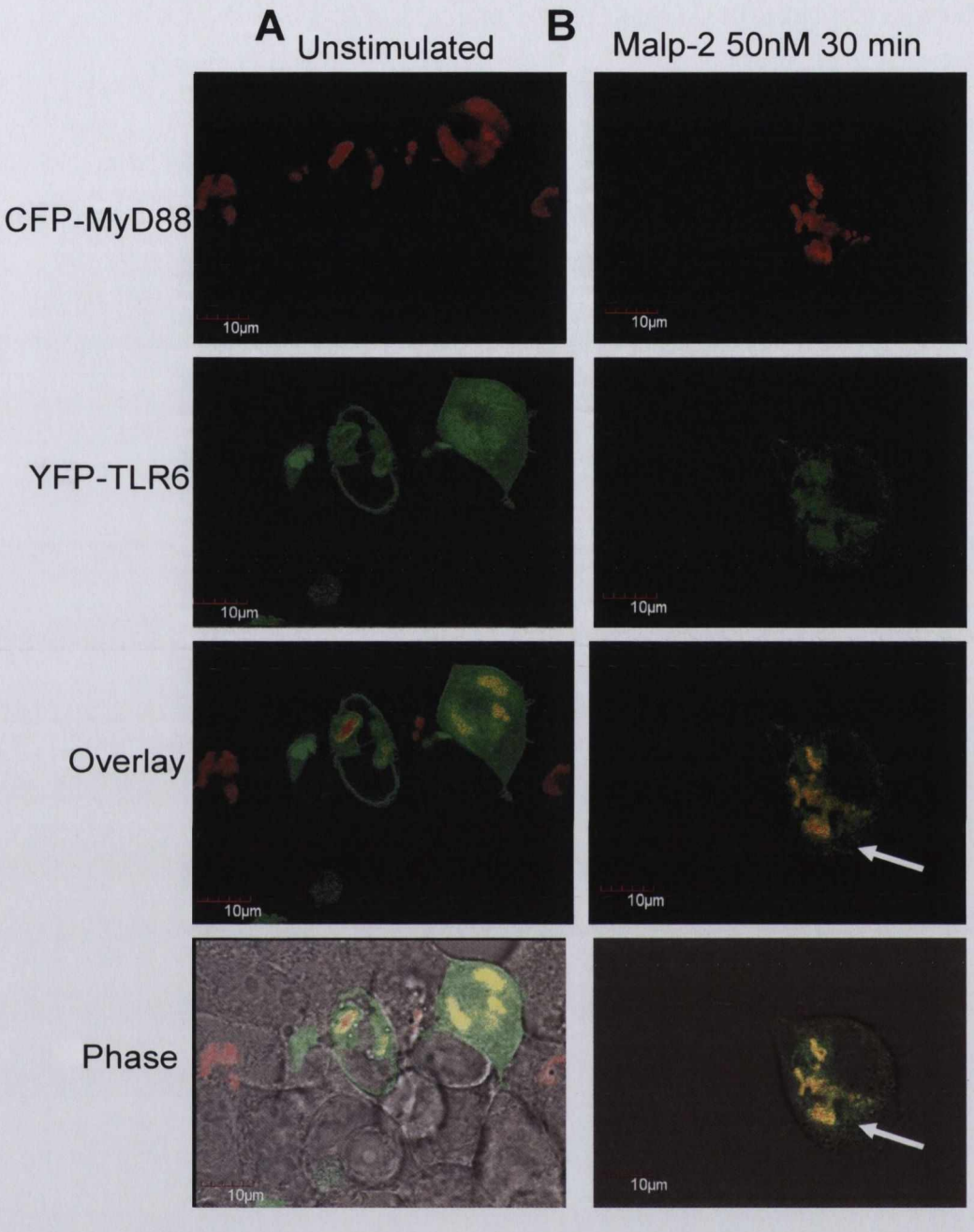

Figure 3.5. **MyD88 and TLR6 co-localise upon stimulation with 50 nM Malp-2**

HEK-293T cells were transiently transfected with 3 µg YFP-TLR6 and CFP-MyD88. 48 h post transfection the cells were examined using the Olympus Fluoview FV1000 Imaging system (A).The cells were then stimulated with 50 nM Malp-2 for 30 min and re-examined by confocal microscopy (B). An average of 20% of cells examined expressed both proteins. The cells shown are representative of three independent experiments.

3.2.3 Live cell imaging of the co-localisation patterns of Mal with TLR1, 2, 4 and 6 yielded no clear results due to transfection issues

The HEK-293T cells were also co-transfected with YFP-TLR1, TLR2, TLR4 or TLR6 and CFP-Mal and examined by live cell imaging using confocal microscopy. It proved very difficult to locate cells with both plasmids present. The plasmids were transfected in at different times and concentrations in an attempt to optimise this assay without success.

Endogenous antibodies raised against TLR1, TLR2 and TLR6 were also used in conjunction with CFP-Mal in THP1 cells but the antibodies were not suitable for use in confocal microscopy.

Cells stably transfected with YFP-TLR1, TLR2, TLR4 and TLR6 would be required to optimise this assay further.

3.2.4 Flag-tagged TLR2 and TLR6 are expressed after transfection into HEK-293Ts, but flag-TLR1 is not

To further determine where MyD88 and Mal interacted in the TLR1/2 and TLR2/6 signalling complexes co-immunoprecipitation assays using over-expression of the TLRs of interest and MyD88 and Mal were carried out.

To ensure the plasmids used to perform the co-immunoprecipitations could be expressed in HEK-293T cells the cells were transfected with flag-TLR1, TLR2 and TLR6, lysed in sample loading buffer and analysed by Western blotting with the flag antibody. As

shown in Figure 3.6A flag-TLR2 and TLR6 were expressed by the cells but flag-TLR1 was not.

The flag-TLR1 plasmid was then transfected into the HEK-293T cells at increasing concentrations to optimise its expression but as shown in Figure 3.6B the TLR1 was not detected by Western blotting. For this reason only flag-TLR2 and TLR6 were used in the co-immunoprecipitation assays.

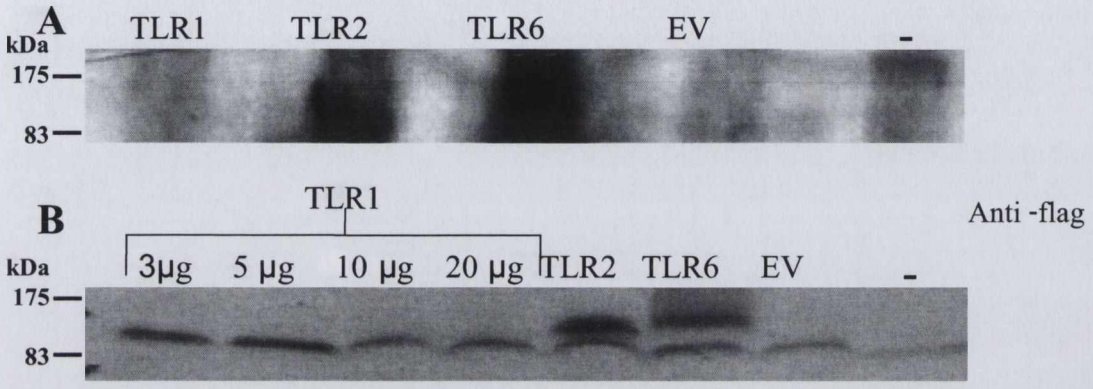

Figure 3.6. **Flag-TLR2 and 6 express in HEK-293T cells but flag-TLR1 does not** HEK-293T cells were transiently transfected with 3 µg flag-TLR1, flag-TLR2, flag-TLR6 or empty vector (EV) (A) or increasing concentrations of 3, 5, 10 and 20 µg Flag-TLR1, 3 µg TLR2, TLR6 and EV (B). 24 h post transfection the cells were lysed directly into 5x sample loading buffer, sonicated and tested for the expression of the Flag-tagged TLRs via Western blotting using the anti-flag antibody.

3.2.5 Co-immunoprecipitation assays reveal no clear interactions due to the presence of non-specific interactions in the control samples

Flag-TLR2 and Myc-MyD88 or Ha-Mal were transiently transfected into HEK-293T cells and co-immunoprecipitated. As shown in Figure 3.7A TLR2 and MyD88 did interact (lane 4, bottom panel) when the cell lysates were incubated with the flag antibody and Western blotted for the presence of Myc-MyD88. TLR2 was pulled down with the flag antibody (lane 4, top panel) and MyD88 was pulled down with the Myc antibody (lane 3, bottom panel). However, bands were also seen in the negative control lanes (lane 2, both panels) demonstrating that the flag antibody was non-specifically pulling down proteins.

As shown in Figure 3.7B a similar result was seen for the interaction between TLR2 and Mal. Lane 4 in the bottom panel revealed an interaction when the lysates were incubated with the flag antibody and the presence of Ha-Mal was examined. TLR2 was again pulled down with the flag antibody (lane 4, top panel). Mal was pulled down with the anti-Ha antibody (lane 3, bottom panel). However, non-specific interactions were also present (lane 2, both panels).

Due to the presence of bands in the negative control lanes no conclusive result was seen in the TLR2 co-immunoprecipitation assays.

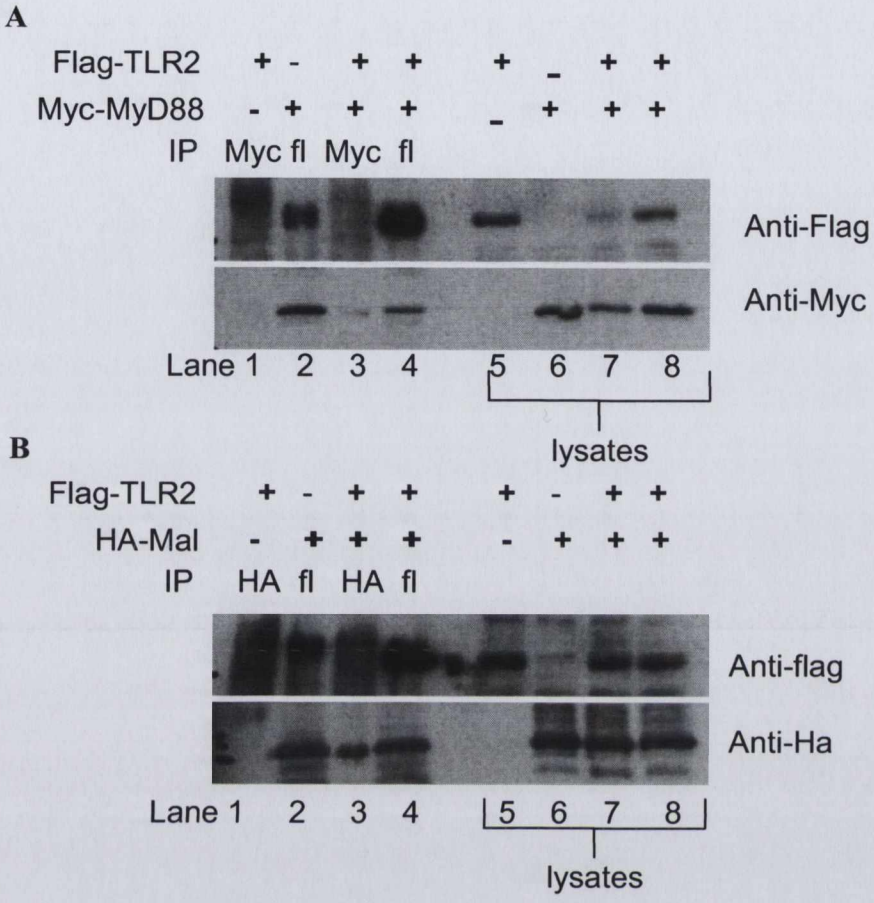

Figure 3.7. **Co-immunoprecipitation of flag-TLR2 and MyD88 or Mal does not reveal interacting partners due to non-specific interactions**

HEK-293T cells were transiently transfected with 3 μg flag-TLR2, Myc-MyD88 and EV (A) or flag-TLR2, Ha-Mal and EV (B). 24 h post transfection the cells were lysed in high stringency lysis buffer and co-immunoprecipitated with Flag, Myc or Ha antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-flag, anti-Myc or anti-Ha antibodies. These data are representative of three experiments.

Flag-TLR6 and Myc-MyD88 or Ha-Mal were transiently transfected into HEK-293T cells and co-immunoprecipitated. As illustrated in Figure 3.8A TLR6 and MyD88 did not interact when the samples were incubated with the flag antibody and Western blotted with the Myc antibody for the presence of MyD88 (lane 4, bottom panel). Flag TLR6 was pulled down with the flag antibody (lane 4, top panel) and MyD88 was pulled down with the Myc antibody (lane 3, bottom panel). However, non-specific bands were seen in the negative control lanes (lanes 1 and 2, both panels).

As demonstrated in Figure 3.8B TLR6 and Mal appeared to interact (lane 4, bottom panel). TLR6 was pulled down with the flag antibody in the same sample (lane 4, top panel) and Mal was pulled down with the Ha antibody (lane 3, bottom panel). Unfortunately non-specific bands were seen in the negative controls again (lane 2, both panels).

Due to the presence of bands in the negative control lanes no conclusive result was revealed in the TLR6 co-immunoprecipitation assays.

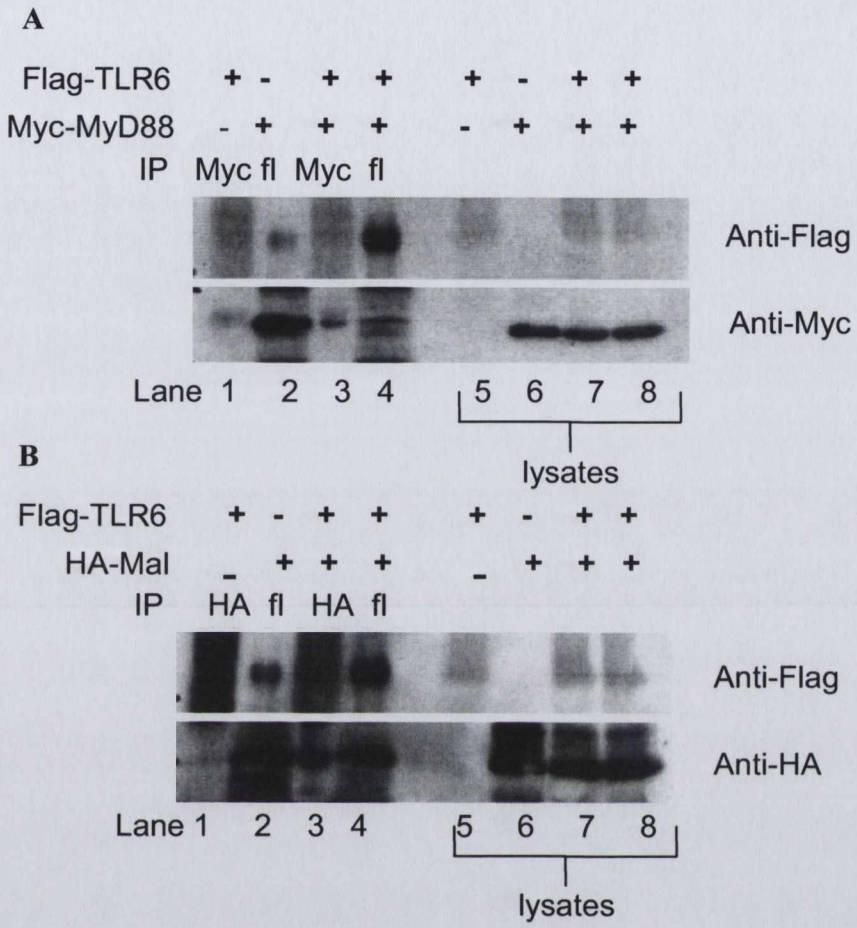

Figure 3.8. **Co-immunoprecipitation of flag-TLR6 and MyD88 or Mal does not reveal interacting partners due to non-specific interactions**

HEK-293T cells were transiently transfected with 3 μg flag-TLR6, Myc-MyD88 and EV (A) or flag-TLR6, Ha-Mal and EV (B). 24 h post transfection the cells were lysed in high stringency lysis buffer and co-immunoprecipitated with Flag, Myc or Ha antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-flag, anti-Myc or anti-Ha antibodies. These data are representative of three experiments.

3.2.6 GST-MyD88 and GST-Mal can interact with over-expressed MyD88 and Mal

When doing the co-immunoprecipitation assays I found it difficult to isolate large amounts of the over-expressed TLRs perhaps as they are transmembrane proteins and hence are difficult to remove from the cell membrane in large quantities. Due to this and the non-specific antibody interactions observed I next generated GST-MyD88 and GST-Mal and carried out GST-pulldown assays.

The GST-EV, GST-MyD88 and GST-Mal plasmids were firstly transformed into BL21 bacterial cells and the proteins were isolated by attaching them to glutathione-sepharose 4B beads. I analysed the expression levels by SDS-PAGE with coomassie staining. As shown in Figure 3.9A the GST (~25kDa) vector expressed at the highest level (lanes 1-3), GST-MyD88 (~58kDa) expressed at a lower level (lanes 4-6) and GST-Mal (~47kDa) expressed at a high level (lanes 7-9). A 1 μg/ml stock of GST was used to estimate the concentration of the fusion proteins (lane 10). The lower molecular weight bands seen in lanes 4 to 9 are most likely degradation products of the GST-MyD88 and Mal fusion proteins.

To ensure the fusion proteins were correctly folded I transiently transfected Myc-MyD88 or Ha-Mal into HEK-293T cells and incubated the lysates with GST, GST-MyD88 or GST-Mal. As illustrated in Figure 3.9B Myc-MyD88 was pulled down by GST-MyD88 (lane 2) but not GST-Mal (lane 3). The lack of interaction between MyD88 and GST-Mal in this assay may be due to the low level of Myc-MyD88 expression in the samples as is shown by the non-specific bands in lanes 10 to 12. The interaction between MyD88 and Mal may not be as robust as the MyD88 homodimer interaction. Ha-Mal, on the other

hand, was pulled down with both GST-MyD88 and GST-Mal as demonstrated in Figure 3.9C lanes 2 and 3.

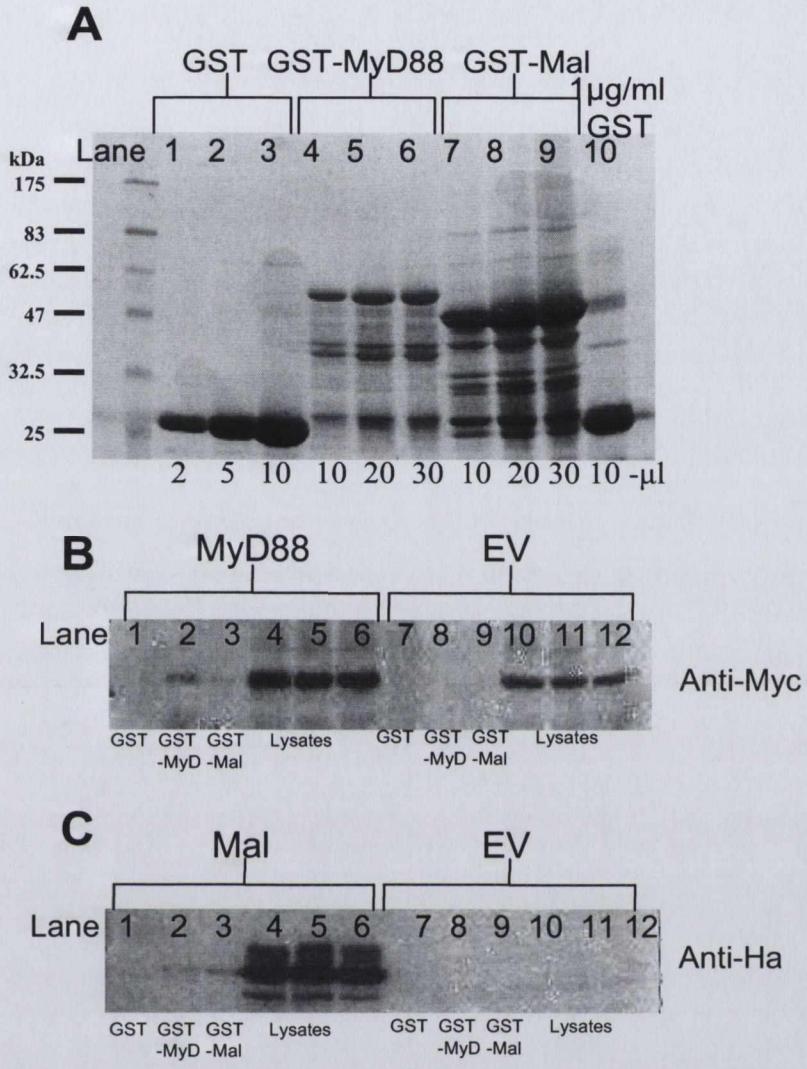

Figure 3.9. **GST-MyD88 and GST-Mal can pull down MyD88 and Mal**

BL21 bacterial cells were transformed with pGex-4T2, pGex-4T2-MyD88 and Mal. The bacteria were lysed and the GST, GST-MyD88 and GST-Mal were isolated by incubation with glutathoine S-transferase beads for 3 h at 4^0C and analysed by SDS-PAGE and coomassie staining (A). HEK-293T cells were transiently transfected with 3 µg Myc-MyD88 and EV (B) or Ha-Mal and EV (C). 24 h post transfection the cells were lysed in low stringency lysis buffer and incubated with GST, GST-MyD88 or GST-Mal for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using anti-Myc or anti-Ha antibodies.

3.2.7 GST-MyD88 and GST-Mal can not pull down endogenous TLR1, TLR2 or TLR6

As the GST-MyD88 and GST-Mal were capable of pulling MyD88 and Mal down their ability to pull down endogenous TLR1, TLR2 or TLR6 from THP1 cells was next tested. As demonstrated in Figure 3.10A GST-MyD88 and GST-Mal were not able to pull down TLR1 (lanes 2 and 3). They were also unable to pull down TLR2 as seen in Figure 3.10B lanes 2 and 3, or TLR6 as shown in Figure 3.10C lanes 2 and 3.

The inability to see any interacting partners in these assays may have been due to difficulties in isolating large amount of the TLRs of interest from the plasma membrane of the THP1 cells. I increased the cell numbers used and the length of cell lysis in an attempt to optimise this assay, with no success. The endogenous antibodies were also difficult to optimise as they required a high concentration of TLR present in the lysates for the appearance of a band. Therefore an interacting band may have been too weak to be seen by Western blotting.

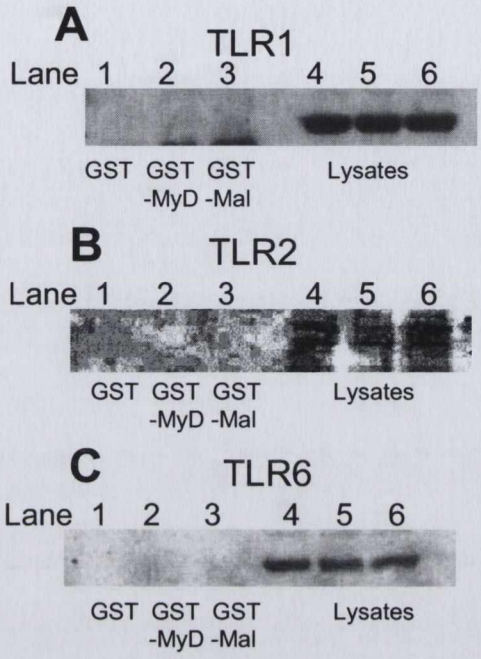

Figure 3.10. **GST-MyD88 and GST-Mal cannot pull down endogenous TLR1, TLR2 or TLR6 in THP-1 cells**

$2x10^7$ THP1 cells per point were lysed in 1% CHAPs lysis buffer and incubated with GST, GST-MyD88 or GST-Mal for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using anti-TLR1 (A), anti-TLR2 (B) or anti-TLR6 (C) antibodies. These data are representative of three experiments.

3.2.8 GST-MyD88 and GST-Mal can not pull down over-expressed TLR2 or TLR6

As I was unable to pull down endogenous TLRs with GST-MyD88 and GST-Mal I returned to over-expression of the TLRs of interest. I transfected flag-TLR2 or flag-TLR6 into the HEK-293T cells and carried out a GST-pulldown assay.

As seen in Figure 3.11A GST-MyD88 and GST-Mal were unable to pull down flag-TLR2 (lanes 2 and 3). TLR6 was also not pulled down by GST-MyD88 or GST-Mal as shown in Figure 3.11B lanes 2 and 3.

In these assays I again found it difficult to consistently isolate large amounts of the TLRs of interest. In an attempt to optimise this I used several lysis buffers and transfected in increasing amounts of the TLRs. I also increased the length of cell lysis to no avail. As with the endogenous co-immunoprecipitation assays I did not consistently see similar levels of TLR expression and therefore the bands revealing any interactions were difficult to identify by Western blotting.

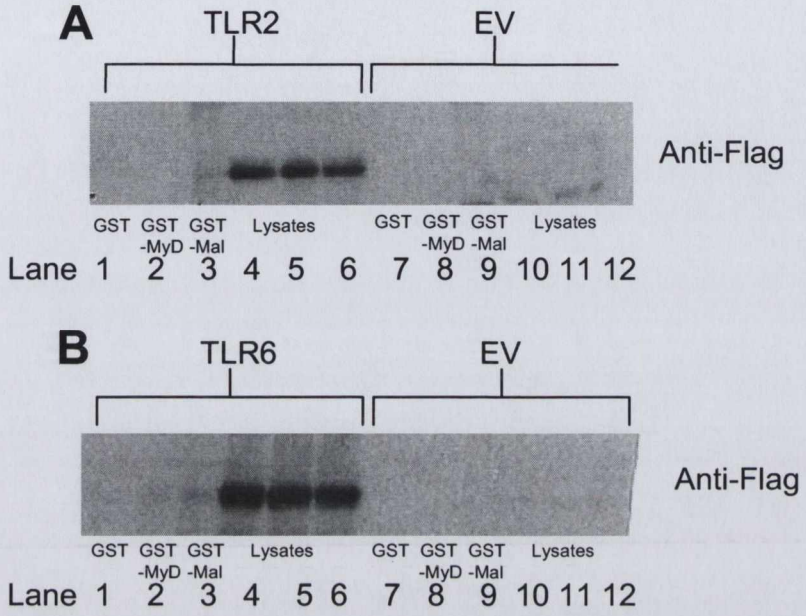

Figure 3.11. **GST-MyD88 and GST-Mal cannot pull down over-expressed TLR2 or TLR6**

HEK-293T cells were transiently transfected with 3 μg (A) flag-TLR2, (B) flag-TLR6 or EV. 24 h post transfection the cells were lysed in high stringency lysis buffer and incubated with GST, GST-MyD88 or GST-Mal for 3 h at 4^0C. The beads were washed three times and analysed by SDS-PAGE and Western blotting using the anti-flag antibody. These data are representative of three experiments.

3.2.9 GST-MyD88 and GST-Mal do not interact with TLR2 or TLR6 after membrane fractionation

I next attempted to increase the concentration of TLR2 and TLR6 in the cell lysates used in the GST- pulldown assay by carrying out membrane fractionation on the cell lysates from the HEK-293T cells in order to remove the cytosolic fraction.

As demonstrated in Figure 3.12A TLR2 was found in the membrane fraction (lane 1) and the whole cell lysates (lane 3) of the HEK-293T cells but not the cytosolic fraction (lane 2). Similarly TLR6 was found in the membrane fraction in Figure 3.12B (lane 1) and the whole cell lysate (lane 3) but not the cytosolic fraction (lane 2).

As I was able to isolate the TLRs to the membrane fraction I next incubated this fraction with GST, GST-MyD88 or GST-Mal to attempt to pull down the TLRs. As shown in Figure 3.12C GST-MyD88 was unable to pull down TLR2 (lane 2) but GST-Mal could interact with TLR2 (lane 3). Unfortunately I was unable to consistently repeat this. Figure 3.12D shows that GST-MyD88 and GST-Mal were unable to pull down TLR6 (lanes 2 and 3, respectively).

Therefore increasing the concentration of TLR2 or TLR6 through membrane fractionation did not aid in revealing the interactions between them and GST-MyD88 or GST-Mal.

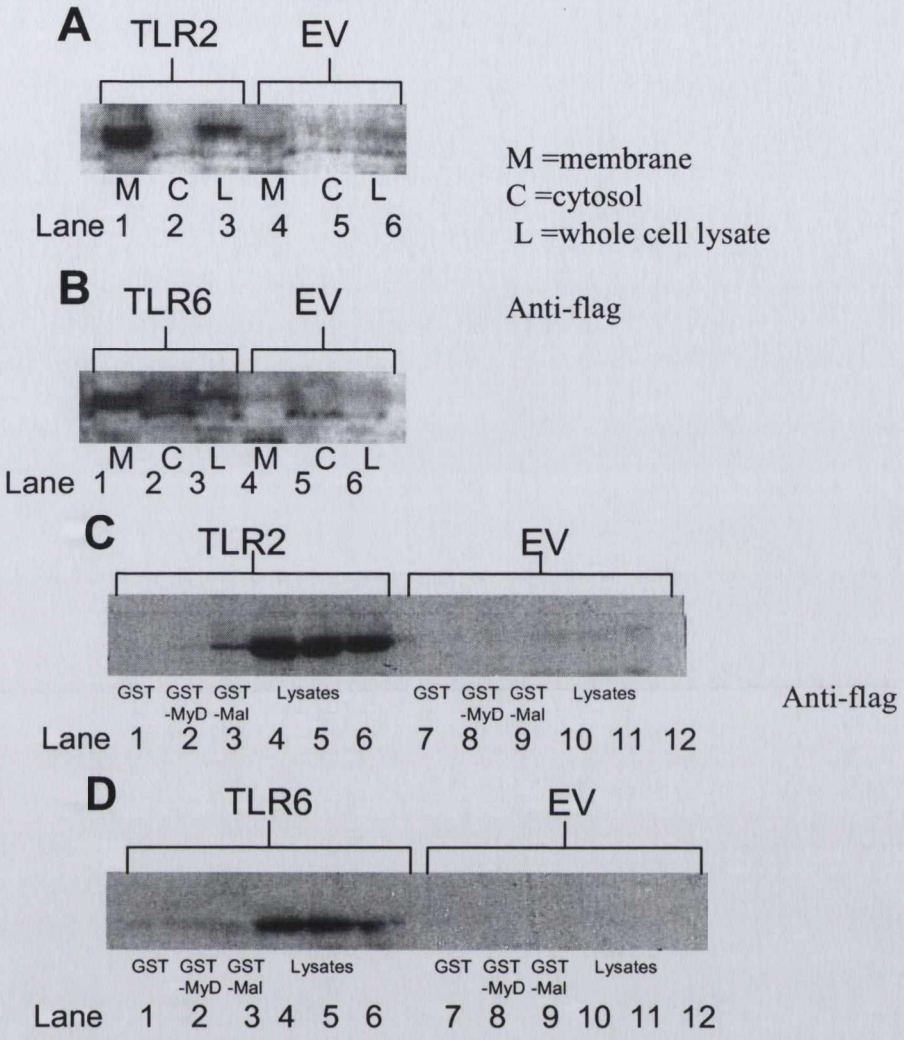

Figure 3.12. **Membrane fractionation increased the concentration of TLR2 and TLR6 in cell lysates but did not reveal clear interacting partners in a GST-pulldown assay with GST-MyD88 or GST-Mal**

HEK-293T cells were transiently transfected with 3 μg flag-TLR2 and EV (A & C) or flag-TLR6 and EV (B & D). 24 h post transfection the cells were lysed in membrane lysis buffer, douce homogenised 35 times and centrifuged at 50 Krpm for 50 min. The pellets were lysed in 5x sample loading buffer and analysed by Western blotting (A & B). The pellets were lysed in high stringency lysis buffer and incubated with GST, GST-MyD88 or GST-Mal for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-flag antibody (C & D).

3.2.10 Generation of GST-TLR1, TLR2 and TLR6 fusion proteins

When examining the TLR2 and TLR6 interactions with MyD88 and Mal by GST-pulldown it was very difficult to isolate a large amount of the TLRs from the cell membranes making the GST-pulldowns difficult to optimise. Due to these difficulties I next generated GST-fusion proteins containing the TIR domains of TLR1, TLR2 and TLR6.

The sequences of the TIR domains of TLR1, TLR2 and TLR6 were retrieved from the NCBI database and primers with an *EcoR* I and a *Sal* I at either end were designed. The TIR domains were then amplified out of the flag or YFP-tagged TLR vectors and levels of amplification can be seen in Figure 3.13A. The PCR products were removed from the gel, restriction digested with the *EcoR* I and *Sal* I enzymes and ligated into the pGex-4T2 vector shown in Figure 3.13B.

The vectors were transformed into DH5-α bacterial cells, the DNA was isolated and again restriction digested with the *EcoR* I and *Sal* I enzymes to ensure the insert had gone into the pGex vector. As shown in Figure 3.13C a 500 bp band was seen for all the TLRs. Several samples of DNA were then sequenced to ensure no mutations occurred during the PCR process and as shown in Figure 3.14 the sequences of the three GST-TIR domains generated were identical to the TIR domain sequences from the NCBI database when aligned using ClustalW2 (Thompson et al., 1994).

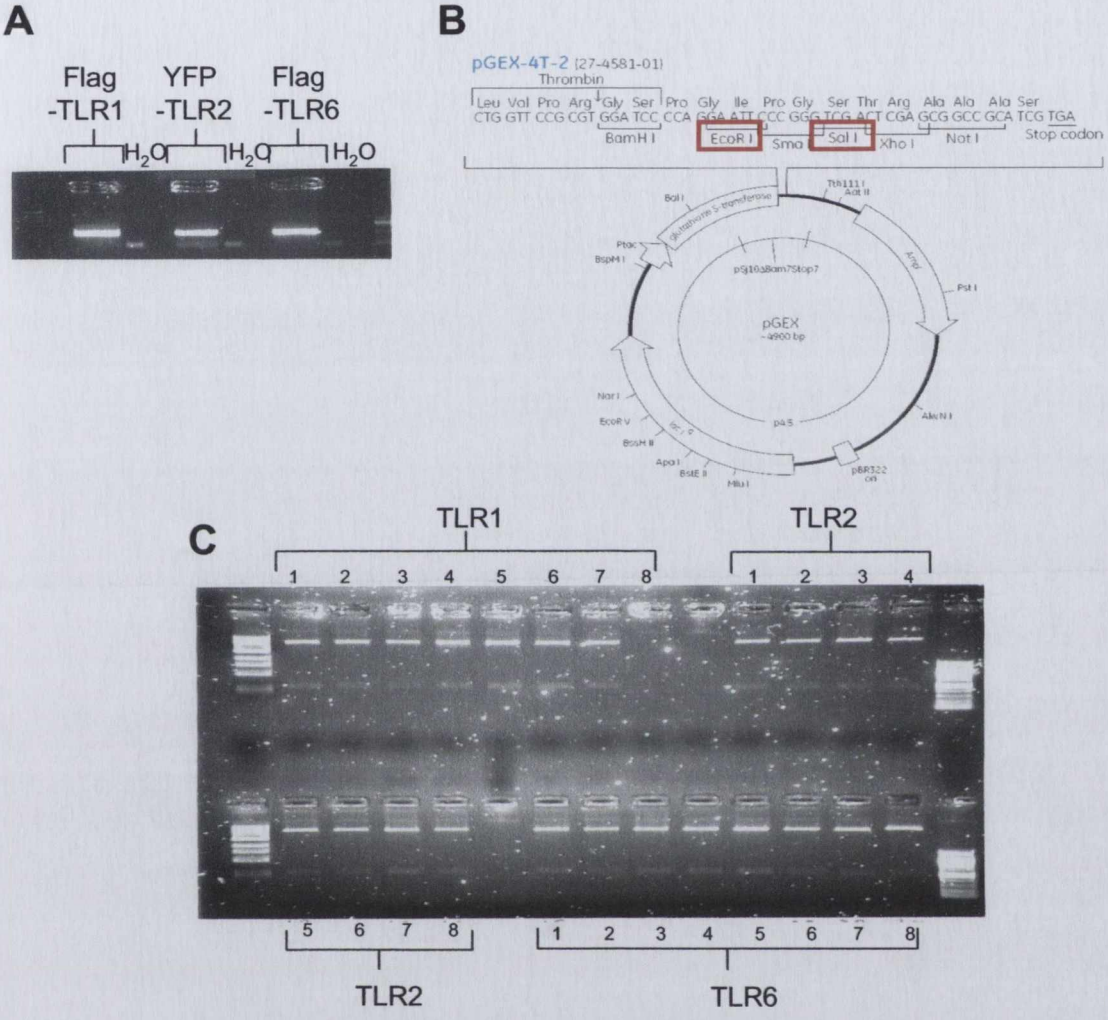

Figure 3.13. **TLR1, TLR2 and TLR6 TIR domains were sub-cloned from YFP and flag vectors into the pGex-4T2 vector**

The TIR domains of flag-TLR1, YFP-TLR2 and flag-TLR6 were amplified out of their plasmids (A), digested with the *EcoR* I and *Sal* I restriction enzymes, and ligated into the pGex-4T2 vector (B). The plasmids were then grown in DH5-α cells, the DNA was isolated and restriction digests were carried out to confirm the presence of the inserts (C).

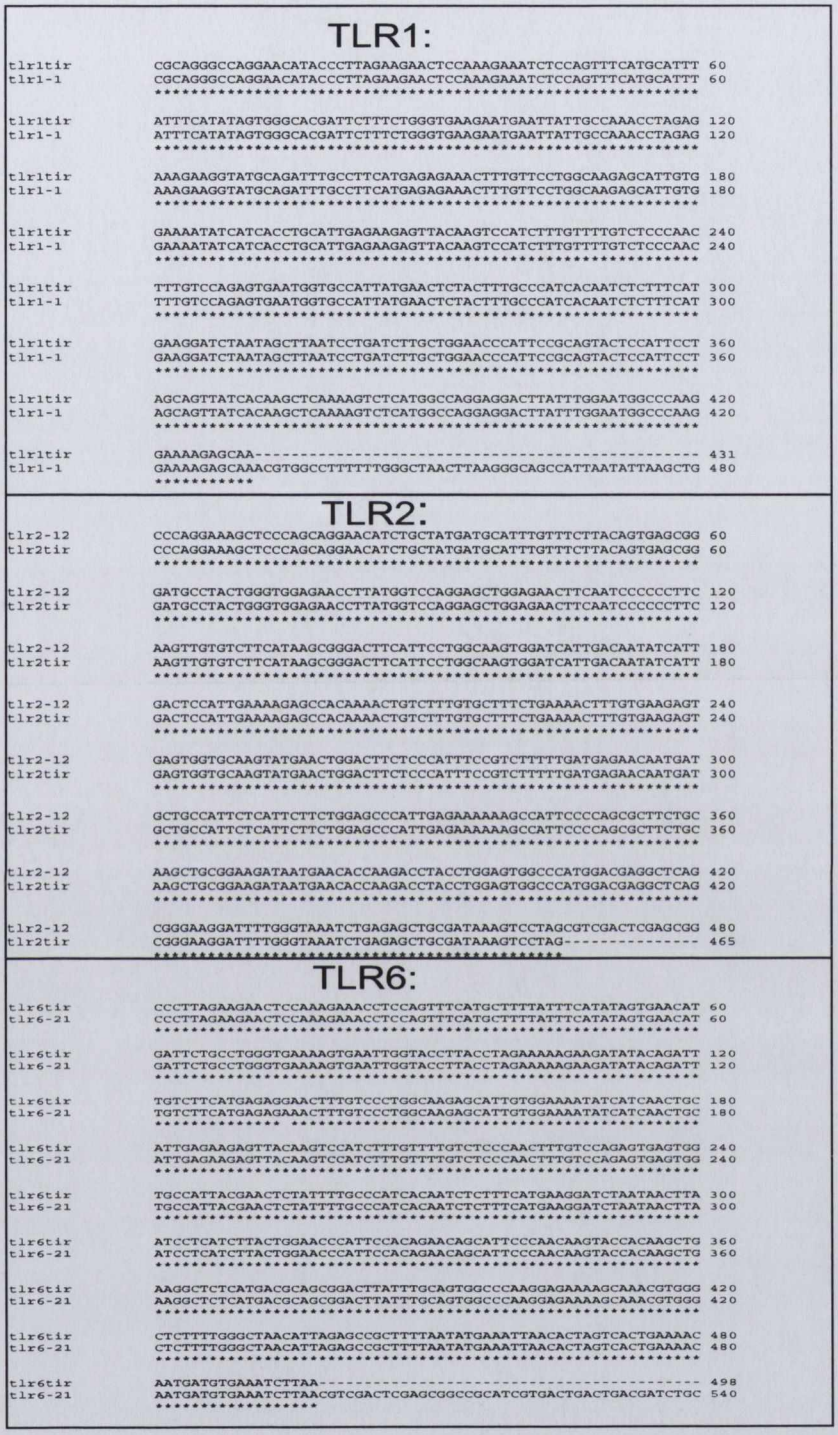

Figure 3.14. **The TIR domains of TLR1, TLR2 and TLR6 sub-cloned into the pGex-4T2 are complementary to the TLR TIR domain sequences in the NCBI database**

The pGex-4T2 vectors were sequenced and the results were compared with the known sequences of TLR1, TLR2 and TLR6. Sequence alignments were carried out using ClustalW2 (Thompson et al., 1994).

The GST-TLR1, TLR2, TLR4 and TLR6-TIR plasmids were transformed into Rosetta gami 2 bacterial cells and the protein was isolated by incubating the lysed bacteria with glutathione-sepharose 4B beads. 5, 10 and 20 µl of each protein were analysed by SDS-PAGE and coomassie staining.

As shown in Figure 3.15 all the GST-fusion proteins were expressed at different levels. From these stained gels it was decided to use 0.5 µl GST, 2 µl GST-TLR1, 20 µl GST-TLR2 and TLR6 and 100 µl GST-TLR4 for the GST-pulldown assays.

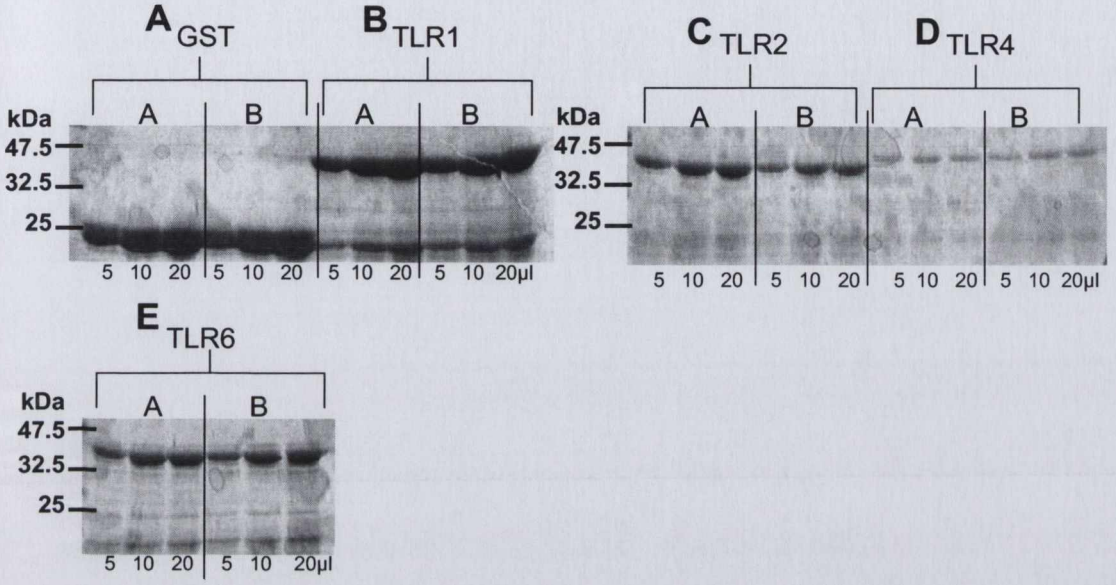

Figure 3.15. **GST, GST-TIR TLR1, TLR2, TLR4 and TLR6 are expressed in bacteria and can be isolated from them**

Rosetta gami 2 bacterial cells were transformed with the pGex-4T2 plasmids containing GST, GST-TIR-TLR1, TLR2, TLR4 or TLR6. A single colony of each was grown over night at 37^0C, transferred to a large culture and grown at 37^0C until the OD at 600 nm was 0.5. 100 µM IPTG was added to each culture and the bacteria were incubated overnight at 18^0C. The cells were pelleted, lysed and the GST-fusion proteins were isolated by incubation with glutathione-sepharose 4B beads at 4^0C for 3 h. 5, 10 and 20 µl of GST (A), GST-TLR1 (B), GST-TLR2 (C), GST-TLR4 (D) and GST-TLR6 (E) were analysed by SDS-PAGE with coomassie staining.

3.2.11 MyD88 interacts with TLR2, TLR4 and TLR6, Mal interacts with TLR2 and TLR4 only in a GST-pulldown assay

I next used the GST-TIR domain fusion proteins in GST-pulldown assays with Myc-MyD88 and Ha-Mal. HEK-293Ts were transiently transfected with either MyD88 or Mal and the lysates were incubated with GST, GST-TLR1, TLR2, TLR4 or TLR6. As illustrated in Figure 3.16A Myc-MyD88 was pulled down with TLR2 (lane 3, top panel), TLR4 (lane 2, bottom panel) and TLR6 (lane 3, bottom panel) but not with TLR1 (lane 2, top panel) as that band was comparable to the GST band in lane 1 and as such was considered background. No non-specific bands were seen when the empty vector samples were tested (lanes 4 to 6, both panels) or in the presence of the GST-EV control (lane 1, both panels).

As shown in Figure 3.16B Ha-Mal was pulled down with TLR2 (lane 3, top panel) and TLR4 (lane 2, bottom panel) but not with TLR1 (lane 2, top panel) or TLR6 (lane 3, bottom panel). All controls were correct (lanes 1 and 4 to 6, both panels).

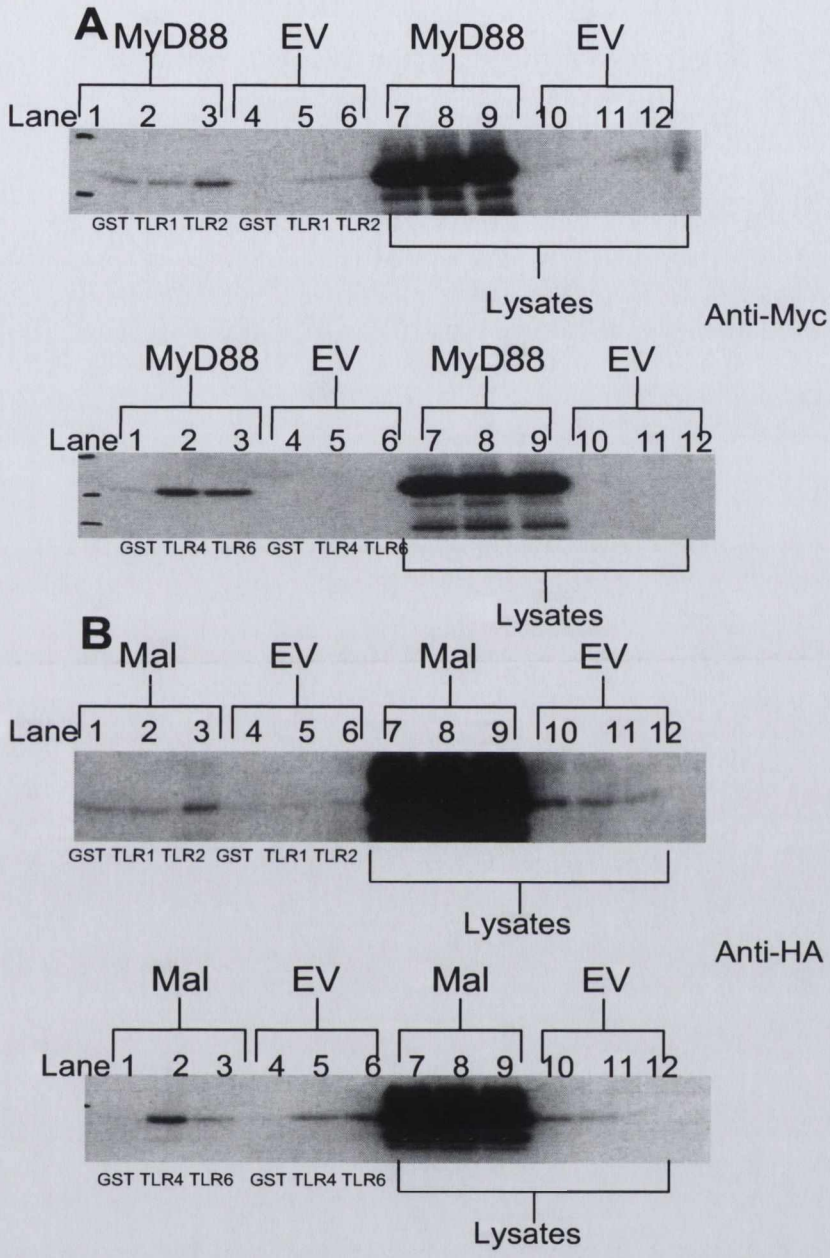

Figure 3.16. **MyD88 interacts with TLR2, TLR4 and TLR6 but Mal interacts with TLR2 and TLR4 only**

HEK-293T cells were transiently transfected with 3 μg Myc-MyD88 (A), Ha-Mal (B) or EV. 24 h post transfection the cells were lysed in low stringency lysis buffer, pre-cleared twice in glutathione-sepharose 4B beads for 45 min and incubated with GST, GST-TLR1, TLR2, TLR4 or TLR6 for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using anti-Myc or anti-Ha antibodies.

3.2.12 MyD88 interacts with GST-TLR1 in a concentration-dependent manner

To further examine if TLR1 could interact with MyD88 increasing amounts of GST-TLR1 were incubated with Myc-MyD88. As shown in Figure 3.17A 5 and 7 µl GST-TLR1 could pull down a small amount of Myc-MyD88 (lanes 2 and 3). When the amount of TLR1 was increased to 10 and 12 µl as in Figure 3.17B (lanes 2 and 3) Myc-MyD88 was again pulled down but to a greater extent. Finally 15 and 20 µl GST-TLR1 also pulled down Myc-MyD88 as shown in Figure 3.17C lanes 2 and 3. The empty vector control lanes showed no non-specific interactions (lanes 1 and 4 to 6, all panels).

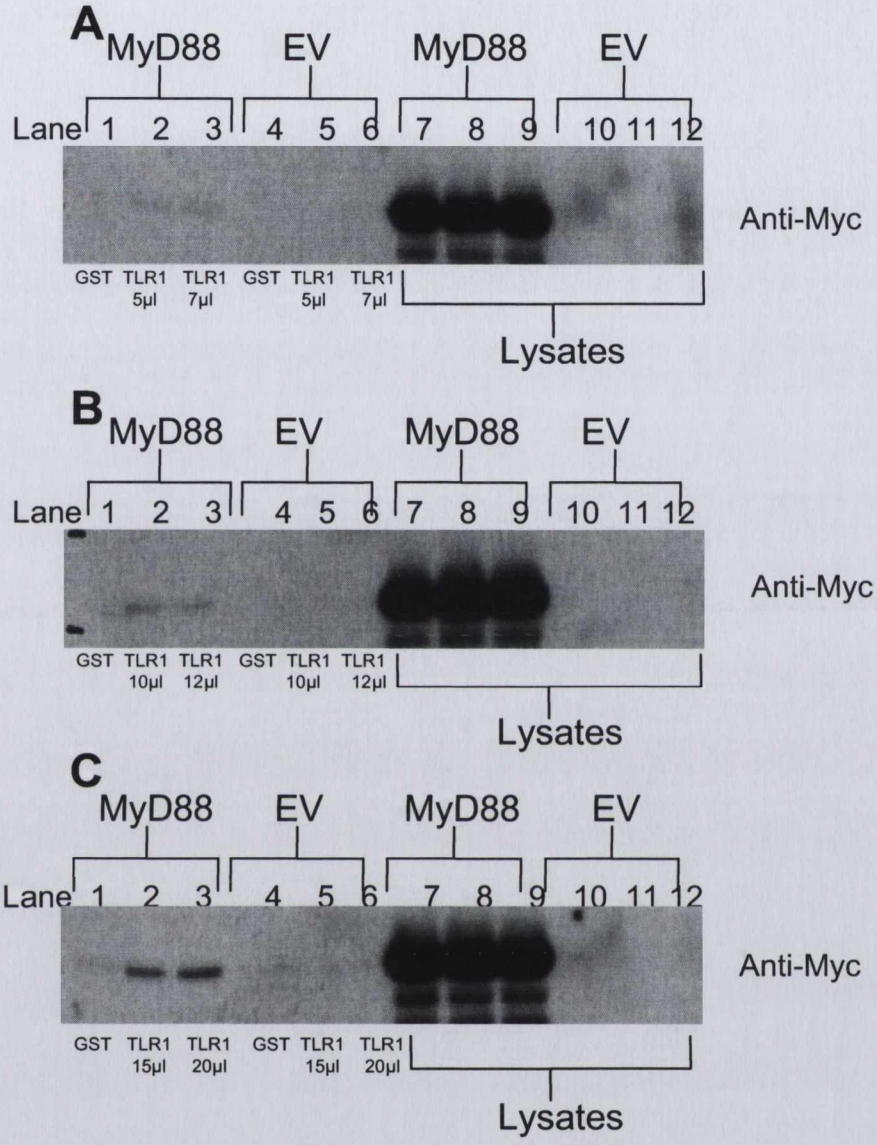

Figure 3.17. **MyD88 interacts with TLR1 in a concentration-dependent manner** HEK-293T cells were transiently transfected with 3 µg Myc-MyD88 or EV. 24 h post transfection the cells were lysed in low stringency lysis buffer, pre-cleared in glutathione-sepharose 4B beads twice for 45 min and incubated with GST, or 5, 7 (A), 10, 12 (B), 15 or 20 µl (C) GST-TLR1 for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-Myc antibody.

3.2.13 GST-TLR1 does not interact with Caspase1 and the GST-TLRs do not interact non-specifically with all TIR containing proteins

To ensure the interaction between TLR1 and MyD88 was specific and not due to the large amount of TLR1 incubated with the cell lysates I next repeated the GST-pulldown assay with TLR1 and MyD88, Mal, TRIF and Caspase1 as the control.

As demonstrated in Figure 3.18A TLR1 pulled down Myc-MyD88 (lane 2), TLR1 also pulled down Ha-Mal in Figure 3.18B (lane 2). TLR1, however, was unable to pull down Caspase1 as shown in Figure 3.18C (lane 2) verifying the interactions between TLR1 and MyD88 and, surprisingly, Mal were specific. There were some non-specific interactions as shown by the empty vector control lanes (lanes 1, 3 and 4, all panels).

To ensure that the GST-pulldown interactions between TLR1, TLR2, TLR4 and TLR6 and MyD88 and Mal were specific and not due to a non-specific TIR-TIR interaction I next tested their ability to pull down TRIF, an adaptor protein not directly linked to any of the TLRs of interest. As shown in Figure 3.19A GST-TLR1 and GST-TLR2 did not pull down Ha-TRIF (lanes 2 and 3, respectively). GST-TLR4 and GST-TLR6 also did not pull down TRIF as shown by Figure 3.19B lanes 2 and 3. All controls were as expected (lanes 1 and 4 to 6, both panels).

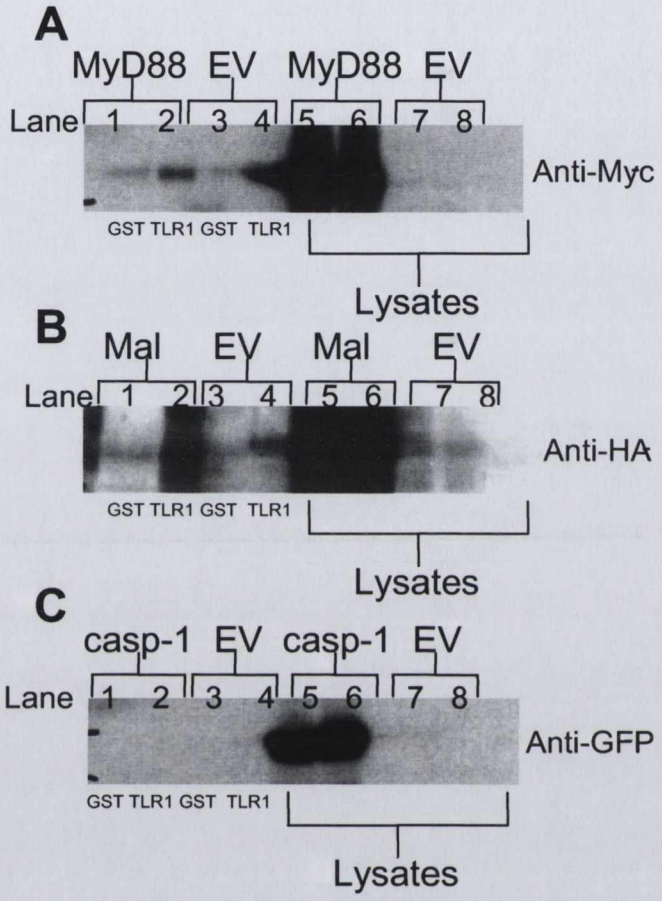

Figure 3.18. **GST-TLR1 interacts with MyD88 and Mal but not Caspase 1**

HEK-293T cells were transiently transfected with 3 μg Myc-MyD88 (A), Ha-Mal (B), GFP-Caspase 1 (C) and EV for 24 h, lysed in low stringency lysis buffer and incubated with GST-fusion protein containing the TIR domain of TLR1, for 3 h at 4^0C. The samples were washed three times and analysed by Western blotting with anti-Myc, Ha and GFP antibodies.

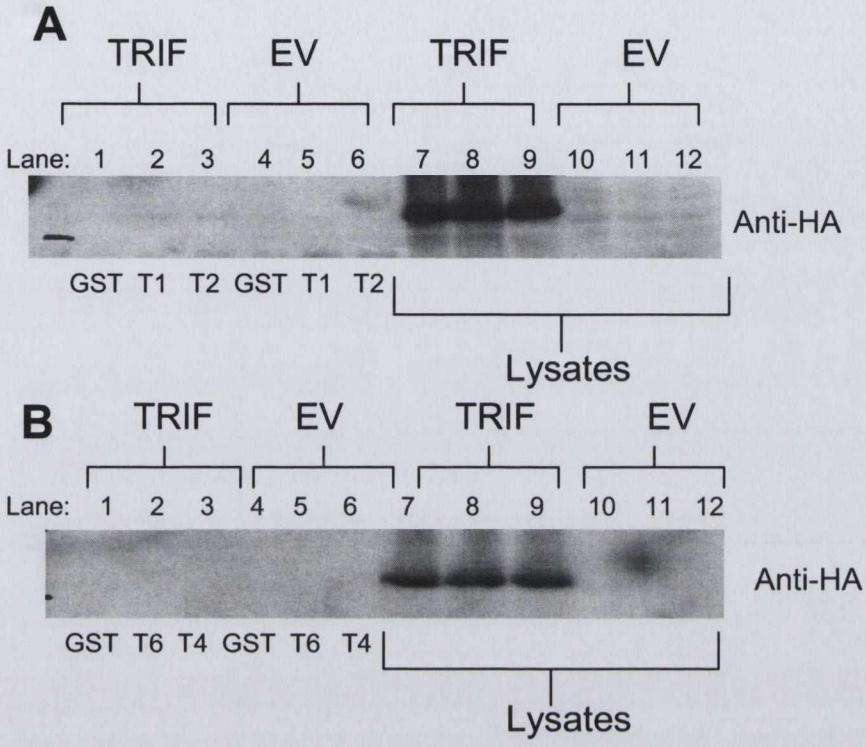

Figure 3.19. **TRIF does not interact with TLR1, TLR2, TLR4 and TLR6**

HEK-293T cells were transiently transfected with 3 μg Ha-TRIF or EV for 24 h, lysed in low stringency lysis buffer, pre-cleared twice in glutathione-sepharose 4B beads for 45 min and incubated with GST-fusion proteins containing the TIR domain of TLR1, TLR2 (A), TLR4 and TLR6 (B) for 3 h at 4^0C. The samples were washed three times and analysed by Western blotting with anti-Ha antibodies. These data are representative of three experiments.

3.2.14 MyD88 interacts with TLR1, TLR2, TLR4 and TLR6, Mal interacts with TLR1, TLR2 and TLR4 only

As I had been able to show TLR1 could interact with MyD88 and Mal if a higher amount of GST-TLR1 was used I next repeated the GST-pulldown with TLR1, TLR2, TLR4, TLR6, MyD88 and Mal. 1 µl of GST-EV, 20 µl of GST-TLR1, TLR2 and TLR6 and 100 µl of GST-TLR4 were incubated with the cell lysates containing either MyD88 or Mal.

As shown in Figure 3.20A TLR1 and TLR2 pulled down Myc-MyD88 (lanes 2 and 3, top panel). TLR4 and TLR6 also pulled down MyD88 (lane 2 and 3, bottom panel). As illustrated in Figure 3.20B Mal was pulled down with TLR1, TLR2 (lane 2 and 3, top panel) and TLR4 (lane 2, bottom panel) but not TLR6 (lane 3, bottom panel). All empty vector controls were blank (lanes 1 and 4 to 6, all panels).

These assays therefore revealed for the first time the interactions between TLR1 and MyD88 and Mal and between TLR6 and MyD88. Direct interactions between TLR2 and MyD88 and TLR4 and MyD88 were also revealed casting doubt over the bridging role of Mal to these TLRs.

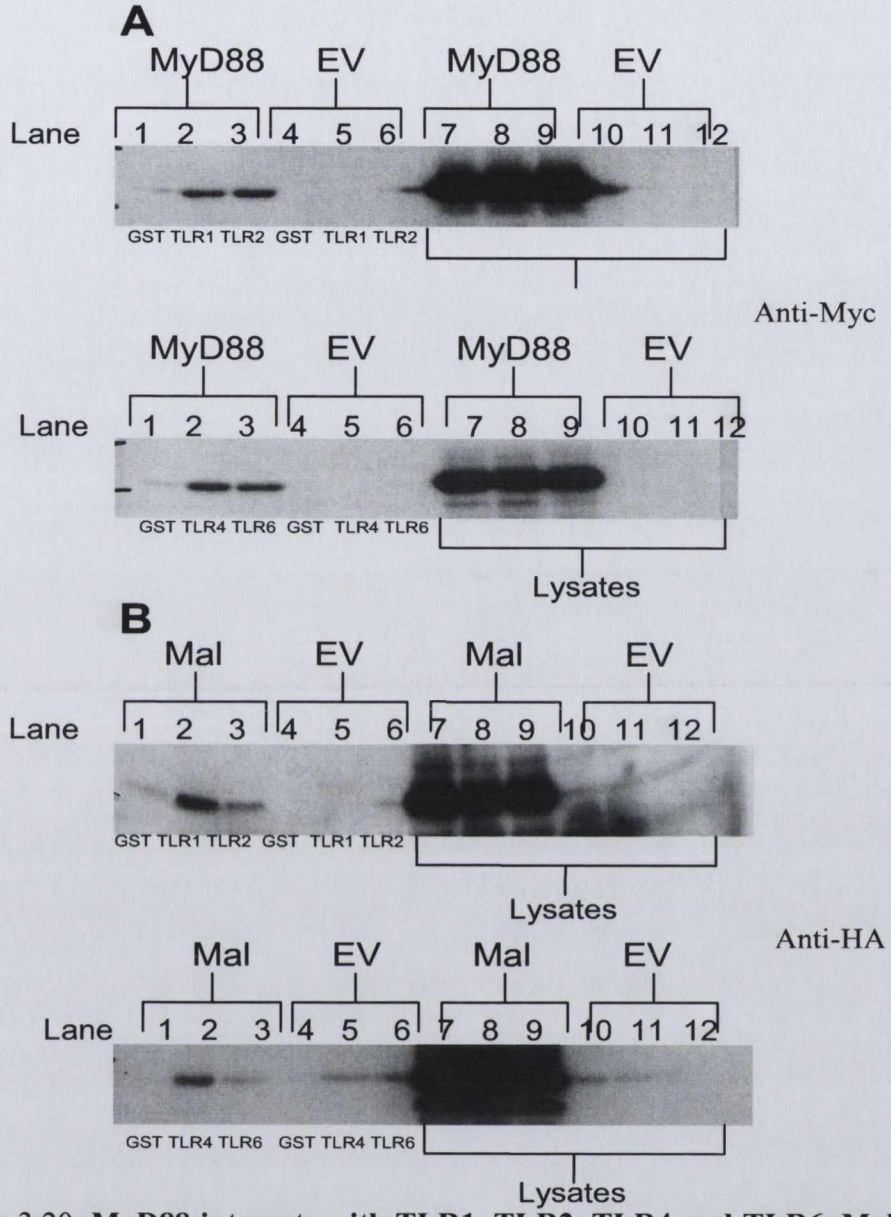

Figure 3.20. **MyD88 interacts with TLR1, TLR2, TLR4 and TLR6, Mal interacts with TLR1, TLR2 and TLR4 but not TLR6**

HEK-293T cells were transiently transfected with 3 μg Myc-MyD88 (A) or Ha-Mal (B) for 24 h, lysed in low stringency lysis buffer, pre-cleared twice in glutathione-sepharose 4B beads for 45 min and incubated with GST-fusion proteins containing the TIR domain of TLR1, TLR2, TLR4 and TLR6 for 3 h at 4^0C. The samples were washed three times and analysed by Western blotting with anti-Myc and anti-Ha antibodies. These data are representative of three experiments.

3.3 Results: Signalling experiments

As revealed by the interaction experiments above TLR1, TLR2 and TLR4 interact with both Mal and MyD88 and TLR6 only interacts with MyD88. The next aim of the project was therefore to clarify if Mal and MyD88 were required for the production of cytokines and activation of down stream signalling molecules in response to TLR1/2, TLR2/6 and TLR4 stimulation with Pam_3CSK4, Malp-2 and LPS respectively. In order to do this macrophages and dendritic cells from wild type, MyD88- and Mal-deficient mice were used.

3.3.1 MyD88 is absolutely required for TLR2 signalling but Mal is not in immortalised macrophages

To clarify the roles Mal and MyD88 play in TLR2 and TLR4 signalling immortalised bone marrow derived macrophages from wild type, MyD88- and Mal-deficient mice were tested for their ability to produce IL6 upon TLR stimulation. The macrophages were treated with the ligands indicated in Figure 3.21 for 18 h and tested for the production of IL6 by ELISA.

As shown in Figure 3.21A stimulation of the wild type macrophages (black bars) at all concentrations of the TLR1/2 ligand Pam_3CSK4 tested (20, 50 and 200 nM) resulted in the production of IL6. Stimulation of the MyD88-deficient macrophages (grey bars) did not induce the production of IL6 at any concentration tested. The Mal-deficient macrophages (white bars) were capable of the induction of IL6 at all three ligand concentrations tested.

At ligand concentrations of 20 and 50 nM the level of IL6 was much lower than that seen for the wild type cells but a 4 to 5 fold induction still was seen in the Mal-deficient macrophages. At the 200 nM concentration the Mal-deficient macrophages were capable of inducing IL6 production to levels closer to the amounts produced by the wild type cells with 13 fold induction in the Mal-deficient cells compared to 16 fold induction in the wild type cells.

As shown in Figure 3.21B a similar trend was seen in these cells when treated with the TLR2/6 ligand Malp-2 at 20, 50 and 200 nM concentrations. The wild type macrophages produced IL6 at all ligand concentrations tested, the MyD88-deficient macrophages could not produce IL6 in response any concentration of Malp-2 and the Mal-deficient cells induced IL6 production at all three concentrations of ligand although to a lower level than the wild type cells. Again at the 200 nM concentration the Mal-deficient macrophages produced IL6 at levels close to that of the wild type cells (30 fold compared to 45 fold in the wild type cells).

I next treated the wild type, MyD88- and Mal-deficient macrophages with 50, 75 and 100 ng/ml LPS for 18 h and tested for the production of IL6. As shown in Figure 3.21C the wild type cells produced IL6 at all concentrations of LPS tested. The MyD88-deficient macrophages were unresponsive at all three concentrations of LPS as was seen above for the TLR2 ligands. The Mal-deficient macrophages were also unable to produce IL6 in response to LPS which differed from the results seen with TLR2 ligand stimulation.

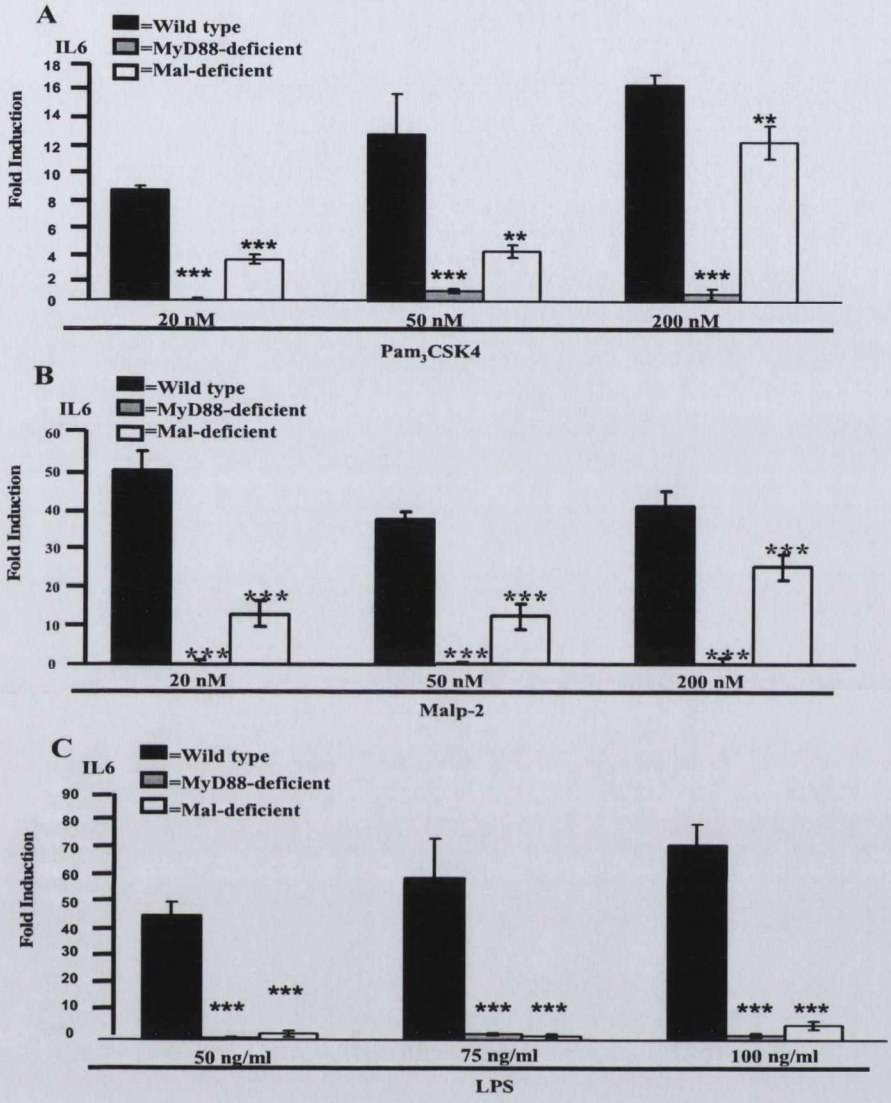

Figure 3.21. **IL6 production in response to TLR2 ligands is absolutely dependent on MyD88 but not Mal and in response to LPS, the TLR4 ligand, is dependent on both**

Immortalised macrophages derived from the bone marrow of wild type, MyD88- and Mal-deficient mice were treated with 20, 50 and 200 nM of either Pam_3CSK4 (A) or Malp-2 (B) and 50, 75, and 100 ng/ml LPS (C). After 18 h incubations IL6 production was measured by ELISA. Data are expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. These data are representative of three experiments. ***, $p < 0.005$, **, $p < 0.01$; significant differences between wild type, MyD88- and Mal-deficient macrophages.

3.3.2 TLR1-, TLR2- and TLR6-deficient macrophages respond to TLR2 ligands as anticipated

To ensure the Pam_3CSK4 and Malp-2 were in fact TLR1/2 and TLR2/6 ligands primary wild type, TLR1-, TLR2- and TLR6-deficient bone marrow derived macrophages were tested for the production of IL6 after 18 h stimulations with these ligands.

As shown in Figure 3.22A the TLR1- and TLR2-deficient (pale grey and white bars) cells were unable to produce IL6 in response to Pam_3CSK4 at all concentrations tested (20, 50, and 200 nM), as anticipated. The wild type (black bars) and TLR6-deficient macrophages (dark grey bars) induced the production of IL6 at comparable levels at all ligand concentrations tested.

As shown in Figure 3.22B the TLR2- and TLR6-deficient macrophages were unable to produce IL6 in response to Malp-2 at all concentrations tested (20, 50 and 200 nM). The wild type and TLR1-deficient macrophages induced the production of IL6 at all ligand concentrations tested.

As a control the cells were also treated with 50 ng/ml LPS and 25 μg/ml PolyIC for 18 h. As shown in Figure 3.22C the TLR1-, TLR2- and TLR6-deficient macrophages produced IL6 to similar or increased levels when compared to the wild type cells.

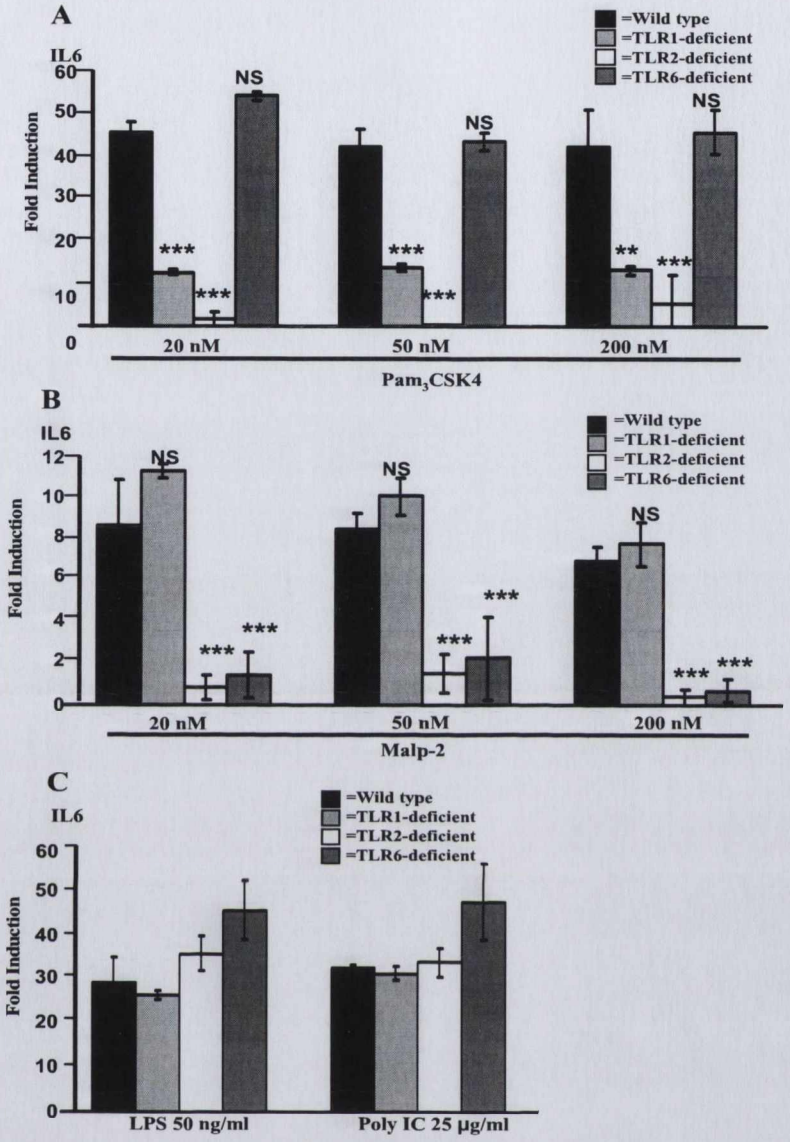

Figure 3.22. **IL6 production in response to Pam_3CSK4 is TLR1/2-dependent and in response to Malp-2 is TLR2/6-dependent**

Primary macrophages derived from the bone marrow of wild type, TLR1-, TLR2- or TLR6-deficient mice were treated with 20, 50 and 200 nM of either Pam_3CSK4 (A) or Malp-2 (B), 50 ng/ml LPS and 25 µg/ml PolyIC (C). After 18 h incubations IL6 production was measured by ELISA. Data are expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. These data are representative of three experiments. ***, $p < 0.005$, **, $p < 0.01$, NS $p > 0.05$; significant differences between wild type and TLR1-, TLR2- and TLR6-deficient macrophages.

3.3.3 *Salmonella typhimurium* does not require Mal for signalling in primary macrophages

To further confirm that Mal-deficient cells retained the ability to produce cytokines such as IL6 in response to TLR2 stimulation, as outlined above, wild type and Mal-deficient primary macrophages were treated at various multiplicities of infection with *Salmonella typhimurium* (a known TLR2 activator) (Weiss et al., 2004). The production of IL6 and TNF-α were then tested by ELISA.

As shown in Figure 3.23A stimulation of the wild type primary macrophages (black bars) at multiplicities of infection (MOI) of 1, 10 and 30 resulted in a concentration-dependent increase in the level of IL6 production. The Mal-deficient primary macrophages (white bars) also showed concentration-dependent induction of IL6. They were, however, unable to produce IL6 at a level comparable to the wild type cells at the low MOI of 1. At the higher MOIs of 10 and 30 the Mal-deficient primary macrophages did produce IL6 at a similar level to that seen for the wild type primary macrophages.

A comparable result was seen when TNF-α production was examined as outlined in Figure 3.23B. Both the wild type and Mal-deficient macrophages produced IL6 in response to *S. typhimurium* at an MOI of 1 and 10. At the MOI of 1 the Mal-deficient macrophages (grey bars) did not produce TNF-α to the same level as the wild type macrophages (white bars). When the MOI was increased to 10 the Mal-deficient cells regained their ability to produce TNF-α to the same level as the wild type cells.

These data again showed a lack of requirement for Mal in TLR2 signalling with a somewhat ligand concentration-dependence on the activity of the Mal-deficient cells.

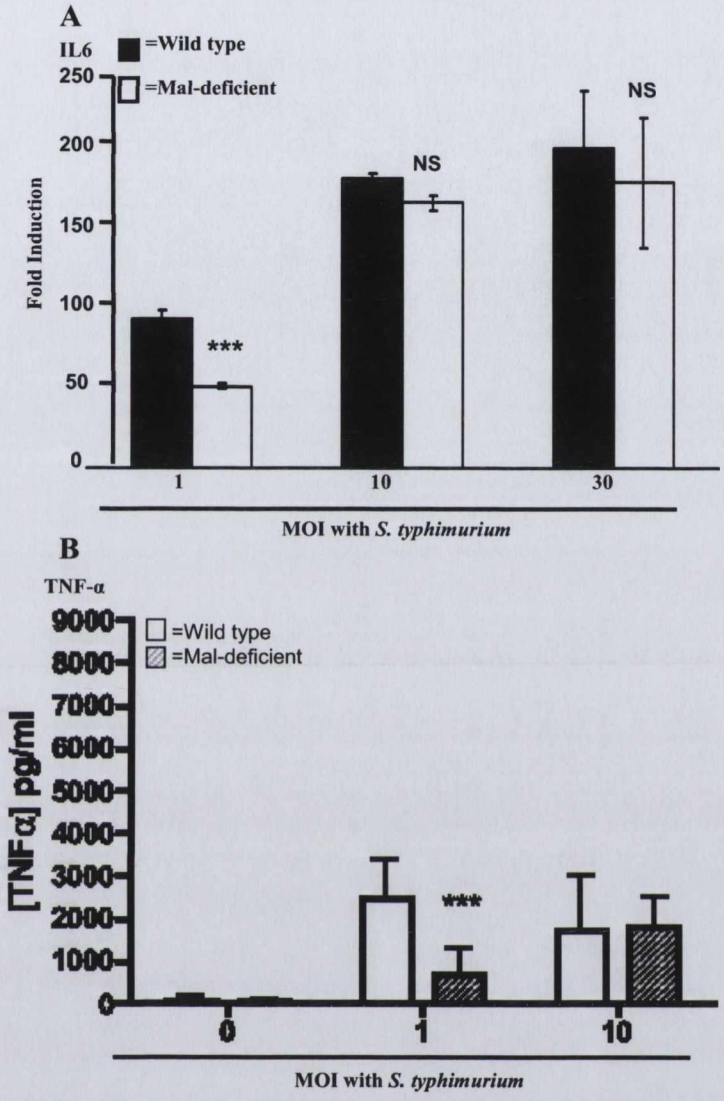

Figure 3.23. **IL6 and TNF-α production in response to *Salmonella typhimurium* is Mal-independent in primary macrophages**

Primary macrophages derived from the bone marrow of wild type and Mal-deficient mice were treated with *Salmonella typhimurium* at multiplicities of infection (MOI) of 1, 10 and 30. After 18 h incubations IL6 (A) and TNF-α (B) production were measured by ELISA. In (A) the data are expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. These data are representative of three experiments. ***, $p < 0.005$, NS $p > 0.05$; significant differences between wild type and Mal-deficient macrophages.

3.3.4 Activation of downstream signalling molecules in response to TLR2 stimulation is MyD88-dependent but relatively Mal-independent

In order to confirm the the total requirement of MyD88 and the lack of requirement of Mal in TLR2 signalling the signals downstream of both adaptor molecules were examined. The phosphorylation of p38 and c-jun N-terminal kinase (JNK) and the degradation of IκB-α were examined after stimulation of the immortalised wild type, MyD88- and Mal-deficient macrophages with the TLR2 ligands at 0, 5, 10, 15, 30 and 45 min time points.

As shown in Figure 3.24A upon Pam_3CSK4 stimulation at a concentration of 200 nM, the wild type cells induced the phosphorylation of p38 and JNK and the degradation of IκB-α after 5-10 min stimulation and these molecules continued to be activated to the 45 min time point. The MyD88-deficient macrophages were unresponsive to the Pam_3CSK4 at 200 nM as no activation of signalling was seen for the three molecules tested. The Mal-deficient macrophages also induced the phosphorylation of p38 and JNK and the degradation of IκB-α upon stimulation with 200 nM Pam_3CSK4 although this activation was delayed somewhat, occuring from 15-30 min.

The relative intensities of the phosphorylated p38 were quantified and graphed to demonstrate the activation of the wild type and Mal-deficient macrophages at this concentration of Pam_3CSK4. As similar patterns of activation were seen for JNK and IκB-α their relative intensties were not plotted.

As shown in Figure 3.24B upon Pam_3CSK4 stimulation at a lower concentration of 50 nM the wild type macrophages were still capable of activating the three signalling

molecules tested within 5-10 min and continued to activate them to the 45 min time point. The MyD88-deficient macrophages were unresponsive to the 50 nM Pam_3CSK4 stimulation for all signalling molecules tested. The Mal-deficient macrophages were less responsive than the wild type cells as stimulation with 50 nM Pam_3CSK4 did not result in the phosphorylation of p38 or JNK or degradion of IκB-α. The relative intensites of the phosphorylated p38 bands were again plotted to further demonstate the trend seen in the Western blots.

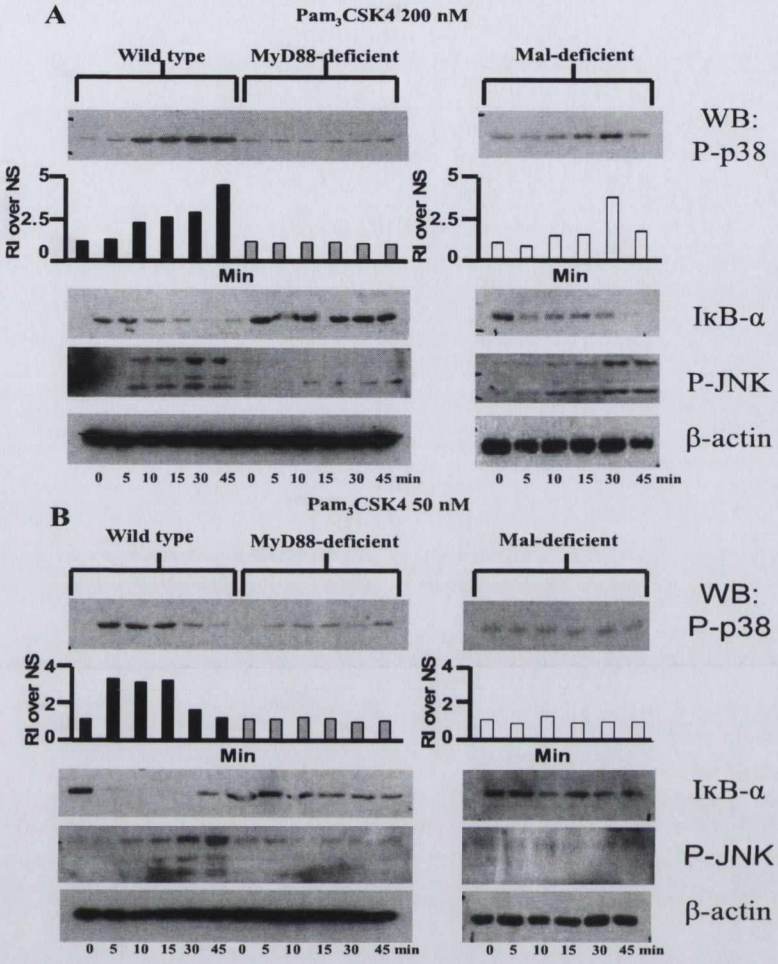

Figure 3.24. **Downstream signal activation in response to the TLR1/2 ligand Pam_3CSK4 is MyD88-dependent at all concentrations but Mal-dependent at the 50 nM ligand concentration only**

Wild type, MyD88- and Mal-deficient immortalised macrophages were treated for the indicated times with either (A) 200 nM Pam_3CSK4 or (B) 50 nM Pam_3CSK4. The cell lysates were collected and p38 phosphorylation, IκB-α degradation and JNK phosphorylation were determined by Western blot. β-Actin was included as a loading control. Densitometric analysis of band intensities was determined for the P-p38 blots, where each band was normalised to its β-actin and the relative intensity (R.I.) of the bands over the non-stimulated control (set at 1) were calculated. These data are representative of three experiments.

The wild type, MyD88- and Mal-deficient macrophages were also tested for the phosphorylation of p38 and JNK and the degradation of IκB-α after stimulation with 200 and 50 nM concentrations of the TLR2/6 ligand Malp-2 for 0, 5, 10, 15, 30 and 45 min.

As shown in Figure 3.25A stimulation of the wild type macrophages with 200 nM Malp-2 resulted in the phosphorylation of p38 and JNK and the degradation of IκB-α after 5-10 min and this continued to the 45 min time point. The MyD88-deficient macrophages were again unable to activate the three signalling molecules at this concentration. The phosphorylation of p38 and JNK and the degradation of IκB-α in the Mal-deficient macrophages in response to the 200 nM concentration of Malp-2 occurred in a manner similar to the wild type cells.

As was seen for the 50 nM concentration of Pam_3CSK4 the ability to activate the signalling molecules was decreased in the Mal-deficient macrophages at the lower concentration of 50 nM Malp-2 (Figure 3.25B). Stimulation of the Mal-deficient cells did, however, still result in the activation of all three signalling molecules. The wild type cells were responsive at 5-10 min and the MyD88-deficient cells remained unresponsive. The relative intensites of p38 phosphorylation were again plotted to demonstrate the trend seen in the Western blots.

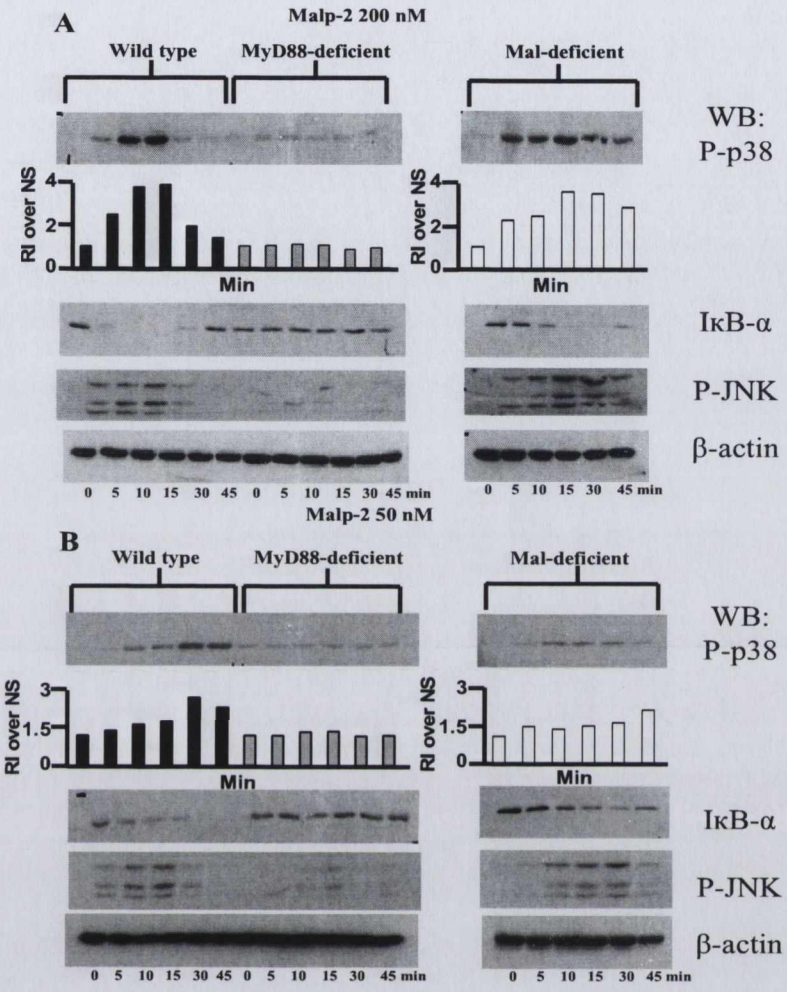

Figure 3.25. **Downstream signal activation in response to the TLR2/6 ligand Malp-2 is MyD88-dependent but Mal-independent**

Immortalised wild type, MyD88- and Mal-deficient macrophages were treated for the indicated times with either (A) 200 nM Malp-2 or (B) 50 nM Malp-2. The cell lysates were collected and p38 phosphorylation, IκB-α degradation and JNK phosphorylation were determined by Western blot. β-Actin was included as a loading control. Densitometric analysis of band intensities was determined for the P-p38 blots, where each band was normalised to its β-actin and the relative intensity (R.I.) of the bands over the non-stimulated control (set at 1) were calculated. These data are representative of three experiments.

3.3.5 Activation of down stream molecules in response to TLR4 stimulation is MyD88- and Mal-independent

The macrophages were also tested for the phosphorylation of p38 and JNK and the degradation of IκB-α in response to 50 ng/ml LPS at 0, 5, 10, 15, 30, and 45 min time points. As shown in Figure 3.26 stimulation of the wild type macrophages resulted in activation of all three signalling molecules after 5-10 min similar to the TLR2 ligand responses. Stimulation of the MyD88-deficient macrophages also led to the activation of all three in a manner similar to the wild type cells. LPS treatment of the Mal-deficient macrophages also resulted in the activation of all three molecules although there was a delay with activation beginning at the 30 min time point. The relative intensities of p38 phosphorylation were again plotted to show the trend.

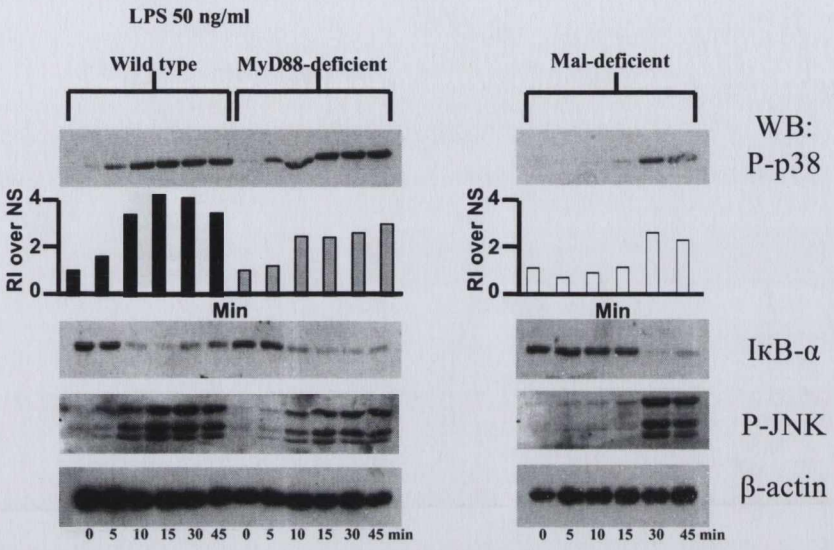

Figure 3.26. **Downstream signals in response to LPS, the TLR4 ligand, are relatively normal in the absence of MyD88 or Mal**

Immortalised macrophages from wild type, MyD88- and Mal-deficient mice were treated for the indicated times with 50 ng/ml LPS. The cell lysates were collected and p38 phosphorylation, IκB-α degradation and JNK phosphorylation were determined by Western blot. β-Actin was included as a loading control. Densitometric analysis of band intensities was determined for the P-p38 blots, where each band was normalised to its β-actin and the relative intensity (R.I.) of the bands over the non-stimulated control (set at 1) were calculated. These data are representative of three experiments.

3.3.6 Mal is required for TLR4 signalling but not TLR2 signalling in primary macrophages

To confirm that TLR2 signalling did not rely on Mal and that TLR4 had a total dependence on Mal, as shown above using the immortalised macrophages, I next used primary macrophages from wild type and Mal-deficient mice. These cells were treated with the TLR1/2, TLR2/6 and TLR4 ligands as indicated in Figure 3.27 and IL6 and TNF-α production were analysed by ELISA.

As shown in Figure 3.27A stimulation of wild type primary macrophages (black bars) and Mal-deficient macrophages (white bars) with 200 nM Pam_3CSK4 resulted in 70 and 40 fold induction of IL6 respectively. Similarly when the cells were stimulated with 200 nM Malp-2 30 and 10 fold induction of IL6 was seen. These results shows little dependence on Mal for the production of IL6 in response to Pam_3CSK4, the TLR1/2 ligand and Malp-2, the TLR2/6 ligand, in primary macrophages.

Stimulation of the wild type macrophages with 100 ng/ml LPS resulted in 90 fold induction of IL6 whereas the Mal-deficient cell stimulation only resulted in 2 fold induction of IL6. This huge reduction in the level of IL6 produced in the absence of Mal demonstrated the requirement for Mal in TLR4 signalling.

TNF-α production was also tested in these cells in response to 200 nM Pam_3CSK4, 200 nM Malp-2 and 100 ng/ml LPS and a similar result was seen as outlined in Figure 3.27B. Stimulation of the wild type cells with 200 nM Pam_3CSK4 resulted in 100 fold induction of TNF-α and stimulation of the Mal-deficient cells resulted in 50 fold induction of TNF-α. There was a halving of induction of IL6 in the Mal-deficient macrophages but

they did still produce TNF-α. Stimulation of the wild type macrophages with 200 nM Malp-2 resulted in 50 fold induction of TNF-α and 15 fold induction in the absence of Mal. This again revealed that cells can produce cytokines in response to TLR2 stimulation in the absence of Mal.

The cells were also examined for production of TNF-α in response to the TLR4 ligand LPS. Stimulation of the Mal-deficient cells with 100 ng/ml LPS resulted in only 6 fold induction of TNF-α in comparison to 130 fold induction in the wild type cells. Thus a requirement of Mal for TLR4 signalling but not TLR2 signalling was again seen.

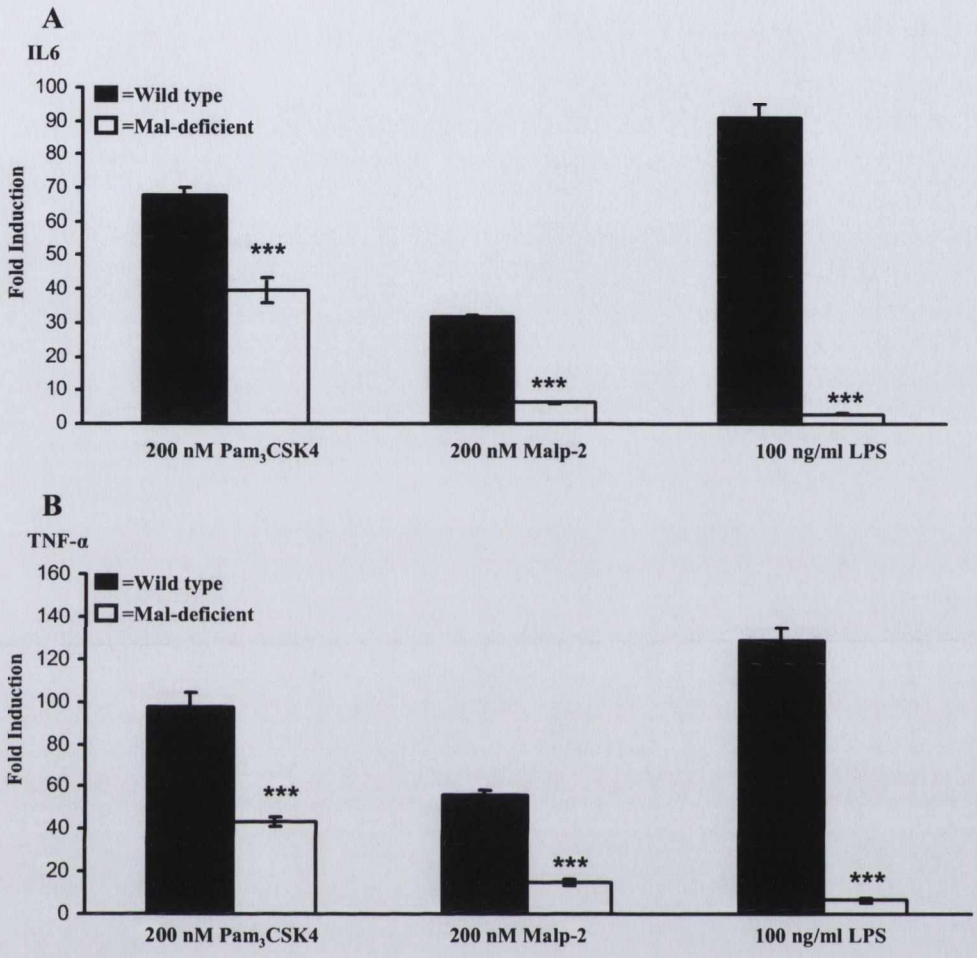

Figure 3.27. **IL6 and TNF-α production are less Mal-dependent in response to TLR2 ligands than in response to the TLR4 ligand LPS**

Primary macrophages derived from the bone marrow of wild type and Mal-deficient mice were treated with 200 nM of either Pam_3CSK4 or Malp-2 and 100 ng/ml LPS. After 18 h incubations IL6 (A) and TNF-α (B) production were measured by ELISA. Data are expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. These data are representative of three experiments. ***, $p < 0.005$; significant differences between wild type and Mal-deficient macrophages.

3.3.7 Mal is not essential for TLR2 signalling in primary dendritic cells

To assess whether the total requirement of Mal in TLR4 signalling and the non essential nature of Mal in response to TLR2 stimulation was macrophage-specific or a more global feature of TLR2 signalling I next used primary wild type and Mal-deficient dendritic cells. These cells were tested for their ability to produce IL6 and TNF-α in response to Pam_3CSK4, Malp-2 and LPS as shown in Figure 3.28.

As shown in Figure 3.28A stimulation of the wild type and Mal-deficient dendritic cells with 200 nM Pam_3CSK4 and Malp-2 resulted in 25 and 20 fold induction of IL6 respectively. This again showed the lack of requirement for Mal in TLR2 signalling. However, somewhat surprisingly, stimulation of the wild type and Mal-deficient cells with 100 ng/ml LPS also resulted in 25 and 20 fold induction of IL6 respectively. The ability of the Mal-deficient dendritic cells to produce IL6 was odd as it has not been seen previously and Mal was thought to be totally required for TLR4 signalling in all other assays used in this project.

The production of TNF-α was also examined in these cells as outlined in Figure 3.28B. The dendritic cells produced very low levels of TNF-α in response to the TLR2 ligands so a clear result was not as evident. However, in response to 200 nM Pam_3CSK4 stimulation the wild type cells produced a 2.5 fold induction of TNF-α and the Mal-deficient cells produced a 1.5 fold induction. There was a decrease in TNF-α production in the absence of Mal but detectable amounts were still produced. Stimulation of the wild type cells with 200 nM Malp-2 resulted in 1.5 fold induction of TNF-α and a 0.5 fold induction in the Mal-deficient cells. Again these levels are low but the absence of Mal still allows the

production of TNF-α. The levels of TNF-α produced in the absence of Mal were 60% and 33% of that produced by the wild type cells for Pam_3CSK4 and Malp-2 stimulation respectively.

The cells were also tested for the production of TNF-α in response to 100 ng/ml LPS. The cells were more responsive to LPS stimulation than to Pam_3CSK4 and Malp-2 stimulation with 7 fold induction of TNF-α in the wild type cells. This dropped to 1.5 fold induction in the Mal-deficient macrophages. This was only 20% of the amount of TNF-α produced by the wild type cells thus showing a more of a requirement for Mal in TLR4 signalling than TLR2 as outlined above in the macrophages.

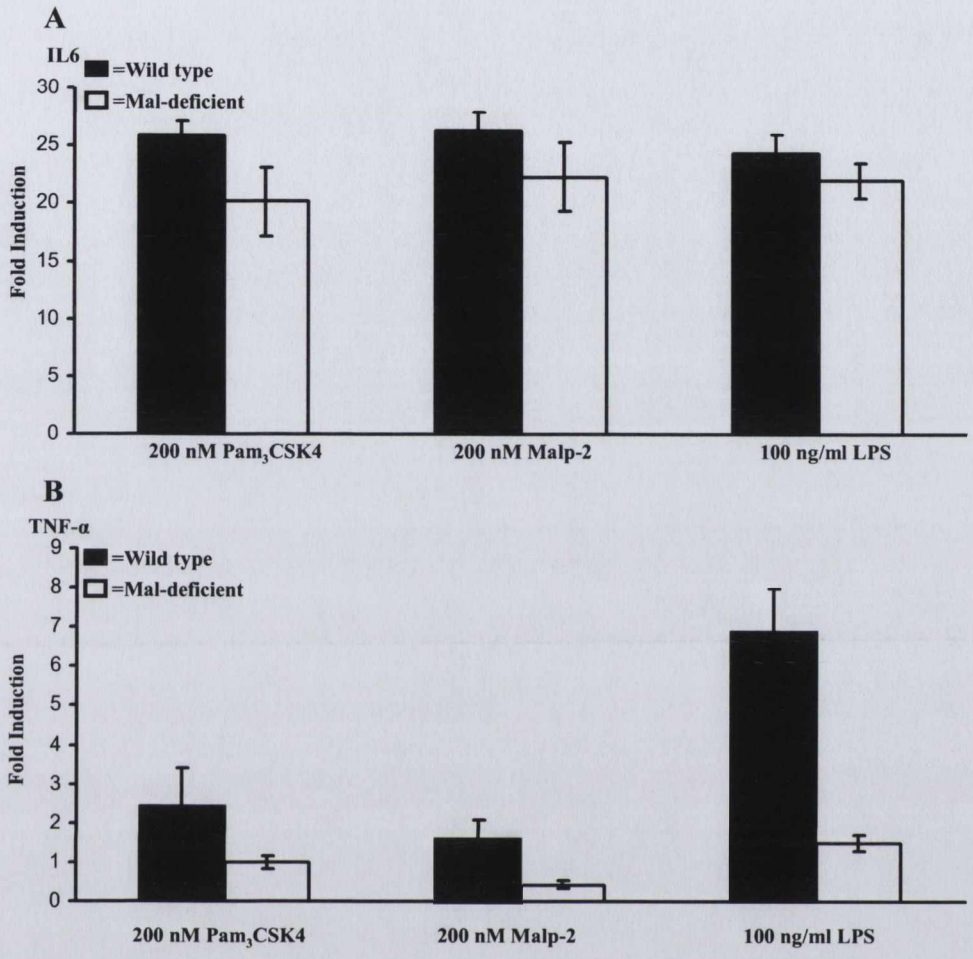

Figure 3.28. **In primary dendritic cells IL6 and TNF-α production in response to TLR2 ligands is Mal-independent, and in response to TLR4 stimulation IL6 production is Mal-independent and TNF-α production is Mal-dependent**

Primary dendritic cells derived from the bone marrow of wild type and Mal-deficient mice were treated with 200 nM of either Pam_3CSK4 or Malp-2 and 100 ng/ml LPS. After 18 h incubations IL6 (A) and TNF-α (B) production were measured by ELISA. Data are expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. These data are representative of two experiments.

3.4 Discussion

This project was inspired by the fact that the precise interactions between Mal and MyD88 the TLR1/2, TLR2/6 and TLR4 signalling complexes has never been fully investigated. It is known that both TLR2 and TLR4 signalling require Mal and MyD88 with Mal thought of as a bridging adaptor for these TLRs allowing for the activation of the MyD88-dependent pathway (Kagan and Medzhitov, 2006; Yamamoto et al., 2002a). The ability of Mal and MyD88 to directly bind TLR1 and TLR6 had never been examined and as such this became the starting point of the project.

I began by carrying out a yeast two-hybrid assay using the TIR domains of human TLR1, TLR2, TLR4 and TLR6 and full length Mal and MyD88. In this experiment the novel interactions between TLR1 and MyD88 and TLR6 and MyD88 were revealed. In this assay TLR2 and TLR4 bound both MyD88 and Mal. The ability of TLR2 and TLR4 to bind MyD88 was somewhat unexpected as Mal was previously though to be the link between these two TLRs and MyD88. Therefore in the absence of Mal TLR2 and TLR4 can still interact with MyD88.

To complement the interactions revealed in the yeast two-hybrid assay I next examined the co-localisation of YFP-TLR1, TLR2, TLR4 and TLR6 with CFP-MyD88 using live cell imaging via confocal microscopy. This assay revealed that TLR1, TLR2, TLR4 and TLR6 could co-localise with MyD88 but only upon stimulation with their respective ligands. No co-localisation was seen prior to stimulation with MyD88 mainly localised in the cytosol and the TLRs mainly on the plasma membrane. The co-localisation of TLR1, TLR2 and TLR6 with MyD88 after stimulation occurred largely within the cell

and not on the plasma membrane. The internalisation of TLR2 has previously been shown with TLR2 moving to phagosomes in response to stimulation with zymosan (Underhill et al., 1999a). TLR1 and TLR6 have also been found in phagosomes upon stimulation (Ozinsky et al., 2000) therefore TLR1 and TLR6 can also internalise to initiate signalling.

The co-localisation of TLR4 with MyD88, on the other hand, occurred at the plasma membrane and within 5 min of stimulation with LPS. This co-localisation was lost after 20 min stimulation with the movement of TLR4 into the cell. This supports work by Kagan et al. showing TLR4 is internalised to endosomes to trigger TRIF-dependent signalling (Kagan et al., 2008).

The novel TLR1 and TLR6 interactions with MyD88 were again shown in these experiments. MyD88 also co-localised with TLR2 and TLR4 in the absence of Mal thus further confirming that Mal may not be a bridging adaptor for these TLRs as previously thought.

With regards to the live cell imaging assay investigating the co-localisation of YFP-TLR1, TLR2, TLR4 and TLR6 with CFP-Mal I was unable to optimise this due to transfection and co-expression issues with the CFP-Mal vector. Using endogenous TLR1, TLR2 and TLR6 antibodies in these experiments in an attempt to alleviate the transfection issues provided no insight as the antibodies were not suitable for confocal microscopy. Cells stably expressing fluorescently labelled TLR1, TLR2, TLR4 or TLR6 would be required to reveal any co-localisation of Mal and the TLRs of interest in this assay.

I next attempted to further confirm the interactions seen for the TLRs of interest using co-immunoprecipitation assays with over-expressed TLRs, MyD88 and Mal. Unfortunately these experiments did not clarify the interactions due to issues with non-

specific antibody binding and reproducibility. I tried to optimise these experiments by carrying out pre-incubations with the protein A/G-plus agarose beads used in the experiments to remove any non-specific binding of the proteins to the beads. I varied the concentrations of antibody used to immunoprecipitate the proteins of interest in an effort to remove the non-specific antibody interactions. I also used several different lysis buffers in the hope of isolating more of the TLRs from the membrane of the HEK-293T cells and therefore revealing any interactions. However, none of my attempts to optimise these experiments were successful.

I therefore moved on to GST-pulldown assays using GST-MyD88 and GST-Mal to pull down TLR1, TLR2 and TLR6. To begin I attempted to pull down endogenous TLR1, TLR2 and TLR6 from THP1 cells but this was unsuccessful as no interactions were seen. The endogenous antibodies used were difficult to optimise as high concentrations of protein and antibody were required to see any protein in the whole cell lysates and therefore the ability to see any interaction bands in the pull down was very difficult.

I therefore returned to over-expressing the TLRs of interest in HEK-293T cells and examining the lysates for any interactions with the adaptors. I encountered problems with this assay due to the difficulty in isolating large enough quantities of the membrane bound TLRs to see any interactions by Western blotting. Even after concentrating the TLRs with membrane fractionation I was unable to detect any interactions by Western blotting.

Due to these technical difficulties I next decided to generate GST-TIR domain fusion proteins for the TIR domains of TLR1, TLR2 and TLR6 and thus eliminating the issue of removing the TLRs from the plasma membrane in the experiments. I designed primers at each end of the TIR domains with an *EcoR* I and a *Sal* I restriction site at either

end. I then amplified the TIR domains out of the YFP or flag vectors and inserted them into the pGex-4T2 vector. After isolating the proteins from bacterial cells the fusion proteins were used to pull down over-expressed MyD88 and Mal from the HEK-293T cell lysates. These experiments revealed that MyD88 interacted with TLR1, TLR2, TLR4 and TLR6. Interactions between Mal and TLR2, TLR4 and, surprisingly, TLR1 were also revealed.

To confirm that the TLR1 interactions with MyD88 and Mal were specific I also attempted to pull down Caspase1 with GST-TLR1. As it did not interact with TLR1 I believe these experiments have revealed TLR1 as a binding partner of MyD88 and Mal and as such this study is the first demonstration of MyD88 interacting with TLR1 and TLR6 and of Mal interacting with TLR1.

To further confirm that the interactions seen were specific and not just due to non-specific TIR-TIR interactions I also tested the ability of the GST-TLRs to pull down TRIF. As TRIF has not been implicated in TLR2 or directly in TLR4 signalling (as TRAM is the bridging adaptor for TRIF in TLR4 signalling) it acted as a good negative control and as such did not interact with any of the GST-TLRs tested. Thus the interacting partners revealed by the GST-pulldown assays were correct and specific.

The ability of TLR1 to directly bind Mal, as seen in the GST-pulldown assay, was not shown in the yeast two-hybrid experiments. This may be due to the artificial nature of the two-hybrid assay using yeast to express mammalian proteins and expecting the proteins to interact as normal. As the GST-pulldown assay involves expressing mammalian proteins in a mammalian system (HEK-293T cells) it is a more reliable method for the examination of protein-protein interactions.

As I had revealed interesting and novel binding partners involved in TLR1/2, TLR2/6 and TLR4 signal transduction I continued my investigations by examining the cytokines and signalling molecules activated in the absence of MyD88 or Mal in response to the TLR1/2 ligand Pam_3CSK4, the TLR2/6 ligand Malp-2 and the TLR4 ligand LPS.

The initial experiments were carried out using immortalised macrophages generated from wild type, MyD88- and Mal-deficient mice. By examining IL6 production in response to Pam_3CSK4, Malp-2 and LPS I revealed a total requirement for MyD88 in all three signalling complexes. This was not unexpected as MyD88 is known to have a crucial role in TLR2 and TLR4 signalling (Takeuchi et al., 2000b) and interacted with all four TLRs in the yeast two-hybrid assay, live cell imaging and the GST-pulldown assays.

A non essential requirement for Mal in response to the TLR2 ligands was revealed using these cells. The Mal-deficient macrophages were capable of producing IL6 in response to all concentrations of the TLR2 ligands tested. The level of IL6 produced in the absence of Mal was decreased when compared to the wild type cells at all three concentrations. However, the wild type cells did not respond in a concentration-dependent manner suggesting they were fully activated at all three ligand concentrations used. The Mal-deficient cells, on the other hand, did reveal concentration-dependence with the levels of IL6 production increasing as the ligand concentration increased. Therefore these experiments revealed that TLR2 stimulation is not fully dependent on Mal but is totally dependent on MyD88.

An absolute requirement for Mal in response to TLR4 stimulation was seen in these experiments. In the absence of Mal the levels of IL6 produced by the macrophages was hugely decreased. The levels produced were comparable to the MyD88-deficient

macrophages thus revealing the absolute requirement for both Mal and MyD88 in TLR4 signal transduction.

To ensure the TLR1/2 ligand Pam_3CSK4 and the TLR2/6 ligand Malp-2 were activating the correct TLR complexes I next tested the induction of IL6 in response to these ligands in macrophages from wild type, TLR1-, TLR2- and TLR6-deficient mice. The ligands acted as anticipated and the cells were as described as all types responded to LPS and PolyIC.

I subsequently examined the ability of the Mal-deficient macrophages to produce cytokines in response to a whole pathogen rather than a ligand. I tested IL6 and TNF-α production in response to *Salmonella typhimurium,* a known TLR2 activator (Weiss et al., 2004), to further confirm the lack of requirement of Mal in TLR2 signalling. Treatment of the Mal-deficient macrophages with *S. typhimurium* at a low multiplicity of infection (MOI) of 1 resulted in the production of both IL6 and TNF-α although at decreased levels in comparison to the wild type macrophages. At the MOIs of 10 or 30 the Mal-deficient macrophages produced IL6 and TNF-α at levels similar to the wild type cells thus confirming the lack of requirement of Mal in TLR2 signalling.

In the initial signalling experiments it seemed that the lack of requirement of Mal in TLR2 signalling was more clearly revealed at the higher ligand concentrations and MOIs. At the lower ligand concentrations and MOIs the Mal-deficient cells appeared somewhat depleted in their ability to respond therefore suggesting a somewhat concentration-dependency on Mal in TLR2 signalling.

I next examined the activation of the downstream signalling molecules p38, JNK and IκB-α in response to the TLR2 ligands at two concentrations (50 and 200 nM). These

experiments also revealed that Mal is not essential for TLR2 signalling. A slight concentration-dependency on Mal was again revealed. Stimulation with 200 nM of Pam_3CSK4 or Malp-2 in the absence of Mal allowed for the phosphorylation of the downstream signalling proteins p38, JNK and the degradation of IκB-α. The 50 nM ligand stimulation resulted in a loss of this activation in TLR1/2 signalling and a modest impairment in TLR2/6 signalling. For all concentrations of TLR2 ligands MyD88 again revealed its absolute requirement to allow the activation of downstream signalling molecules, and thus, is the central activator of TLR2 signalling.

Examination of these cells after LPS stimulation resulted in normal downstream signal activation in the absence of MyD88 and delayed activation in the absence of Mal. As TLR4 can also recruit TRAM and hence TRIF to activate the TRIF-dependent signalling pathway this signal molecule activation was not unexpected. It is, however, unclear why the activation of these signalling molecules in the absence of MyD88 or Mal did not result in the production of IL6. In the case of MyD88 this anomaly has previously been seen and was attributed to the TRIF-dependent pathway (Kawai et al., 1999).

To confirm the results observed in the immortalised macrophages I next tested the production of IL6 and TNF-α in response to the TLR2 and TLR4 ligands in primary wild type and Mal-deficient macrophages. A similar trend was seen in these cells with regards Pam_3CSK4 and Malp-2 stimulation. In response to 200 nM stimulation with the TLR2 ligands the wild type and Mal-deficient macrophages induced the production of IL6 and TNF-α. The level of both cytokines produced by the Mal-deficient cells was decreased in comparison to the wild type cells but the cytokines were still detectable. As such the lack of requirement of Mal in TLR2 signalling was further validated.

The primary macrophages also further confirmed the total dependency on Mal in TLR4 signalling. Stimulation of the Mal-deficient macrophages with 100 ng/ml LPS resulted in a massive decrease in IL6 and TNF-α production when compared to the wild type cells.

As all the experiments to this point had been carried out in macrophages I next tested whether the non-essential nature of Mal in TLR2 signalling was a global feature of TLR2 signalling. To do this, primary dendritic cells were generated from wild type and Mal-deficient mice and the production of IL6 and TNF-α in response to the TLR2 and TLR4 ligands were examined. The requirement for Mal in response to TLR2 stimulation was again shown to be non essential in these cells as IL6 and TNF-α production were only marginally impaired in response to Pam_3CSK4 stimulation in the Mal-deficient cells. Malp-2 was shown to more Mal-dependent and lower levels of IL6 and TNF-α were produced in the Mal-deficient cells in comparison with the wild type cells. TLR4 signalling was again shown to be Mal-dependent with regards TNF-α production. A surprising result was seen, however, when IL6 was examined in the Mal-deficient dendritic cells. There was no loss in IL6 production in the Mal-deficient dendritic cells in comparison to the wild type cells thus demonstrating no Mal-requirement in these cells for LPS induced IL6 production. It was not clear why the dendritic cells did not require Mal in response to LPS stimulation to produce IL6 but did require it for the production of TNF-α.

When taken together these results demonstrate a more complicated method of TLR2 signalling through Mal than was previously thought. I therefore propose the following updated role for Mal in TLR2 and TLR4 signalling as outlined in Figure 3.29. TLR2 and TLR4 utilise Mal and MyD88 to signal. Previous work revealing the presence of

a PIP2 binding domain in Mal suggested that it was required by TLR2 and TLR4 to bridge them to MyD88. Another line of evidence for the bridging role of Mal come from a study which revealed that MyD88 and TLR4 are electro-positive in regions thought to be important for their ability to interact whereas Mal is largely electro-negative in these regions suggesting Mal to be the more likely binding partner of TLR4 (Dunne et al., 2003). In the study by Dunne et al. however, TLR4 and MyD88 were shown to interact in a region distinct from that of the TLR4 and Mal interaction. It was also revealed that a TLR2 and MyD88 interaction occurs at a site distinct from that seen for the interaction of TLR4 and MyD88.

In my interaction experiments I have also shown that MyD88 can interact directly with TLR2 and TLR4. Therefore MyD88 does not require Mal for its interactions with these TLRs. MyD88 was also shown to interact with TLR1 and TLR6 and is therefore utilised by all four TLRs tested.

The signalling experiments revealed, however, that the ability of MyD88 to directly interact with TLR4 did not aid in its ability to produce IL6 in response to LPS. This was revealed in the Mal-deficient cells as they did not produce IL6 upon LPS stimulation demonstrating that even though MyD88 can directly interact with TLR4 it cannot initiate signal transduction in the absence of Mal. There was also no IL6 produced in the MyD88-deficient cells confirming that both MyD88 and Mal are both crucial for TLR4 signalling to IL6. The lack of MyD88 and Mal did not completely abolish downstream signalling, however, due to the activation of the TRIF-dependent pathway.

A very different response was seen in TLR2 signalling where the ability of MyD88 to directly interact with TLR2 allowed for IL6 production in response to the TLR2 ligands.

This was demonstrated in the Mal-deficient cells where the absence of Mal did not completely abolish IL6 production. TLR2 through its heterodimerisation with TLR1 and TLR6 can bring in additional MyD88 in the absence of Mal and hence the TLR1/2 and TLR2/6 signalling complexes remain responsive to stimulation. In the absence of MyD88, however, no TLR2 signal activation is seen. Therefore MyD88 is central to TLR2 signal transduction but Mal is not.

TLR4 requires the presence of Mal and MyD88 to allow MyD88-dependent signalling as it cannot signal in the absence of either adaptor. TLR2 may act in a manner similar to that of TLR4 as it is the only other TLR that utilises Mal and MyD88 to signal. TLR2 may therefore require Mal for successful activation of the MyD88-dependent signalling pathway. However, as TLR2 and TLR4 recruit Mal to distinct regions of their TIR domains TLR2 may be capable of interacting with MyD88 and initiating MyD88-dependent signal transduction in the absence of Mal whereas TLR4 is not (Dunne et al., 2003).

As TLR1 also recruits Mal it could act similarly to TLR4 and require both MyD88 and Mal for signal activation. This seems unlikely though as TLR1 is very similar to TLR6 with 81% amino acid similarity (Takeuchi et al., 1999b). TLR6 recruits MyD88 but not Mal to activate signal transduction and as such TLR1 may also bind MyD88 and allow signal activation in the absence of Mal.

The location of the signalling complexes revealed in the live cell imaging of the TLRs with MyD88 also provides evidence as to why TLR2 and TLR4 differ in their requirement for MyD88 and Mal to signal. In TLR2 signalling the co-localisation only occurred within the cell. With regards TLR4 signalling, once TLR4 translocated into the

cell the co-localisation seen between it and MyD88, which occurred at the plasma membrane, was lost. A recent study has revealed that TLR2 moves to the phagosome and produces cytokines in response to *Francisella tularensis* (Cole et al., 2008).

A second study by the same group revealed that this TLR2 signalling occurred in a MyD88-dependent but Mal-independent manner (Cole et al., 2010). In this study it was theorised that TLR2 can respond to the prolonged presence of a pathogen or high concentrations of ligand in a Mal-independent manner. Therefore as TLR2 translocates to a phagosome during signal transduction and can respond to *F. tularensis* in the absence of Mal it must not have the absolute requirement for Mal seen in TLR4 signalling. As TLR4 remains on the plasma membrane to induce the MyD88-dependent signal cascade it utilises the presence of both MyD88 and Mal for adequate signalling.

In conclusion I propose that Mal is not essential in TLR2 signalling as Mal-independent recruitment of MyD88 to the TLR2 complex via TLR1 and TLR6 allows for signal transduction which can also occur in the phagosome. TLR4 is totally reliant on Mal and MyD88 to signal as it forms homodimers on the plasma membrane and has a total dependence on the presence of both adaptor proteins to activate MyD88-dependent signal cascades.

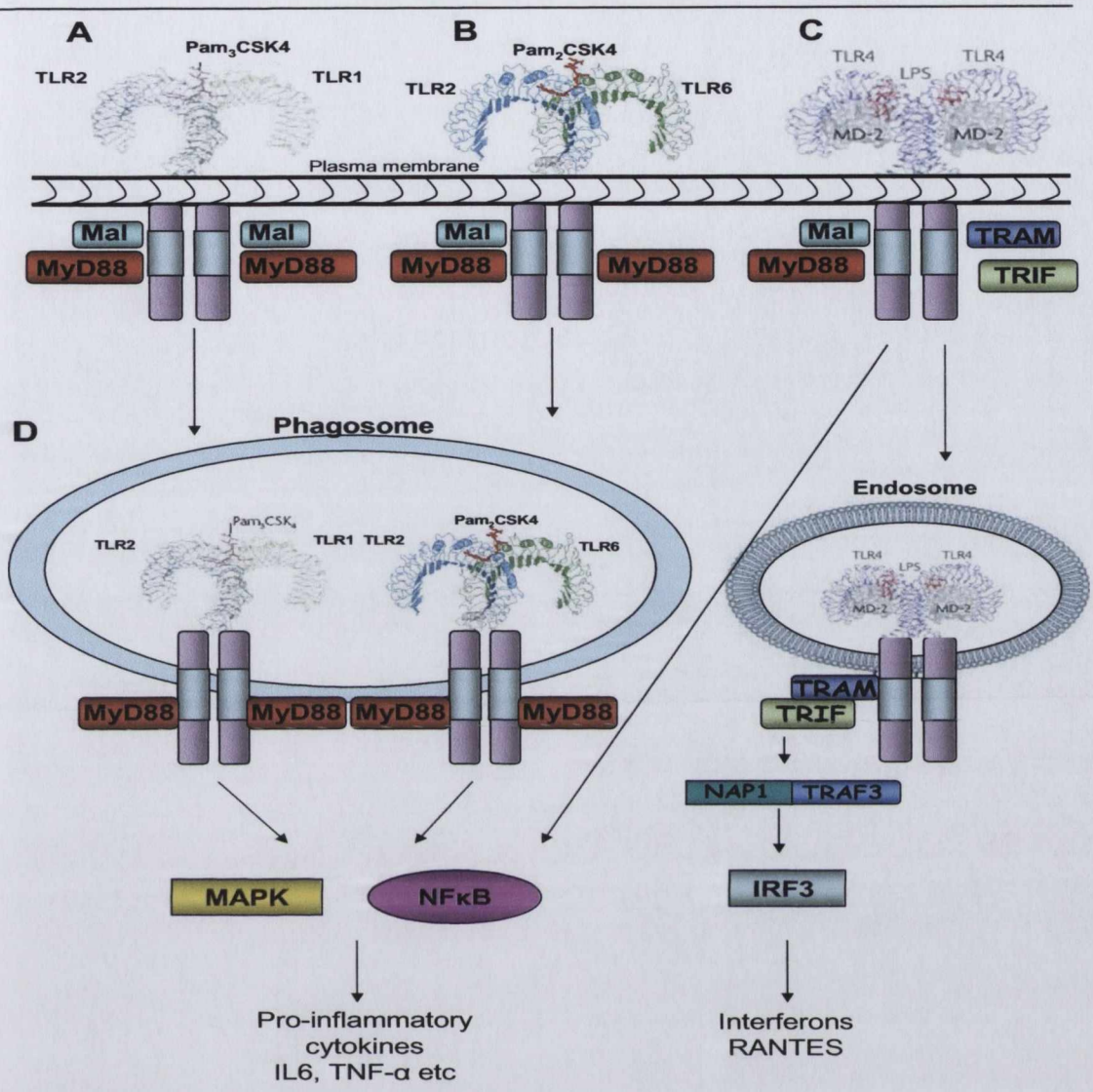

Figure 3.29 **Model of TLR2 and TLR4 signalling**

In response to the binding of Pam_3CSK4 to the TLR1/2 complex Mal and MyD88 are recruited to both TLR1 and TLR2 to initiate signalling (A). In response to Pam_2CSK4 (a ligand similar to Malp-2) binding the TLR2/6 complex MyD88 is recruited to both TLR2 and TLR6 but Mal is recruited to TLR2 only to activate NFκB and MAP-kinases (B). In TLR4 signalling MyD88 and Mal are required for the activation of NFκB and MAP-kinases (C). TLR4 translocates to the endosome to initiate TRIF-dependent signalling resulting in interferon and RANTES production. The TLR1/2 and TLR2/6 complexes can also move to phagosomes and signal in the absence of Mal (D).Adapted from (Hennessy et al., 2010; Kang et al., 2009).

Chapter Four

Mal is an inhibitor of TLR3 signalling

4.1 Introduction

As outlined in chapter 1, TLR3 is the receptor responsible for the recognition of dsRNA from viral pathogens (Alexopoulou et al., 2001). TLR3, along with TLR7, TLR8 and TLR9, is found in the endosomes of cells and as such signal activation occurs within the endosome and not on the plasma membrane (de Bouteiller et al., 2005; Heil et al., 2003; Lund et al., 2003). This is due to the fact that a virus will not present dsRNA to the plasma membrane as it must be within the cell to begin replicating. The presence of dsRNA in the endosome results in the homodimerisation of TLR3 and the initiation of signalling in the cytosol of the cell.

The sole adaptor protein linked to TLR3 signalling is TRIF (Yamamoto et al., 2003a). It binds directly to the TIR domain of TLR3 and through several domains recruits the downstream activators of TLR3 signalling. TRAF6 interacts with the N-terminus of TRIF by binding several conserved TRAF6 binding sites. This leads to the activation of NFκB via TRAF6 lysine (K)-63 linked poly-ubiquitination. TRIF also recruits TBK1 at its N-terminus to allow IRF3 phosphorylation (Sato et al., 2003). At the C-terminal TRIF has a RHIM (RIP- homotypic interaction motif) to allow RIP association and the activation of NFκB and apoptosis (Han et al., 2004; Meylan et al., 2004).

Recently a paper emerged stating that a direct interaction also occurs between TLR3 and IRAK2 which allows for TLR3-dependent K-63 linked poly-ubiquitination of TRAF6 thus activating the TLR3 signalling pathway (Keating et al., 2007). This is interesting and somewhat unusual as IRAK2 does not contain a TIR-domain and therefore it is unclear what domain it uses to bind TLR3. In the case of TLR4 signalling IRAK2

interacts with MyD88 to aid in the activation of signal transduction though a direct homotypic death-domain interaction (Muzio et al., 1997). A second interesting finding recently has been the direct interaction between TRIF and TRAF3 therefore linking TRAF3 to TLR3 signalling (Hacker et al., 2006).

In the previous chapter I initially used PolyIC, the TLR3 ligand, as a control during the signalling experiments with the immortalised bone marrow derived macrophages. However, these experiments yielded some unexpected results, where it appeared that a lack of Mal potentiated TLR3 signalling. This therefore warranted further investigation. As a study by Pearlman et al. had shown an inhibitory role for MyD88 in TLR3 signalling (Johnson et al., 2008) the next aim of my project was to investigate the role Mal may play.

4.2 Results: Signalling experiments

To begin my investigations into the role of Mal in the inhibition of TLR3 signalling I examined the production of IL6 in wild type and Mal-deficient macrophages and dendritic cells in response to PolyIC stimulation. I also used two Mal-inhibitor peptides in these experiments.

4.2.1 MyD88 and Mal inhibit IL6 production in response to TLR3 stimulation with PolyIC in immortalised macrophages

I first tested the wild type, MyD88-, Mal- and TRIF-deficient macrophages for the production of IL6 in response to PolyIC stimulation for 18 h. As shown in Figure 4.1 stimulation with 10, 25 and 50 μg/ml PolyIC resulted in a 10 to 15 fold induction of IL6 in wild type macrophages (black bars). The MyD88-deficient (grey bars) and Mal-deficient (white bars) macrophages produced substantially more IL6 upon PolyIC stimulation than the wild type cells. The TRIF-deficient macrophages (dark grey bars) were unable to produce IL6 in response to PolyIC at all concentrations tested confirming the activation was TLR3-dependent.

As a control the immortalised macrophages were also stimulated with LPS at 50, 75 and 100 ng/ml as outlined above in Figure 3.21. The MyD88- and Mal-deficient macrophages did not produce IL6 in response to LPS.

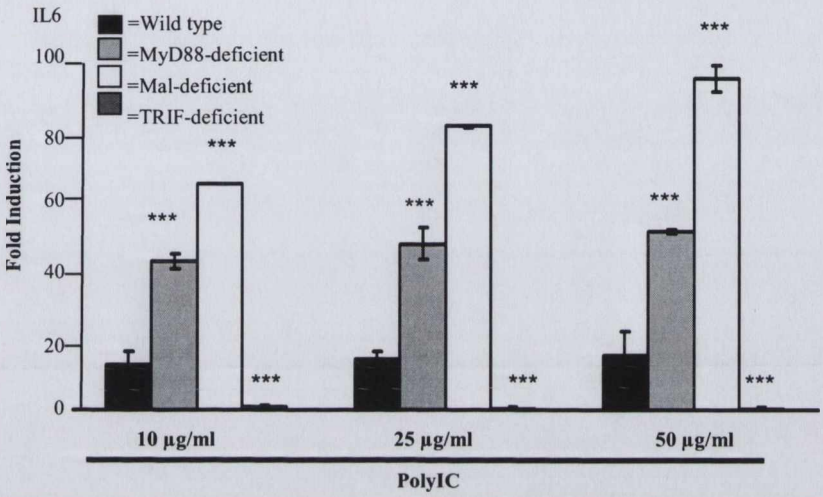

Figure 4.1. **IL6 production is enhanced in response to the TLR3 ligand PolyIC in the absence of MyD88 or Mal**

Immortalised macrophages derived from the bone marrow of wild type, MyD88-, Mal- and TRIF-deficient mice were treated with 10, 25 and 50 µg/ml PolyIC. After 18 h incubations IL6 production was measured by ELISA. Data are expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. These data are representative of three experiments. ***, $p < 0.005$; significant differences between wild type and MyD88-, Mal- and TRIF-deficient macrophages.

4.2.2 A Mal inhibitor peptide enhances IL6 production in wild type macrophages in response to PolyIC stimulation

To ensure the enhancement of IL6 production in the absence of Mal was not an artefact of gene deletion I next treated the immortalised wild type macrophages with a Mal inhibitor peptide. I then examined the ability of the pre-treated cells to produce IL6 upon PolyIC stimulation. The Mal inhibitor peptide consists of amino acids 138-151 of murine Mal with the antennapedia sequence from drosophila positioned at the NH2-terminal end (to allow internalization of the peptide) and has been shown to inhibit TLR4 signalling (Horng et al., 2001).

As shown in Figure 4.2A wild type macrophages pre-treated with a control peptide (black bars) and stimulated with 25, 50 and 75 μg/ml PolyIC yielded a 4 to 6 fold induction of IL6. PolyIC stimulation of the wild type macrophages pre-treated with the Mal inhibitor peptide (white bars) resulted in an enhancement of IL6 production giving a 6 to 10 fold induction. Therefore, by inhibiting Mal in a wild type macrophage, a similar result to the Mal-deficient macrophages was seen.

The wild type macrophages were also stimulated with 100 ng/ml LPS after treatment with the control and Mal inhibitor peptides. As outlined in Figure 4.2B the wild type cells produced IL6 in response to LPS in the presence of the control peptide (black bars) and this IL6 production was inhibited in the presence of the Mal inhibitor peptide (white bars).

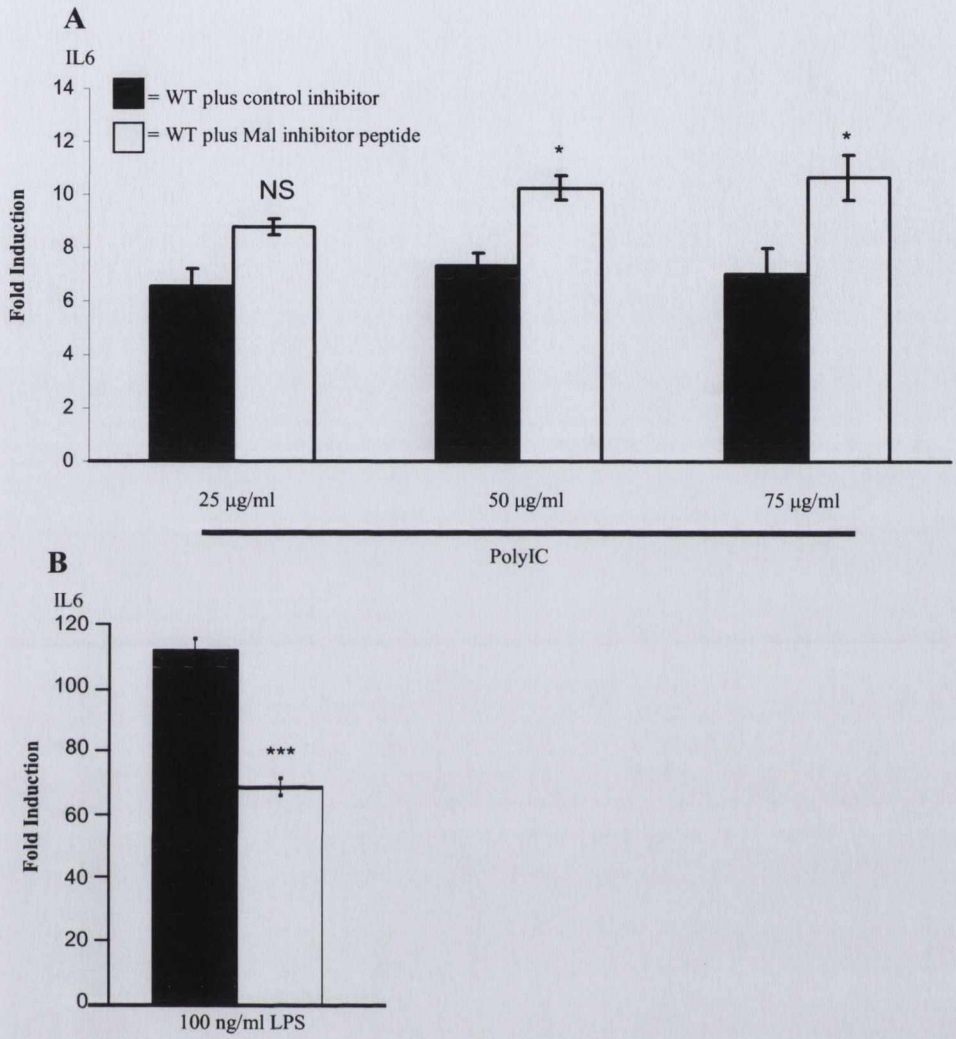

Figure 4.2. **IL6 production is enhanced in response to the TLR3 ligand PolyIC in wild type macrophages treated with a Mal inhibitor peptide**

Immortalised macrophages derived from bone marrow of wild type mice were treated with 1 µg/ml control peptide or Mal inhibitor peptide for 1 h. The cells were then treated with 25, 50 and 75 µg/ml PolyIC (A) or 100 ng/ml LPS (B). After 18 h stimulations IL6 production was measured by ELISA. Data are expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. These data are representative of three experiments. ***, $p < 0.005$, *, $p < 0.05$, NS $p > 0.05$; significant differences between wild type control and wild type Mal inhibitor samples.

4.2.3 Mal inhibits IL6 production in response to TLR3 stimulation with PolyIC in primary macrophages

To further confirm the inhibitory role for Mal in TLR3 signalling I next tested IL6 production in response to PolyIC stimulation in primary wild type and Mal-deficient bone marrow derived macrophages. As shown in Figure 4.3 stimulation with 10, 25 and 50 μg/ml PolyIC resulted in an 80 fold induction of IL6 in wild type macrophages (black bars). The Mal-deficient macrophages (white bars) produced greater levels of IL6 (100 and 110 fold) upon PolyIC stimulation than the wild type cells at the 25 and 50 μg/ml concentrations.

As a control the primary macrophages were also stimulated with LPS at 50, 75 and 100 ng/ml as outlined above in Figure 3.27. The Mal-deficient macrophages failed to produce levels of IL6 comparable to the wild type cells in response to LPS.

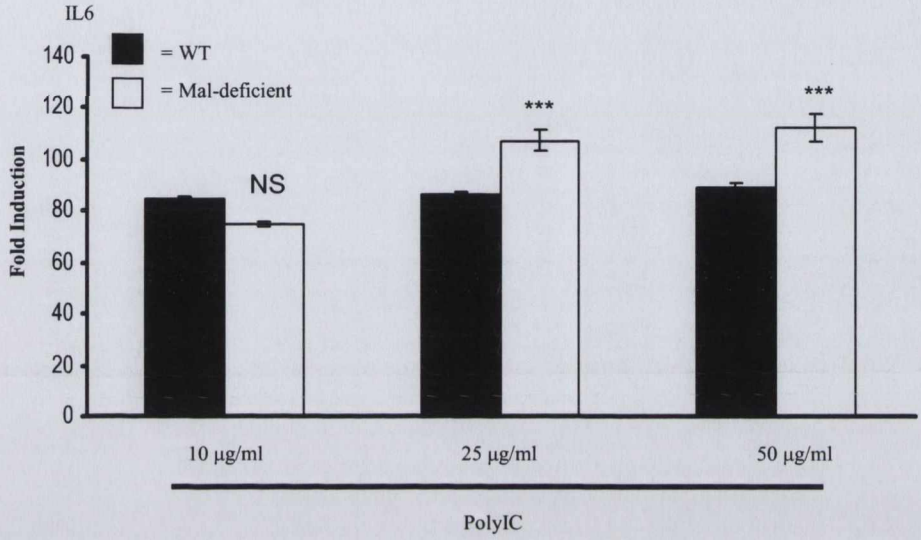

Figure 4.3. **IL6 production is enhanced in response to the TLR3 ligand PolyIC in the absence of Mal in primary macrophages**

Primary macrophages derived from the bone marrow of wild type and Mal-deficient mice were treated with 10, 25 and 50 μg/ml PolyIC. After 18 h incubations IL6 production was measured by ELISA. Data are expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. These data are representative of three experiments. ***, $p < 0.005$, NS $p > 0.05$; significant differences between wild type and Mal-deficient macrophages.

4.2.4 Mal inhibits IL6 production in response to TLR3 stimulation with PolyIC in primary dendritic cells

To assess whether the inhibitory role of Mal in TLR3 signalling was specific to macrophages or a more universal feature of TLR3 signalling I next used primary wild type and Mal-deficient dendritic cells. I stimulated them with several concentrations of PolyIC and examined the production of IL6.

As shown in Figure 4.4 stimulation with 10, 25 and 50 μg/ml PolyIC resulted in 25 fold induction of IL6 in wild type dendritic cells (black bars). The Mal-deficient dendritic cells (white bars) produced IL6 to a greater level (30 to 40 fold) upon PolyIC stimulation than the wild type cells at all three concentrations tested.

As a control the primary dendritic cells were also stimulated with LPS at 50, 75 and 100 ng/ml as outlined above in Figure 3.28 and showed no inhibition in IL6 production in the absence of Mal.

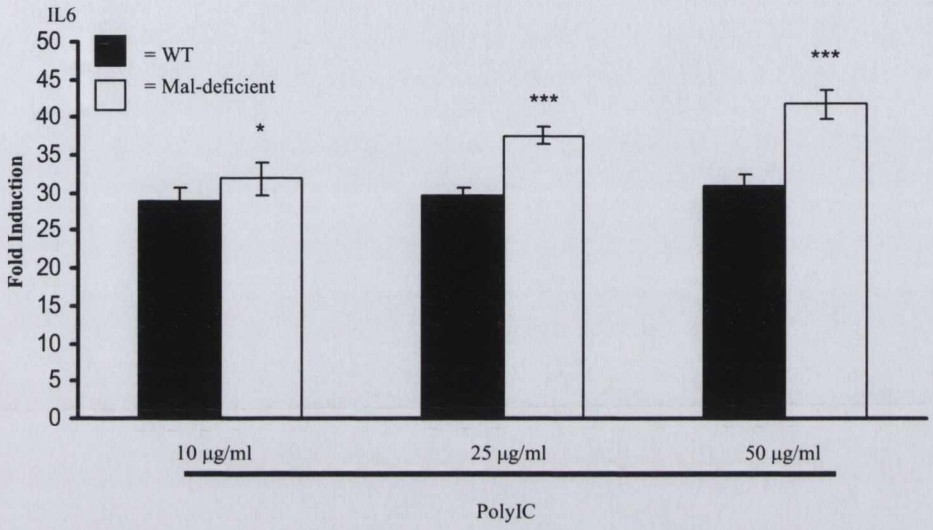

Figure 4.4. **IL6 production is enhanced in response to the TLR3 ligand PolyIC in the absence of Mal in primary dendritic cells**

Primary dendritic cells derived from the bone marrow of wild type and Mal-deficient mice were treated with 10, 25 and 50 µg/ml PolyIC. After 18 h incubations IL6 production was measured by ELISA. Data are expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. These data are representative of three experiments. ***, $p < 0.005$, *, $p < 0.05$; significant differences between wild type and Mal-deficient dendritic cells.

4.2.5 The Mal inhibitor peptide VIPER enhances IL6 and RANTES production in U373 cells in response to PolyIC stimulation

To test if Mal was also inhibitory in TLR3 signalling in a human system I next used the human astrocytoma cell line U373. The cells were treated with viral inhibitory peptide of TLR4 (VIPER, a Mal inhibitor peptide) or a control peptide and stimulated with various TLR ligands.

VIPER is an 11 amino acid long peptide derived from the A46 immunomodulatory protein found in vaccinia virus. It is a specific TLR4 inhibitor which, in a recent study, blocked cytokine production and MAP kinase, NFκB and IRF3 activation in murine and human cell lines in response to LPS stimulation. It also reduced the secretion of IL12/23 p40 in vivo in mice injected with LPS. It interacted directly with Mal and TRAM but not with TLR4. It did not inhibit TLR2 signalling to NFκB and boosted PolyIC-dependent NFκB activation (Lysakova-Devine et al., 2010).

Pre-treatment of U373 cells with the control peptide (black bars) resulted in the production of IL6 in response to 100 nM Pam_3CSK4 and 25 μg/ml PolyIC Figure 4.5A. Pre-treatment of the cells with VIPER (white bars) resulted in the production of IL6 at a comparable level to the control samples in the case of Pam_3CSK4 and an enhancement of IL6 production upon stimulation with PolyIC. As shown in Figure 4.5B the U373 cells also produced IL6 in response to 100ng/ml LPS and this was inhibited in the presence of VIPER.

These cells were also tested for the production of RANTES as shown in Figure 3.5C. A similar result to the IL6 ELISA was seen with an enhancement in RANTES

production in the cells pre-treated with VIPER when compared to the control treated cells in response to PolyIC stimulation. The cells responded as normal to Pam_3CSK4 stimulation and were inhibited for the production of RANTES in response to LPS stimulation.

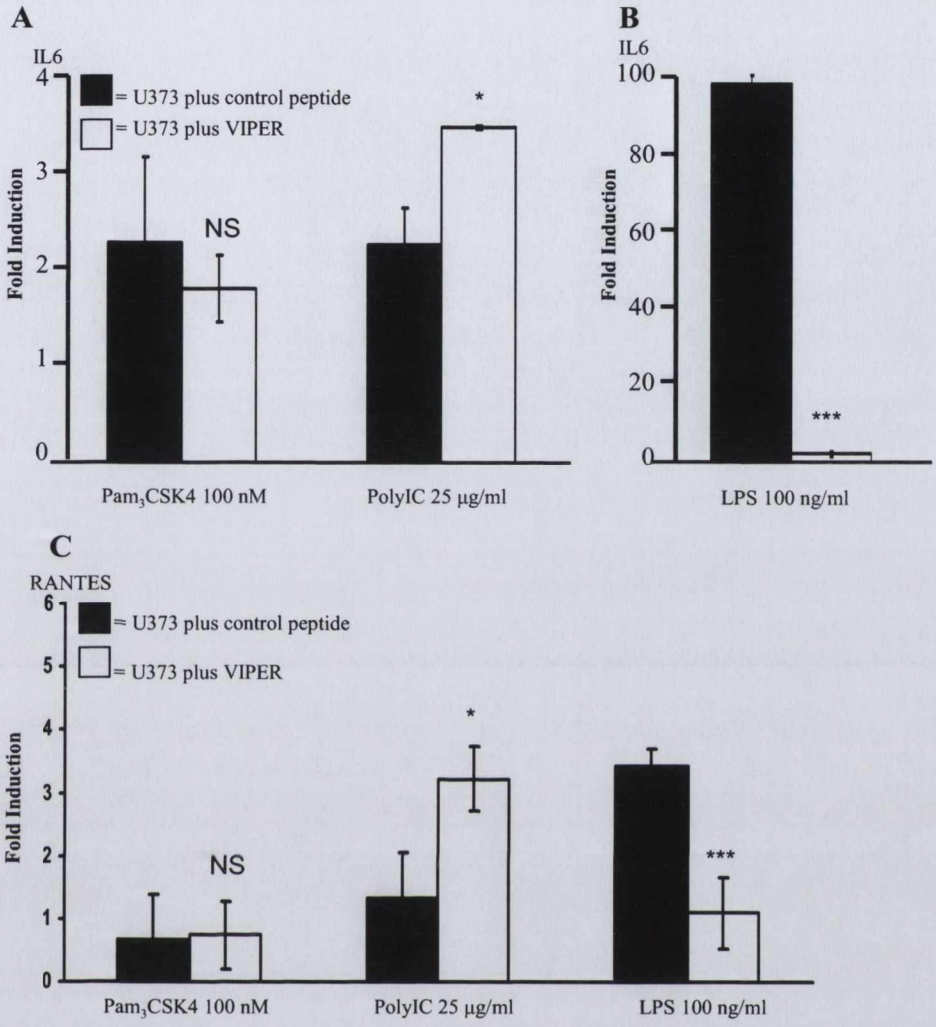

Figure 4.5. **IL6 and RANTES production are enhanced in response to the TLR3 ligand PolyIC in the presence of the Mal inhibitor VIPER in U373 cells**

Human astrocytoma cells (U373s) were treated with 5 µM control peptide or the Mal inhibitor peptide VIPER for 1 h. The cells were then treated with 100 nM Pam_3CSK4, 25 µg/ml PolyIC and 100 ng/ml LPS. After 18 h incubations IL6 (A and B) and RANTES (C) production were measured by ELISA. Data are expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. These data are representative of two experiments. ***, $p < 0.005$, *, $p < 0.05$, NS, $p > 0.05$; significant differences between control and VIPER samples.

4.2.6 MyD88 and Mal inhibit phosphorylation of JNK in response to PolyIC stimulation

To investigate where on the TLR3 signalling pathway MyD88 and Mal inhibit I next examined the activation of the downstream signalling molecules JNK, p38 and IκB-α. The wild type, MyD88-, Mal-, and TRIF-deficient immortalised macrophages were treated with 25 μg/ml PolyIC for 0, 15, 30, 45, 60 and 90 min and the cell lysates were examined by Western blotting.

As outlined in Figure 4.6 when the phosphorylation of JNK was examined the wild type macrophages induced the phosphorylation of JNK after 30 min and continued to activate it through to the 90 min time point. An enhancement in the phosphorylation of JNK in response to PolyIC stimulation was seen in the MyD88- and Mal-deficient macrophages when compared to the wild type cells at the later time points of 60 and 90 min. The use of densitometric analysis demonstrated that the Mal-deficient macrophages had enhanced the induction of phosphorylation of JNK to a greater extent than the MyD88-deficient macrophages in response to PolyIC. The TRIF-deficient cells were unresponsive to PolyIC stimulation at all time points.

The phosphorylation of p38 and the degradation of IκB-α were also examined as demonstrated in Figure 4.7. Stimulation of the wild type macrophages resulted in the phosphorylation of p38 and the degradation of IκB-α after 30 min. The phosphorylation of p38 and the degradation of IκB-α occurred to the same level in the MyD88- and Mal-deficient macrophages as in the wild type cells. Densitometric analysis of the p38 Western blots confirmed this. The TRIF-deficient macrophages were unresponsive to PolyIC

stimulation at all time points tested and as such no phosphorylation of p38 or degradation of IκB-α degradation was evident in these samples.

As a control LPS signalling in the MyD88- and Mal-deficient macrophages was tested. The phosphorylation of JNK and p38 and the degradation of IκB-α were delayed in the absence of Mal but not enhanced. In the MyD88-deficient macrophages no delay in activation of the three signalling molecules was seen. This result was outlined in Figure 3.26.

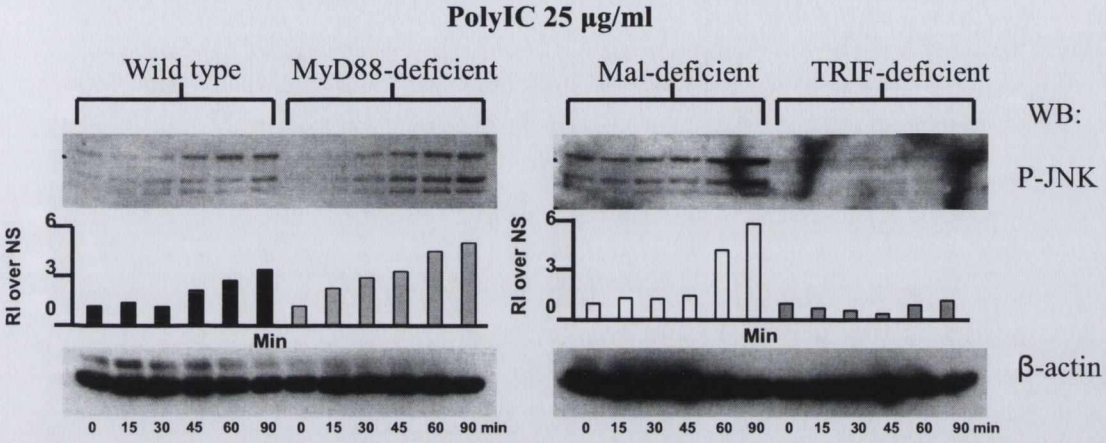

Figure 4.6. **In response to the TLR3 ligand PolyIC, phosphorylation of JNK is enhanced in the absence of MyD88 and Mal**

Bone marrow derived macrophages from wild type, MyD88-, Mal- and TRIF-deficient mice were treated for the indicated times with 25 µg/ml PolyIC. The cell lysates were collected and JNK phosphorylation was determined by Western blot. β-Actin was included as a loading control. Densitometric analysis of band intensities was determined for the P-JNK blots, where each band was normalised to its β-actin and the relative intensity (R.I.) of the bands over the unstimulated control (set at 1) were calculated. These data are representative of three experiments.

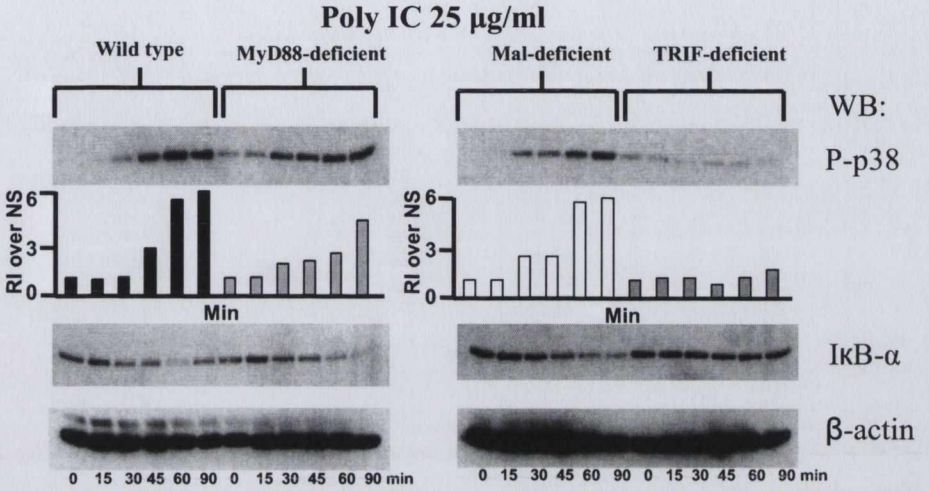

Figure 4.7. **In response to the TLR3 ligand PolyIC, phosphorylation of p38 and IκB-α degradation are relatively normal in the absence of MyD88 and Mal**

Bone marrow derived macrophages from wild type, MyD88-, Mal- and TRIF-deficient mice were treated for the indicated times with 25 µg/ml PolyIC. The cell lysates were collected and p38 phosphorylation and IκB-α degradation were determined by Western blot. β-Actin was included as a loading control. Densitometric analysis of band intensities was determined for the P-p38 blots, where each band was normalised to its β-actin and the relative intensity (R.I.) of the bands over the unstimulated control (set at 1) were calculated. These data are representative of three experiments.

4.2.7 PolyIC stimulation does not lead to enhanced phosphorylation of MKK4 and MKK7 in the absence of Mal

The PolyIC time courses carried out above suggested that the inhibition of TLR3 signalling by Mal was JNK-specific as the phosphorylation of JNK was enhanced in the absence of Mal but the phosphorylation of p38 and IκB-α degradation were not. This JNK specificity had previously been shown for the inhibitory role of MyD88 in TLR3 signalling (Johnson et al., 2008).

I next examined which MAP-kinases upstream of JNK were responsible for its enhanced phosphorylation in the absence of Mal. Map kinase kinase 4 (MKK4) and Map kinase kinase 7 (MKK7) are two kinases known to phosphorylate JNK. MKK4 is thought to also phosphorylate p38 but MKK7 is thought to be JNK-specific (Cuenda, 2000).

The commercially available antibodies raised against the phosphorylated forms of MKK4 and MKK7 were unable to detect endogenous phosphorylated MKK4 or MKK7 in the wild type and Mal-deficient macrophages. Therefore, a JNK2 kinase assay was carried out to determine which of these MAP kinase kinases was responsible for the phosphorylation of JNK upon PolyIC stimulation.

In order to perform the JNK2 kinase assay I first generated the JNK2 K/R (kinase dead form) protein. The kinase dead form of JNK2 was used to ensure no autophosphorylation could occur. The prepared JNK2 K/R proteins are shown in Figure 4.8 in which 1, 2, 5 and 10 μg of JNK2 K/R A, B and BSA were analysed by SDS-PAGE with coomassie staining.

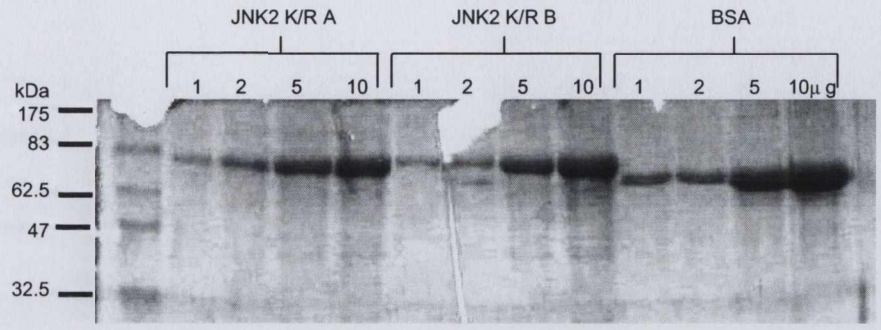

Figure 4.8. **JNK2 K/R is expressed in bacteria and can be isolated from it**

BL21 bacterial cells were transformed with the pGex plasmid containing GST-JNK2 K/R (kinase dead form). A single colony of JNK2 K/R containing bacteria was grown over night at 37^{0}C, transferred to a large culture and grown at 37^{0}C until the OD at 600nm was 0.5. 100 μM IPTG was added to the culture and the bacteria were incubated for 3 h at 37^{0}C. The cells were pelleted, lysed and the GST-fusion proteins were isolated by incubation with glutathione-sepharose 4B beads at 4^{0}C for 3 h. The beads were pelleted at 2,500 rpm for 10 min at 4^{0}C and washed twice in Buffer A. The beads were then washed twice in Buffer A plus 0.27 M sucrose twice and incubated in 30 mM glutathione for 15 min with regular mixing. A Bradford assay was done to determine protein concentration. 1, 2, 5 and 10 μg of JNK2 K/R A and B and BSA were analysed by SDS-PAGE with coomassie staining.

To confirm that the prepared JNK2 K/R protein was correctly folded and capable of being phosphorylated I next stimulated wild type immortalised macrophages with IL1-α and tested for the phosphorylation of MKK4 and MKK7. IL1-α is known to phosphorylate MKK4 and MKK7 (Finch et al., 2001) and as such acted as a good control ligand with which to begin my investigations.

As shown in Figure 4.9A stimulation of wild type macrophages with 50 ng/ml IL1-α for 10 and 30 min resulted in the phosphorylation of MKK4 (lanes 2 and 3). The lack of ^{32}P labelled JNK2 K/R in the IgG control lane (lane 5) demonstrated that the radiolabelled phosphate was specifically incorporated into JNK2 K/R in response to the presence of phosphorylated MKK4. A similar result is outlined in Figure 4.9B were stimulation of the wild type macrophages with 50 ng/ml IL1-α for 10 and 30 min resulted in the phosphorylation of MKK7 (lanes 2 and 3). The phosphorylation of JNK2 K/R by MKK7 was specific due to the lack of ^{32}P labelled JNK2 K/R in the IgG control lane (lane 5).

The Western blots were then probed for the presence of MKK4, MKK7 and JNK2 to ensure equal amounts of these proteins were present in each sample. However, as shown in Figure 4.9C there were slight differences in the levels of JNK2, MKK4 and MKK7 present in each sample.

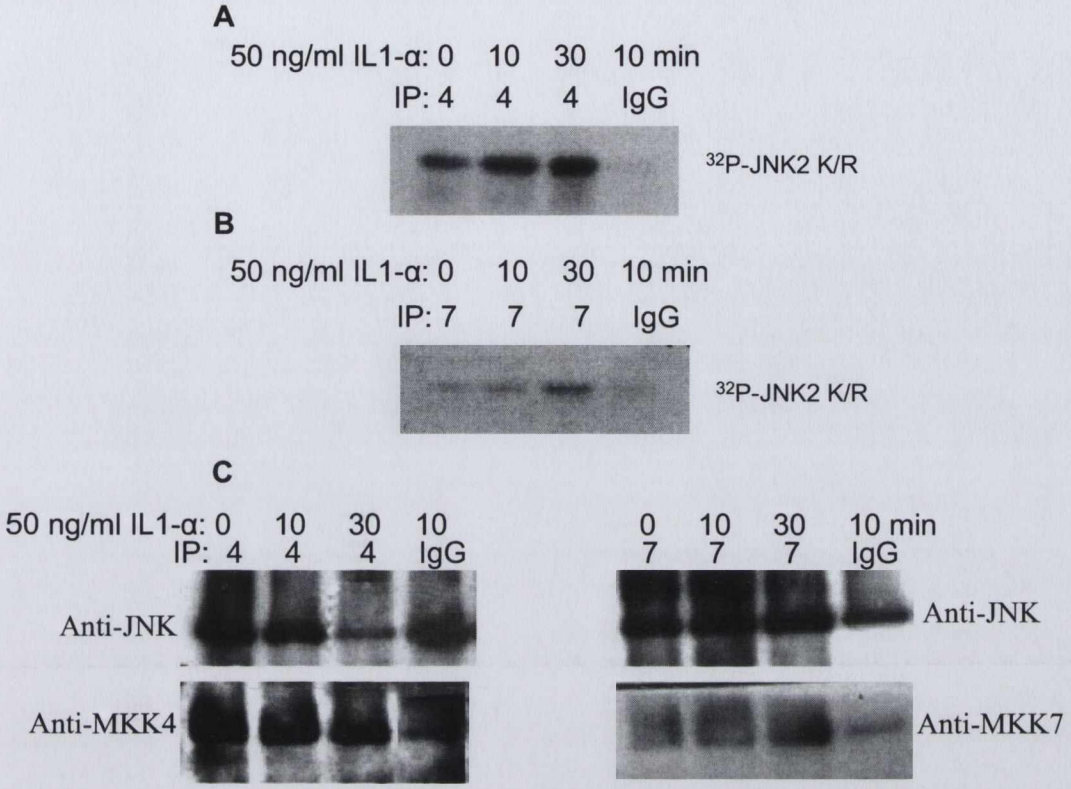

Figure 4.9. **Phosphorylation of MKK4 and MKK7 in wild type macrophages due to IL1-α stimulation leads to the phosphorylation of JNK2 in a kinase assay**

Immortalised macrophages derived from the bone marrow of wild type mice were treated for the indicated times with 50 ng/ml IL1-α. The cells were lysed in low stringency lysis buffer and a Bradford assay was used to determine protein concentration. The lysates were pre-cleared with 20 µl protein A/G-plus agarose beads for 45 min at 4°C. The lysates were incubated overnight at 4°C with 40 µl protein A/G-plus agarose beads and 3 µl anti-MKK4, MKK7 or rabbit IgG. The beads were washed twice in low stringency lysis buffer and twice in kinase buffer. The beads were then incubated for 30 min at 37°C in kinase buffer, 20 nM ATP, 5 µCu ^{32}P-ATP and 2 µg JNK2 K/R at 1,000 rpm. 8 µl 5x sample loading buffer was added and the samples were analysed by Western blotting for the incorporation of ^{32}P-ATP into JNK2 due to MKK4 (A) or MKK7 (B) phosphorylation. The membranes were probed for anti-JNK, MKK4 or MKK7 (C).

To confirm that the JNK2 kinase assay would reveal the MAP kinase kinase involved in JNK phosphorylation in response to PolyIC stimulation in macrophages I next tested the ability of the wild type macrophages to induce the phosphorylation MKK4 and MKK7 in response to a variety TLR ligands.

As outlined in Figure 4.10A phosphorylation of MKK4 occurred in the wild type macrophages when treated for 15 and 30 min with 100 nM Pam_3CSK4, 100 ng/ml LPS and 1 μg/ml R848 (lanes 2 to 7). Stimulation with 3 μg/ml mCpGB and 25 μg/ml PolyIC also resulted in the phosphorylation of MKK4 although at later time points of 45 and 90 min (lanes 8 to 11). The basal level of MKK4 phosphorylation was low as there was no ^{32}P incorporated into JNK2 K/R in the non stimulated sample (lane 1). There was no radio-labelled phosphate incorporated into JNK2 K/R in the IgG control sample (lane 12) demonstrating that the presence of ^{32}P in JNK2 K/R was specific to MKK4 phosphorylation by the TLR ligands tested. Figures 4.10B and C revealed that JNK and MKK4 were expressed at similar levels.

A similar result was seen for the wild type macrophages immunoprecipitated with MKK7 although a weaker signal was seen as demonstrated in Figure 4.11A. There were low levels of MKK7 phosphorylation in response to 15 and 30 min stimulation with 100 nM Pam_3CSK4, 100 ng/ml LPS and 1 μg/ml R848 (lanes 2-7). 45 and 90 min stimulations with 3 μg/ml mCpGB and 25 μg/ml PolyIC also induced the phosphorylation of MKK7 (lanes 8 to 11). A high basal level of MKK7 phosphorylation (lane 1) and the non-specific incorporation of radio-labelled phosphate into JNK2 K/R in the IgG control sample (lane 12) cast doubt over the actual levels of MKK7 phosphorylation in response to TLR ligands. Figures 4.11B and C revealed that JNK and MKK7 were expressed at the similar levels.

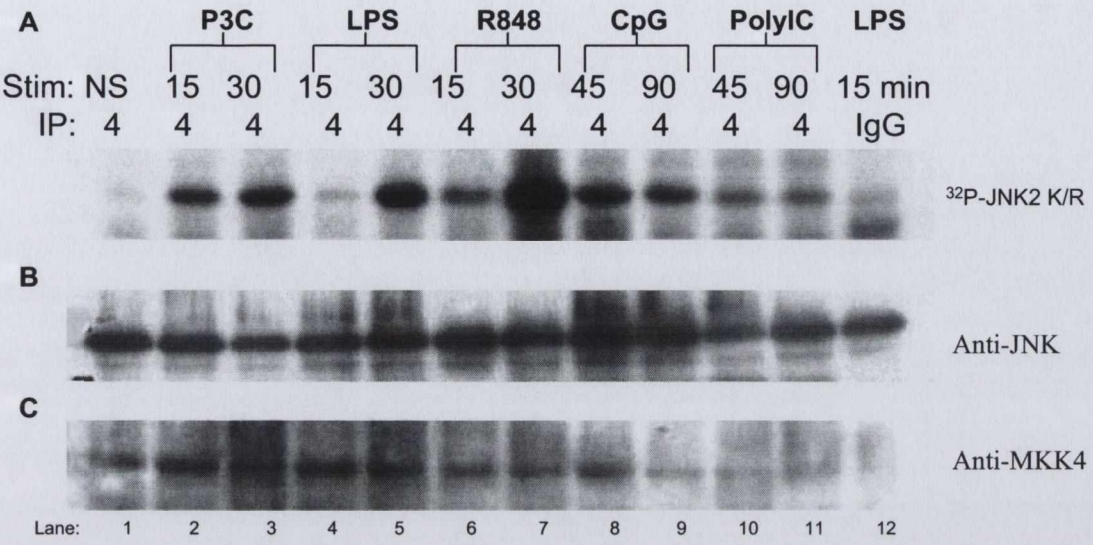

Figure 4.10. **Stimulation of wild type macrophages with TLR ligands leads to the phosphorylation of MKK4 in a JNK2 kinase assay**

Immortalised macrophages derived from the bone marrow of wild type mice were treated for the indicated times with 100 nM Pam_3CSK4, 100 ng/ml LPS, 1 μg/ml R848, 3 μg/ml mCpGB or 25 μg/ml PolyIC. The cells were lysed in low stringency buffer and a Bradford assay was used to determine protein concentration. The lysates were pre-cleared with 20 μl protein A/G-plus agarose beads for 45 min at 4°C. The lysates were incubated overnight at 4°C with 40 μl protein A/G-plus agarose beads and 3 μl anti-MKK4 or rabbit IgG. The beads were washed twice in low stringency lysis buffer and twice in kinase buffer. The beads were then incubated for 30 min at 37°C in kinase buffer, 20 nM ATP, 5 μCu ^{32}P-ATP and 2 μg JNK2 K/R at 1,000 rpm. 8 μl 5x sample loading buffer was added and the samples were analysed by Western blotting for the incorporation of ^{32}P-ATP into JNK2 (A). The samples were then examined for the presence of JNK (B) and MKK4 (C).

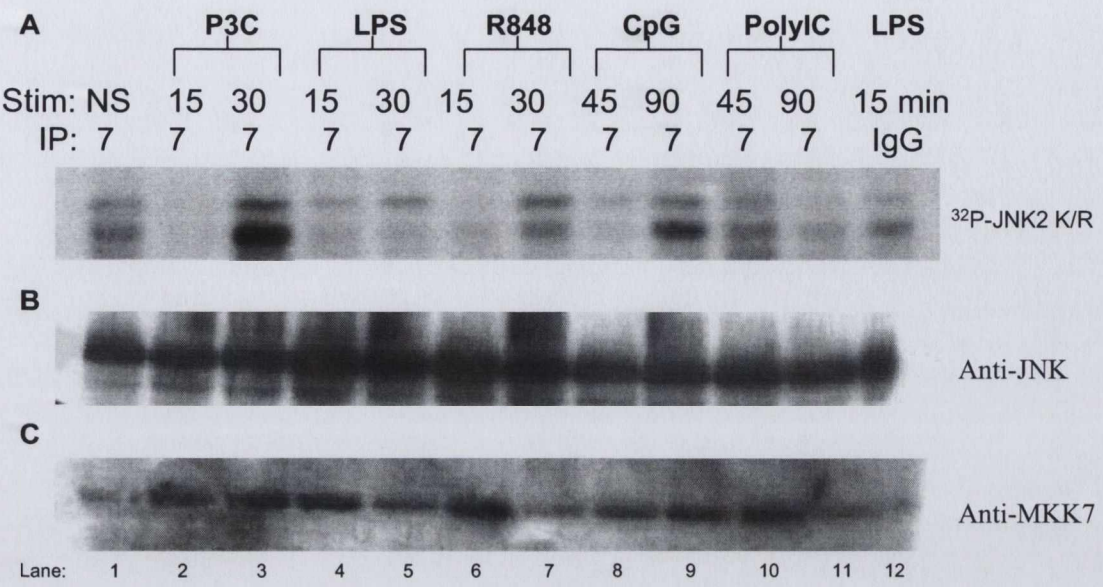

Figure 4.11. **Stimulation of wild type macrophages with TLR ligands leads to the phosphorylation of MKK7 in a JNK2 kinase assay**

Immortalised macrophages derived from the bone marrow of wild type mice were treated for the indicated times with 100 nM Pam_3CSK4, 100 ng/ml LPS, 1 μg/ml R848, 3 μg/ml mCpGB or 25 μg/ml PolyIC. The cells were lysed in low stringency buffer and a Bradford assay was used to determine protein concentration. The lysates were pre-cleared with 20 μl protein A/G-plus agarose beads for 45 min at 4°C. The lysates were incubated overnight at 4°C with 40 μl protein A/G-plus agarose beads and 3 μl anti-MKK7 or rabbit IgG. The beads were washed twice in low stringency lysis buffer and twice in kinase buffer. The beads were then incubated for 30 min at 37°C in kinase buffer, 20 nM ATP, 5 μCu ^{32}P-ATP and 2 μg JNK2 K/R at 1,000 rpm. 8 μl 5x sample loading buffer was added and the samples were analysed by Western blotting for the incorporation of ^{32}P-ATP into JNK2 (A). The samples were then examined for the presence of JNK (B) and MKK7 (C).

I next tested for the phosphorylation of MKK4 and MKK4 in the wild type and Mal-deficient macrophages response to PolyIC stimulation. LPS and R848 were used as controls.

As shown in Figure 4.12A when the wild type macrophages were stimulated with 100 ng/ml LPS or 1 μg/ml R848 for 30 min (lanes 4 and 5) phosphorylation of MKK4 occurred. There was also slight phosphorylation of MKK4 in the wild type cells in response to stimulation with 25 μg/ml PolyIC for 45 and 90 min (lanes 2 and 3) when compared with the non-stimulated sample (lane 1); although the basal level of MKK4 phosphorylation was quite high. There was no non-specific ^{32}P incorporation into JNK2 K/R in the IgG control sample (lane 6).

Stimulation of the Mal-deficient macrophages with LPS and R848 (lanes 10 and 11) induced the phosphorylation of MKK4. Stimulation with 25 μg/ml PolyIC for 45 and 90 min also resulted in MKK4 phosphorylation in the Mal-deficient macrophages (lanes 8 and 9) although the level was not much greater than the non-stimulated control (lane 7). The IgG control lane (lane 12) was clear therefore there was no non-specific incorporation of ^{32}P into JNK2 K/R. There was a no clear enhancement of MKK4 phosphorylation in response to PolyIC in the Mal-deficient macrophages when compared to the wild type cells (lanes 8 and 9 compared to lanes 2 and 3). Figures 4.12B revealed that JNK levels were similar in all samples tested.

When the phosphorylation of MKK7 was examined as outlined in Figure 4.13A similar results were seen. Treatment of the wild type macrophages with 100 ng/ml LPS or 1 μg/ml R848 for 30 min resulted in the phosphorylation of MKK7 (lanes 4 and 5). Stimulation with 25 μg/ml PolyIC for 45 and 90 min also resulted in the phosphorylation of

MKK7 (lanes 2 and 3). The basal level of MKK7 activation was low (lane 1) and the IgG control lane was clear (lane 6).

LPS stimulation of the Mal-deficient macrophages did not result in the phosphorylation of MKK7 (lane 10) when compared to the basal level (lane 7). Stimulation of the Mal-deficient macrophages with 1 μg/ml R848 did result in the phosphorylation of MKK7 (lane 11) as did 25 μg/ml PolyIC (lanes 8 and 9). The IgG control lane was clear (lane 12). There was no enhancement in MKK7 phosphorylation in response to PolyIC stimulation in the Mal-deficient macrophages in comparison to the wild type cells (lanes 8 and 9 compared to lanes 2 and 3). Figures 4.13B revealed that JNK was expressed at the same level in each sample.

The levels of MKK4 and MKK7 immunoprecipitaed from the macrophages were undetectable by Western blotting in these assays perhaps due to the low level of expression of these proteins in these cells.

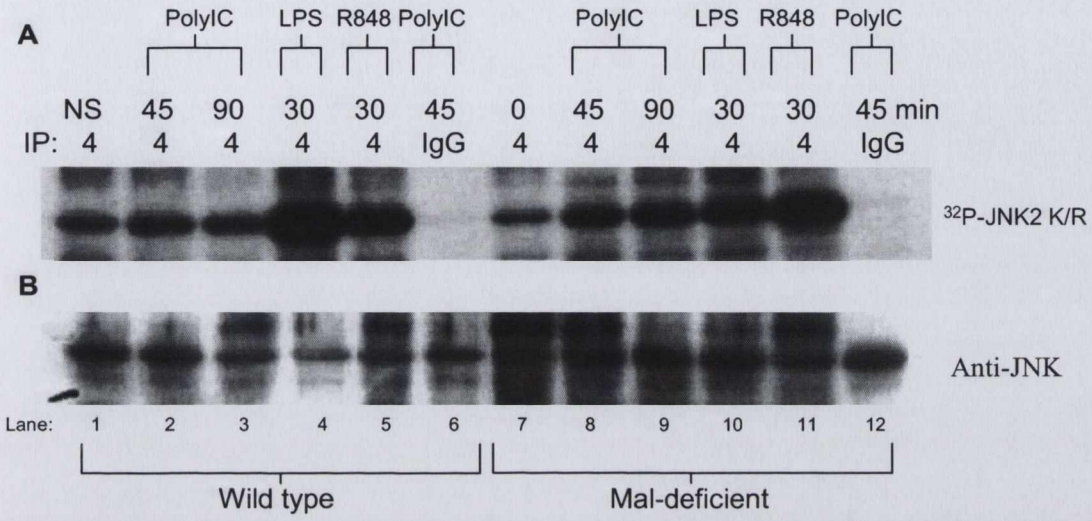

Figure 4.12. **PolyIC induced MKK4 phosphorylation was not enhanced in the absence of Mal in a JNK2 kinase assay**

Immortalised macrophages derived from the bone marrow of wild type and Mal-deficient mice were treated for the indicated times with 25 μg/ml PolyIC, 100 ng/ml LPS or 1 μg/ml R848. The cells were lysed in low stringency buffer and a Bradford assay was used to determine protein concentration. The lysates were pre-cleared with 20 μl protein A/G-plus agarose beads for 45 min at 4°C. The lysates were incubated overnight at 4°C with 40 μl protein A/G-plus agarose beads and 3 μl anti-MKK4 or rabbit IgG. The beads were washed twice in low stringency lysis buffer and twice in kinase buffer. The beads were then incubated for 30 min at 37°C in kinase buffer, 20 nM ATP, 5 μCu ^{32}P-ATP and 2 μg JNK2 K/R at 1,000 rpm. 8 μl 5x sample loading buffer was added and the samples were analysed by Western blotting for the incorporation of ^{32}P-ATP into JNK2 (A). The samples were then examined for the presence of JNK (B).

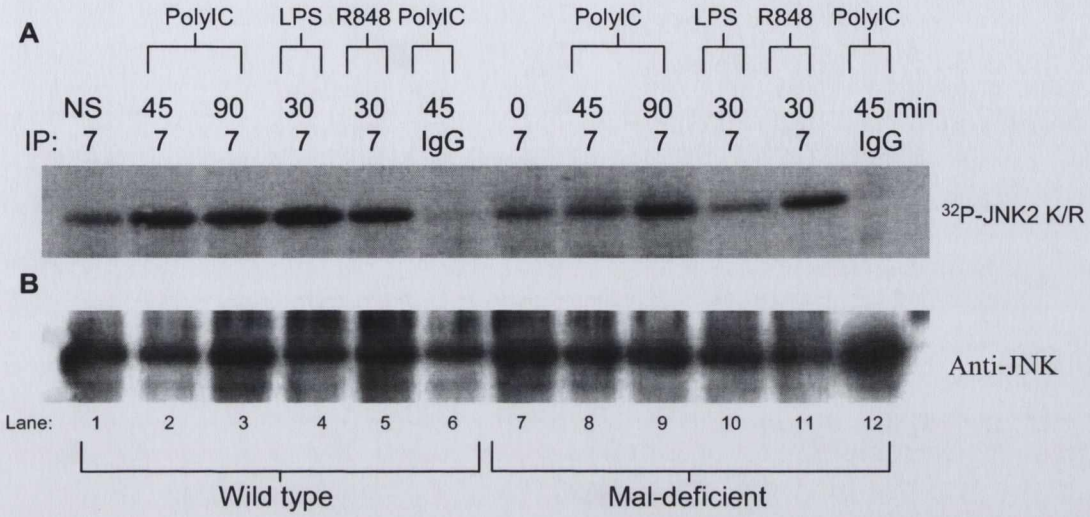

Figure 4.13. **PolyIC induced MKK7 phosphorylation was not enhanced in the absence of Mal in a JNK2 kinase assay**

Immortalised macrophages derived from the bone marrow of wild type and Mal-deficient mice were treated for the indicated times with 25 μg/ml PolyIC, 100 ng/ml LPS or 1 μg/ml R848. The cells were lysed in low stringency buffer and a Bradford assay was used to determine protein concentration. The lysates were pre-cleared with 20 μl protein A/G-plus agarose beads for 45 min at 4°C. The lysates were incubated overnight at 4°C with 40 μl protein A/G-plus agarose beads and 3 μl anti-MKK7 or rabbit IgG. The beads were washed twice in low stringency lysis buffer and twice in kinase buffer. The beads were then incubated for 30 min at 37°C in kinase buffer, 20 nM ATP, 5 μCu ^{32}P-ATP and 2 μg JNK2 K/R at 1,000 rpm. 8 μl 5x sample loading buffer was added and the samples were analysed by Western blotting for the incorporation of ^{32}P-ATP into JNK2 (A). The samples were then examined for the presence of JNK (B).

4.2.8 LPS contamination of PolyIC masks any enhancement of TLR3 signalling in the absence of Mal

Due to the somewhat surprising result in the JNK2 kinase assays where no enhancement of MKK4 or MKK7 phosphorylation was evident in the Mal-deficient macrophages in response to PolyIC stimulation I next re-tested the PolyIC responses by IL6 ELISA. The PolyIC used in the initial signalling experiments (ELISAs and time courses) was purchased from Amersham whereas the PolyIC used in the kinase assays was purchased from Sigma.

As shown in Figure 4.14 the wild type macrophages (black bars) produced IL6 in response to 100 nM Pam_3CSK4, 25 μg/ml PolyIC from Sigma, 100 ng/ml LPS, 1 μg/ml R848 and 3 μg/ml mCpGB as expected. Surprisingly the Mal-deficient macrophages (white bars) failed to produce IL6 in enhanced levels over the wild type cells when stimulated with PolyIC. In fact the Mal-deficient macrophages induced IL6 production at a far lower level than the wild type cells. All other TLR ligands produced the expected responses in the Mal-deficient macrophages with no requirement for Mal in response to Pam_3CSK4, R848 or CpG and no IL6 production in response to LPS.

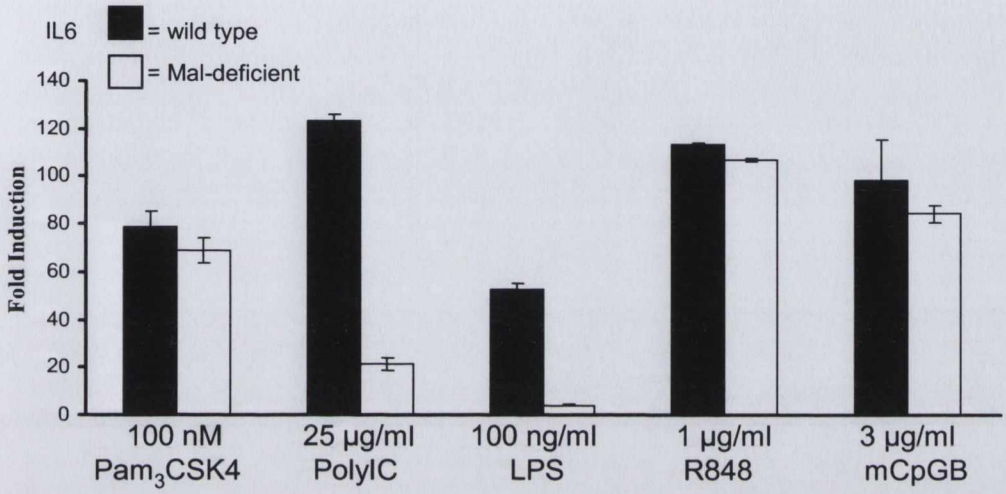

Figure 4.14. **Stimulation of macrophages with PolyIC from Sigma results in decreased IL6 production in the absence of Mal**

Immortalised macrophages derived from the bone marrow of wild type and Mal-deficient mice were treated with 100 nM Pam_3CSK4, 25 µg/ml PolyIC from Sigma, 100 ng/ml LPS, 1 µg/ml R848 or 3 µg/ml mCpGB. After 18 h incubations IL6 production was measured by ELISA. Data are expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. These data are representative of two experiments.

Due to the unexpected lack of enhancement in IL6 production in the Mal-deficient macrophages in response to the Sigma PolyIC I next tested the PolyIC for LPS contamination. I began by incubating the Sigma and Amersham PolyIC, LPS and R848 with 10 μg/ml Polymyxin B for 24 h. Polymyxin B binds LPS and prevents it activating TLR4 (Cardoso et al., 2007) therefore removing any LPS contamination from the ligands.

As described in Figure 4.15A when stimulated with 10 and 25 μg/ml Sigma PolyIC the wild type cells (black bars) produced large amounts IL6. The Mal-deficient macrophages (white bars) also produced IL6 in response to the Sigma PolyIC but at a much lower level. Pre-incubation of the PolyIC with Polymyxin B resulted in far lower levels of IL6 production in the wild type cells and a slight reduction in IL6 levels in the Mal-deficient macrophages as outlined in Figure 4.15B. The enhancement of IL6 production in the absence of Mal was seen in the samples pre-treated with Polymyxin B. This revealed that the loss of enhancement in the IL6 production in the Mal-deficient macrophages as seen in Figures 4.14 was due to LPS contamination.

To ensure earlier results generated using the Amersham PolyIC were not also due to LPS contamination I also treated it with Polymyxin B and examined the production of IL6. As shown in Figure 4.15C the wild type macrophages produced IL6 in response to 10 and 25 μg/ml PolyIC at similar levels with and with out Polymyxin B treatment. The Mal-deficient macrophages also produced IL6 in response to PolyIC stimulation and the levels were greater than that seen for the wild type cells both with and without Polymyxin B treatment. This verified that the data generated with the Amersham PolyIC was accurate.

As a control I also incubated the Polymyxin B with LPS and R848. As outlined in Figure 4.15D the presence of Polymyxin B in the LPS samples completely inhibited IL6

production in the wild type cells. The incubation of R848 with Polymyxin B had no effect on the ability of the wild type or Mal-deficient macrophages to produce IL6.

Therefore the lack of enhancement in signalling through TLR3 in Mal-deficient cells in the kinase assays may have been due to LPS contamination in the Sigma PolyIC. This resulted in boosted responses in the wild type cells as both TLR3 and TLR4 were activated. The Mal-deficient cells on the other hand could only respond to the TLR3 stimulation hence seemed to be less responsive than previous experiments suggested.

Pre-treatment of the Sigma PolyIC with Polymyxin B before stimulation of the macrophages used for the JNK2 kinase assay did not seen like a viable solution to this LPS contamination issue as the level of IL6 production in response to the Sigma PolyIC treated with Polymyxin B was so low (less than 2 fold in the wild type cells in Figure 4.13B). I therefore tested several other PolyIC reagents (Imgenex and Invitrogen) for IL6 production in the wild type and Mal-deficient macrophages. The PolyIC from Imgenex and Invitrogen also had very low activity therefore yielding them unsuitable for use in the JNK2 kinase assays or ELISAs. The generation of a new batch of PolyIC by these companies resulted in a lower molecular weight product and this may be the reason for its lack of ability to activate murine cells. The new batches of PolyIC were still capable of activating human cell lines, as demonstrated by my use on the U373 cells, revealing that shorter PolyIC specifically activates human TLR3 whereas murine TLR3 requires longer PolyIC.

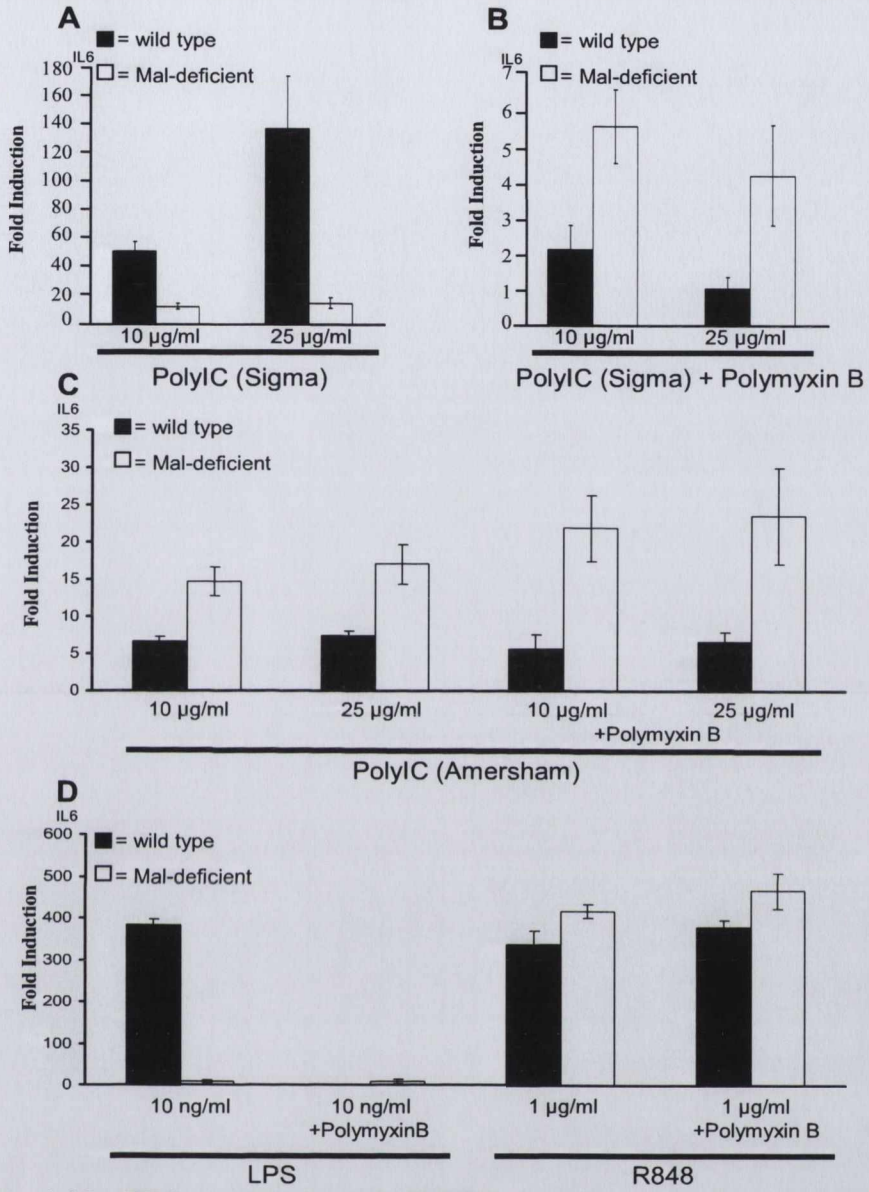

Figure 4.15. **LPS contaminated PolyIC results in a loss of enhanced IL6 production in the absence of Mal**

Macrophages derived from the bone marrow of wild type and Mal-deficient mice were treated with 10 and 25 µg/ml PolyIC from Sigma (A) +/- 10 µg/ml Polymyxin B (B), 10 and 25 µg/ml PolyIC from Amersham +/- 10 µg/ml Polymyxin B (C) or 10 ng/ml LPS and 1 µg/ml R848 +/- 10 µg/ml Polymyxin B (D). After 18 h incubations IL6 production was measured by ELISA. Data are expressed relative to untreated cells, and are the mean ± S.D of triplicate determinations. These data are representative of two experiments.

4.3 Results: Interaction experiments

Due to the lack of PolyIC that would consistently activate the macrophages as required I next attempted to decipher the mechanism by which Mal can inhibit TLR3 signalling by examining, through interaction studies, where in the TLR3 signalling complex Mal was acting as an inhibitor. As TLR3 has been shown to interact directly with IRAK2 (Keating et al., 2007) and Mal and IRAK2 also directly interact (Fitzgerald et al., 2001) I hypothesised that the inhibition of TLR3 signalling by Mal may be due to its sequestration of IRAK2 away from TLR3. To test this theory I first repeated these co-immunoprecipitation assays to confirm these interactions.

4.3.1 Mal and IRAK2 interact in a co-immunoprecipitation assay

To begin my interaction experiments I transfected HEK-293T cells with Myc-IRAK2 and Ha-Mal and co-immunoprecipitation assays were carried out on the cell lysates.

As shown in Figure 4.16 when the cell lysates were incubated with the Myc antibody and tested for the presence of Ha-Mal a band was seen demonstrating an interaction occurred between Mal and IRAK2 (lane 3, bottom panel). The Myc antibody successfully precipitated the Myc-IRAK2 in the same sample (lane 3, top panel). The Myc antibody did not non-specifically precipitate the Ha-Mal (lane 5, bottom panel) and the Ha antibody did not non-specifically precipitate the Myc-IRAK2 (lane 1, top panel). Both proteins were expressed at similar levels (lanes 6 to 10, top and bottom panels).

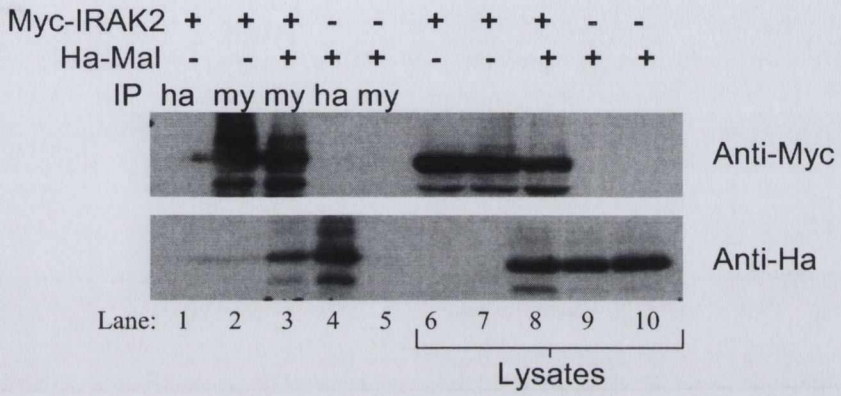

Figure 4.16. **Mal and IRAK2 interact in a co-immunoprecipitation assay**

HEK-293T cells were transiently transfected with 3 μg Myc-IRAK2, Ha-Mal and EV for 24 h, lysed in low stringency lysis buffer and co-immunoprecipitated with either Myc or Ha antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-Myc or anti-Ha antibodies. These data are representative of three experiments.

4.3.2 TLR3 and IRAK2 do not interact in a co-immunoprecipitation assay

I next tested if TLR3 and IRAK2 could interact as has been previously shown (Keating et al., 2007). HEK-293T cells were transiently transfected with flag-TLR3 and Myc-IRAK2 and tested for their ability to interact via a co-immunoprecipitation assay using the anti-flag and anti-Myc antibodies. The co-immunopreciptiation assay was first carried out with the flag antibody and the presence of Myc-IRAK2 was examined. As illustrated in Figure 4.17A using the flag antibody to precipitate TLR3 and Westen blotting for the presence of IRAK2 (lanes 1-4, bottom panel) did not reveal any interaction. TLR3 was pulled down with the anti-flag antibody at concentrations of 5 and 10 μg (lanes 2 and 3, top panel) and the negative controls were blank (lanes 5 and 6, both panels).

The co-immunoprecipiation assay was carried out in the other direction with the Myc antibody and tested for the presence of flag-TLR3. As demonstrated in Figure 4.17B TLR3 and IRAK2 did not interact (lanes 1 and 2, top panel). Myc-IRAK2 was pulled down with the Myc antibody (lanes 1 and 2, bottom panel) All positive and negative controls were as anticipated (lanes 3 to 6 both panels) and the proteins expressed at similar levels in the lysates (lanes 7 to 12 both panels).

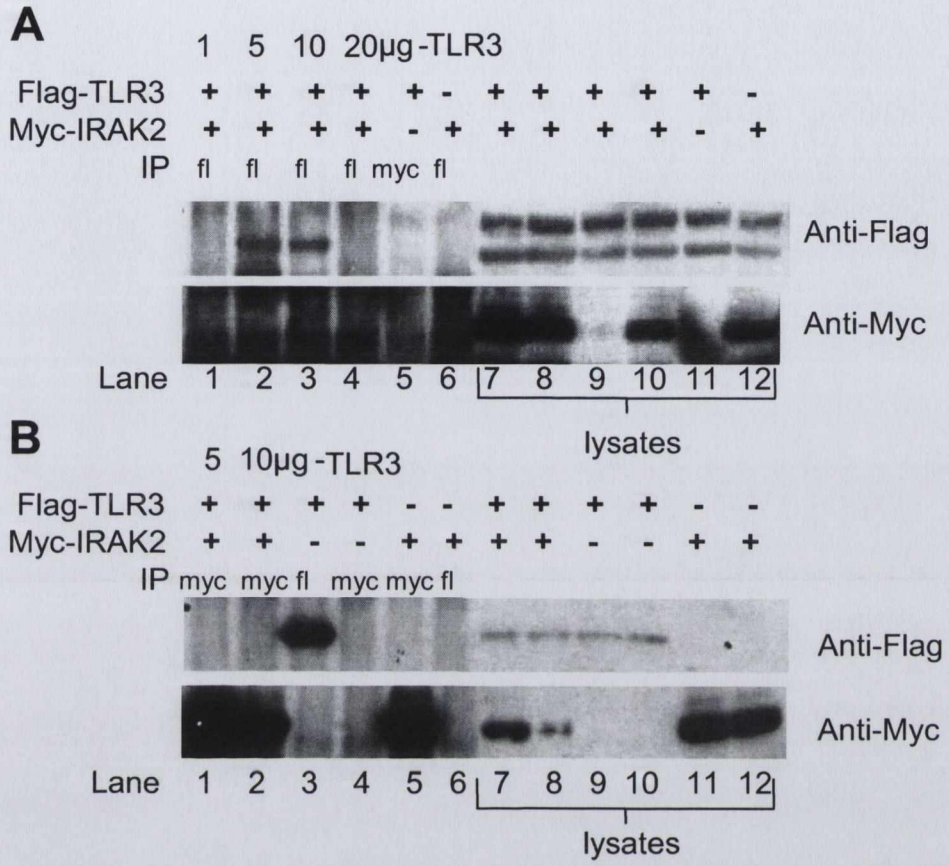

Figure 4.17. **TLR3 and IRAK2 do not interact in a co-immunoprecipitation assay**

HEK-293T cells were transiently transfected with 1, 5, 10 or 20 µg flag-TLR3, 3 µg Myc-IRAK2 and EV (A) or 5 and 10 µg TLR3, 3 µg Myc-IRAK2 and EV (B). 24 h post transfection the cells were lysed in high stringency lysis buffer and co-immunoprecipitated with either Flag or Myc antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-flag or anti-Myc antibodies. These data are representative of three experiments.

4.3.3 TLR3 and IRAK2 do not interact after PolyIC stimulation

I next repeated the co-immunoprecipitation assay but stimulated the cells with 25 μg/ml PolyIC for 3 h before lysis. As illustrated in Figure 4.18A when the samples were immunoprecipitated with the flag antibody and analysed for the presence of Myc-IRAK2 no interaction was seen (lane 1, bottom panel). The flag antibody was able to pull down the flag-TLR3 (lane 1, top panel). The positive and negative controls were as anticipated (lanes 2 to 5, both panels) and the proteins were expressed in the lysates (lanes 6 to 10, both panels).

The co-immunoprecipitation assay was then repeated in the other direction with the samples incubated with the Myc antibody and analysed for the presence of flag-TLR3. As shown in Figure 4.18B there was no interaction between TLR3 and IRAK2 (lane 1, top panel). The Myc antibody pulled down Myc-IRAK2 in the same sample (lane 1, bottom panel). The positive and negative controls were correct (lanes 2 to 5, both panels) and the proteins expressed at similar levels in the whole cell lysates (lanes 6 to 10, both panels).

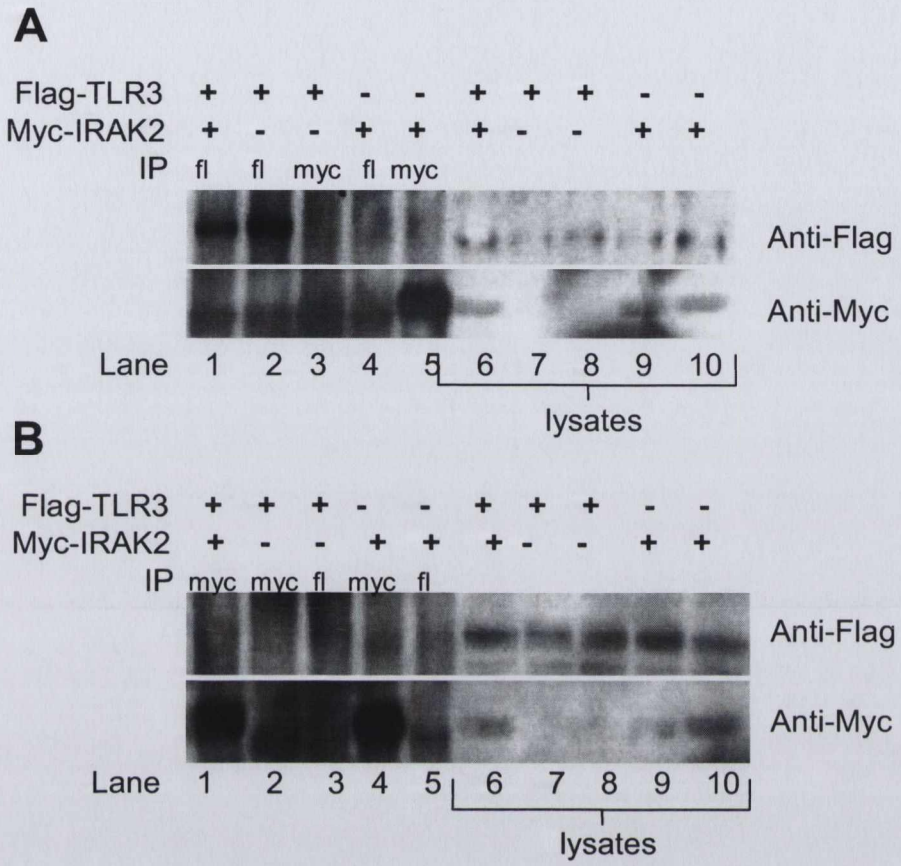

Figure 4.18. **TLR3 and IRAK2 do not interact in a co-immunoprecipitation assay upon PolyIC stimulation**

HEK-293T cells were transiently transfected with 5 μg flag-TLR3, 3 μg/ml Myc-IRAK2 and empty vector (EV). 24 h post transfection the cells were stimulated for 3 h with 25 μg/ml PolyIC. The cells were lysed in high stringency lysis buffer and co-immunoprecipitated with either Flag or Myc antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-flag or anti-Myc antibodies.

4.3.4 TRIF and IRAK2 do not interact

As TLR3 and IRAK2 did not interact I next tested if TRIF and IRAK2 could interact and thus TRIF could act as a bridge between TLR3 and IRAK2 and Mal may inhibit this interaction in TLR3 signalling. HEK-293T cells were transfected with Ha-TRIF and Myc-IRAK2 and co-immunoprecipitation assays were carried out on the cell lysates.

As shown in Figure 4.19A when the samples were incubated with the Ha antibody and tested for the presence of Myc-IRAK2 no interaction was seen (lane 1, bottom panel). The Ha antibody was able to pull down the Ha-TRIF in the same sample (lane 1, top panel). All positive and negative controls were as expected (lanes 2 to 5, both panels) and the proteins expressed at the same level in the whole cell lysates (lanes 6 to 10, both panels).

The co-immunoprecipitation assay was then repeated in the other direction by incubating the cell lysates with the Myc antibody and testing for the presence of Ha-TRIF. As shown in Figure 4.19B no interaction was seen (lane 1, top panel). The Myc antibody could pull down Myc-IRAK2 in the same sample (lane 1, bottom panel). The positive and negative controls were as anticipated (lanes 2 to 5, both panels) and the whole cell lysates (lanes 6 to 10, both panels) showed similar levels of expression of both proteins.

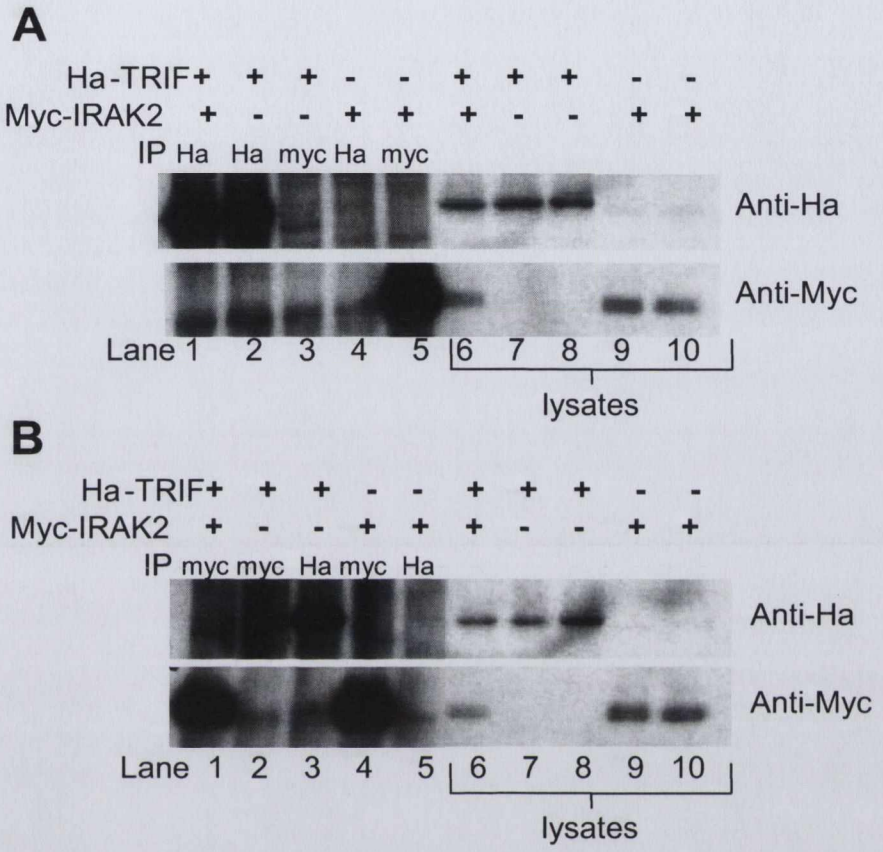

Figure 4.19. **TRIF and IRAK2 do not interact in a co-immunoprecipitation assay** HEK-293T cells were transiently transfected with 3 μg Ha-TRIF, Myc-IRAK2 and EV. 24 h post transfection the cells were lysed in low stringency lysis buffer and co-immunoprecipitated with either Myc or Ha antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-Myc or anti-Ha antibodies.

4.3.5 Generation of GST-TLR3

The co-immunoprecipitation assays using flag-TLR3 were difficult to optimise due to the varying levels of TLR3 expression from experiment to experiment. It was also difficult to isolate TLR3 from the membrane with a lysis buffer that would not break the interactions between proteins during lysis. I therefore decided to generate a GST fusion protein containing the TIR domain of TLR3 and use it to carry out GST-pulldown assays with IRAK2 in an attempt to confirm the interaction shown in the Keating et al. paper.

The sequence of the TIR domain of TLR3 was retrieved from the NCBI database and primers with an *EcoR* I and a *Sal* I at either end were designed. The TIR domain was then amplified out of the flag-TLR3 vector and the level of amplification can be seen in Figure 4.20A. The PCR product was removed from the gel, restriction digested with the *EcoR* I and *Sal* I enzymes and ligated into the pGex-4T2 vector shown in Figure 4.20B.

The vector was transformed into DH5-α bacterial cells, the DNA was isolated and again restriction digested with the *EcoR* I and *Sal* I enzymes to ensure the insert had gone into the pGex vector. As shown in Figure 4.20C a 500 bp band was seen for TLR3. Several samples of DNA were then sequenced to ensure no mutations occurred during the PCR process and as seen in Figure 4.21 when aligned using ClustalW2 (Thompson et al., 1994) the sequence of the GST-TLR3-TIR domain generated was identical to the TIR domain sequence for TLR3 from the NCBI database.

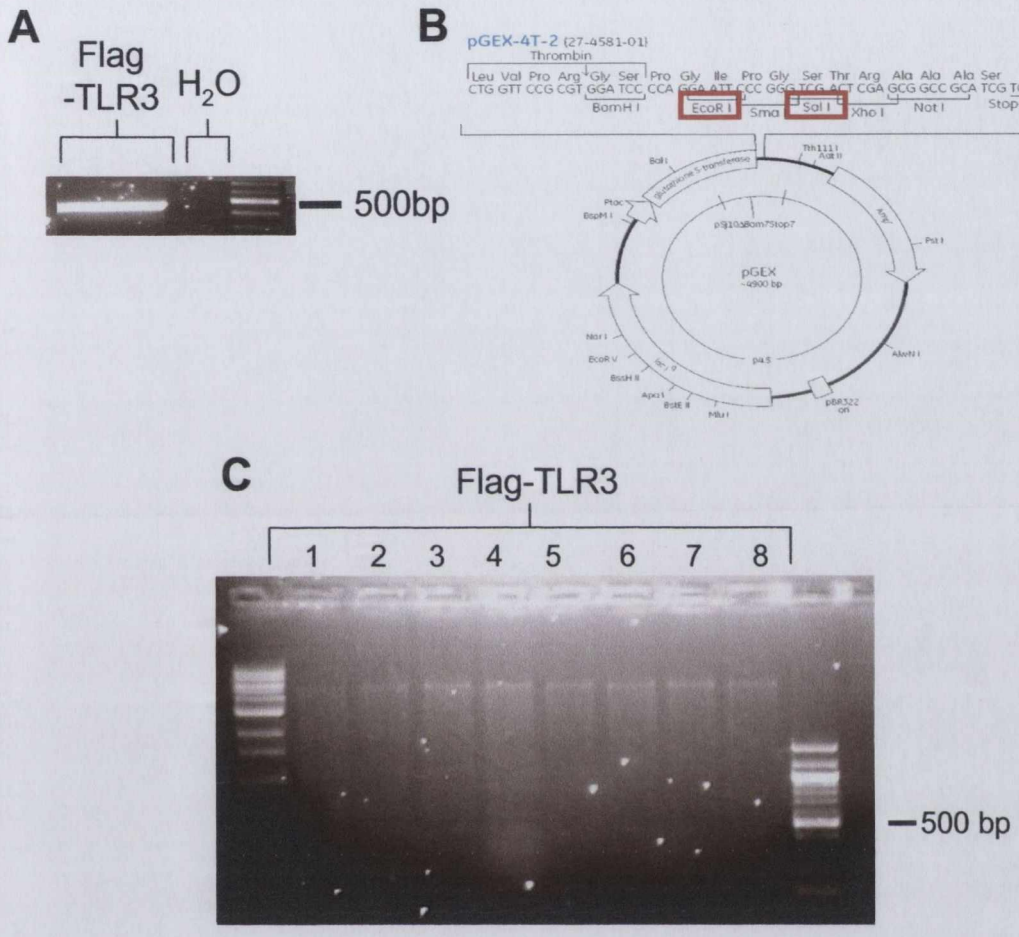

Figure 4.20. **TLR3 TIR domain was subcloned from the flag vector into the pGex-4T2 vector**

The TIR domain of flag-TLR3 was amplified out of its plasmid (A), digested with the *EcoR* I and *Sal* I restriction enzymes, and ligated into the pGex-4T2 vector (B). The plasmid was then grown in DH5-α cells, the DNA was isolated and restriction digests were carried out to confirm the presence of the insert (C).

TLR3

```
TLR3TIR          ---GCAGCATATATAATTCATGCCTATAAAGATAAGGATTGGGTCTGGGAACATTTCTCT 57
TLR3TIR6         CCCGCAGCATATATAATTCATGCCTATAAAGATAAGGATTGGGTCTGGGAACATTTCTCT 60
                    ************************************************************

TLR3TIR          TCAATGGAAAAGGAAGACCAATCTCTCAAATTTTGTCTGGAAGAAAGGGACTTTGAGGCG 117
TLR3TIR6         TCAATGGAAAAGGAAGACCAATCTCTCAAATTTTGTCTGGAAGAAAGGGACTTTGAGGCG 120
                 ************************************************************

TLR3TIR          GGTGTTTTTGAACTAGAAGCAATTGTTAACAGCATCAAAAGAAGCAGAAAAATTATTTTT 177
TLR3TIR6         GGTGTTTTTGAACTAGAAGCAATTGTTAACAGCATCAAAAGAAGCAGAAAAATTATTTTT 180
                 ************************************************************

TLR3TIR          GTTATAACACACCATCTATTAAAAGACCCATTATGCAAAAGATTCAAGGTACATCATGCA 237
TLR3TIR6         GTTATAACACACCATCTATTAAAAGACCCATTATGCAAAAGATTCAAGGTACATCATGCA 240
                 ************************************************************

TLR3TIR          GTTCAACAAGCTATTGAACAAAATCTGGATTCCATTATATTGGTTTTCCTTGAGGAGATT 297
TLR3TIR6         GTTCAACAAGCTATTGAACAAAATCTGGATTCCATTATATTGGTTTTCCTTGAGGAGATT 300
                 ************************************************************

TLR3TIR          CCAGATTATAAACTGAACCATGCACTCTGTTTGCGAAGAGGAATGTTTAAATCTCACTGC 357
TLR3TIR6         CCAGATTATAAACTGAACCATGCACTCTGTTTGCGAAGAGGAATGTTTAAATCTCACTGC 360
                 ************************************************************

TLR3TIR          ATCTTGAACTGGCCAGTTCAGAAAGAACGGATAGGTGCCTTTCGTCATAAATTGCAAGTA 417
TLR3TIR6         ATCTTGAACTGGCCAGTTCAGAAAGAACGGATAGGTGCCTTTCGTCATAAATTGCAAGTA 420
                 ************************************************************

TLR3TIR          GCACTTGGATCCAAAAACTCTGTACATTAA---------------------------- 447
TLR3TIR6         GCACTTGGATCCAAAAACTCTGTACATTAACGTCGACTCGAGCGGCCGCATCGTGACTGA 480
                 ****************************
```

Figure 4.21. **The TIR domain of TLR3 subcloned into the pGex-4T2 is complementary to the TLR TIR domain sequence in the NCBI database**

The pGex-4T2 vector was sequenced and the results were compared with the known sequence of TLR3. Sequence alignments were carried out using ClustalW2 (Thompson et al., 1994).

The GST-EV and GST-TLR3 plasmids were next transformed into Rosetta gami 2 bacterial cells and the proteins were isolated by incubating the lysed bacteria with glutathione-sepharose 4B beads. 5, 10 and 20 µl of the GST-TLR3 and 1 µl of the GST-EV protein were analysed by SDS-PAGE with coomassie staining.

As shown in Figure 4.22 the GST and GST-TLR3 proteins were expressed at different levels. From these stained gels it was decided to use 0.5 µl GST and 20 µl GST-TLR3 for the GST-pulldown assays.

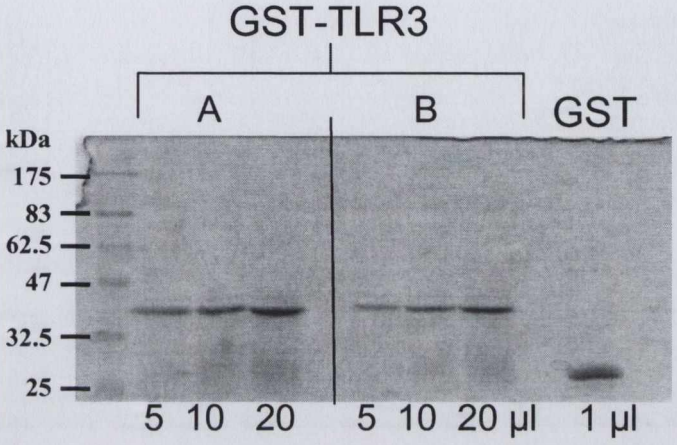

Figure 4.22. **GST and GST-TIR-TLR3 are expressed in bacteria and can be isolated from them**

Rosetta gami 2 bacterial cells were transformed with the pGex-4T2 plasmids containing GST or GST-TIR-TLR3. A single colony of each type was grown over night at 37^{0}C, transferred to a large culture and grown at 37^{0}C until the OD at 600 nm was 0.5. 100 µM IPTG was added to each culture and the bacteria were incubated overnight at 18^{0}C. The cells were pelleted, lysed and the GST-fusion proteins were isolated by incubation with glutathione-sepharose 4B beads at 4^{0}C for 3 h. 1 µl of GST and 5, 10 and 20 µl of GST-TLR3 were analysed by SDS-PAGE with coomassie staining.

4.3.6 GST-TLR3 interacts with TRIF but not IRAK2

I next used the GST-TLR3 TIR domain protein to pull down IRAK2 and, as a control, TRIF. As shown in Figure 4.23A GST-TLR3 pulled down Ha-TRIF (lane 2) and GST-EV did not (lane 1). TRIF was shown to be expressed in similar levels in the whole cell lysates (lanes 5 and 6). There were no bands in the lanes were GST-EV and GST-TLR3 were incubated with empty vector containing lysates (lanes 3 and 4) therefore the interaction seen between TLR3 and TRIF was specific.

As outlined in Figure 4.23B there was no interaction between GST-TLR3 and Myc-IRAK2 (lane 2). All positive and negative controls were as anticipated (lanes 1, 3 and 4) and the IRAK2 was expressed at similar levels in each sample in the whole cell lysates (lanes 5 and 6).

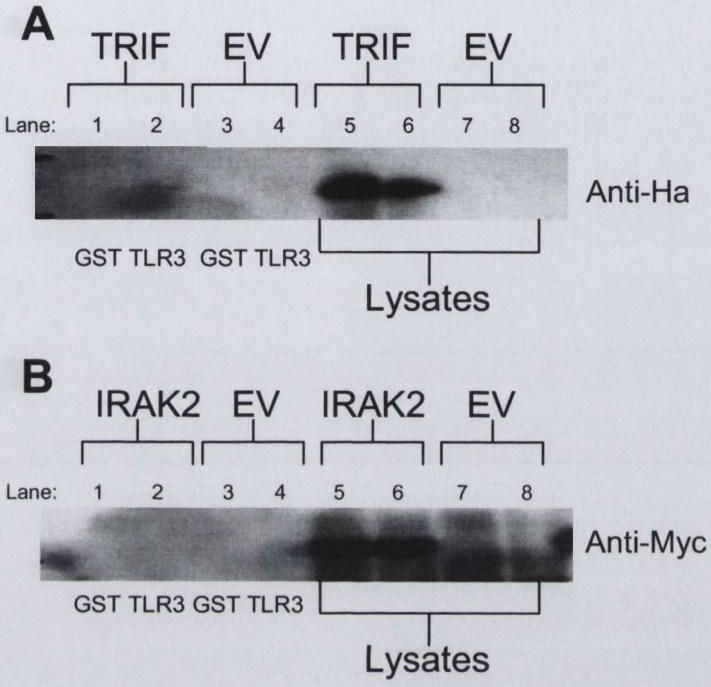

Figure 4.23. **GST-TLR3 interacts with TRIF but not IRAK2**

HEK-293T cells were transiently transfected with 3 μg Ha-TRIF (A), or Myc-IRAK2 (B) and EV for 24 h, lysed in low stringency lysis buffer, pre-cleared twice in glutathione-sepharose 4B beads for 45 min and incubated with GST-fusion protein containing the TIR domain of TLR3, for 3 h at 4^0C. The samples were washed three times and analysed by Western blotting with anti-Ha and Myc antibodies.

4.3.7 GST-TLR3 interacts with MyD88 but not Mal and Caspase1

To confirm the GST-TLR3 was specifically interacting with TRIF and not just binding non-specifically to TIR domain containing proteins I next tested the ability of GST-TLR3 to pull down MyD88, Mal and, as a negative control, Caspase1.

As outlined in Figure 4.24A, somewhat surprisingly, the GST-TLR3 did interact with Myc-MyD88 (lane 2). All negative controls were as expected (lanes 1, 3 and 4) and the MyD88 expressed to the same level in both samples (lanes 5 and 6). GST-TLR3 did not interact with Ha-Mal as shown in Figure 4.24B (lane 2) or Caspase1 (Figure 4.24C, lane 2) suggesting the interaction seen between TLR3 and MyD88 was specific.

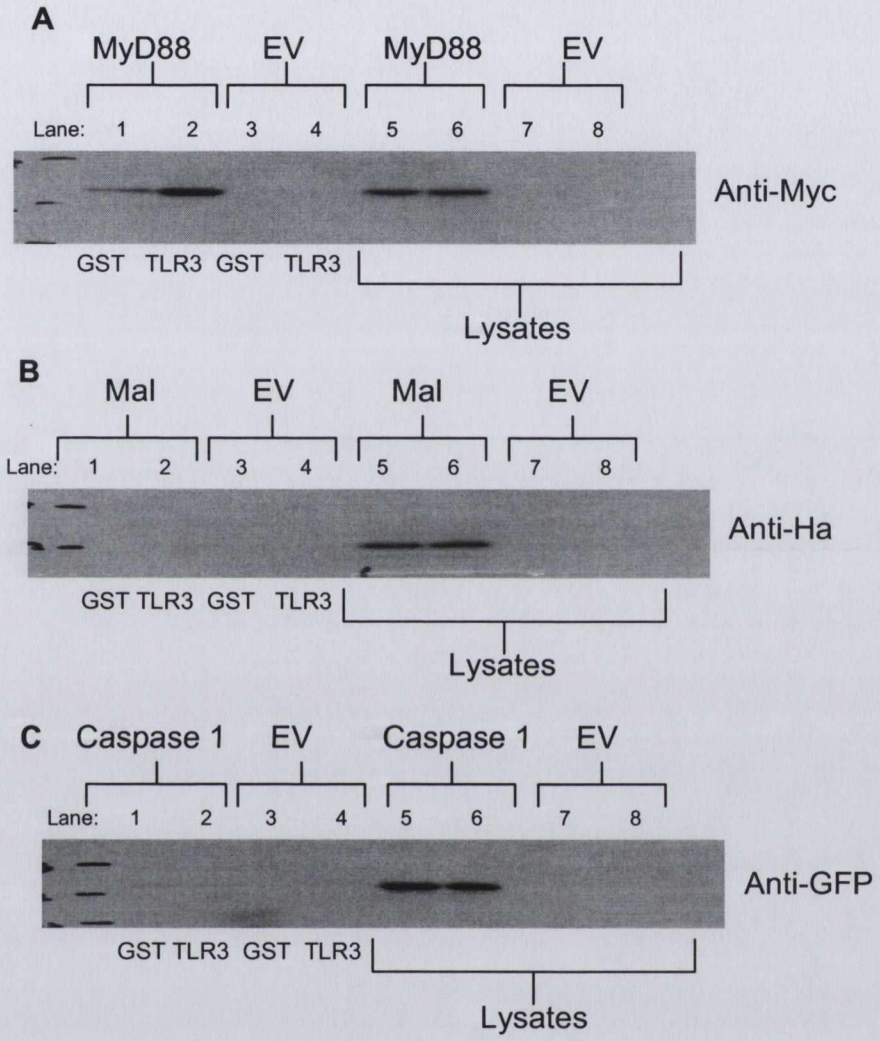

Figure 4.24. **TLR3 interacts with MyD88 but not Mal or Caspase 1**

HEK-293T cells were transiently transfected with 3 μg Myc-MyD88 (A), Ha-Mal (B) or GFP-Caspase1 (C) and EV for 24 h, lysed in low stringency lysis buffer, pre-cleared twice in glutathione-sepharose 4B beads and incubated with GST-fusion protein containing the TIR domain of TLR3, for 3 h at 4^0C. The samples were washed three times and analysed by Western blotting with anti-Myc, Ha and GFP

4.3.8 TRAF3 and TRAF6 interact with IRAK2

A recent study demonstrated that TRAF3 interacted with TRIF and MyD88 (Hacker et al., 2006). TRAF3 is known to be key to interferon production in TLR4 signalling with TRAF6 used for pro-inflammatory signalling and MAP-kinase activity in TLR4 signalling. A study by Tseng et al. revealed that TRAF3 must be degraded in response to TLR4 signalling to allow MAP-kinase activation. The degradation of TRAF3 required TRAF6 and the cIAPs (Tseng et al., 2010). The same may be true for TLR3 signalling where the degradation of TRAF3 allows MAP-kinase activation.

Due to the link between IRAK2 and TLR3 signalling as demonstrated by the Keating et al study (Keating et al., 2007) and the ability of Mal to interact with IRAK2 I hypothesised that TRAF3 could be the bridge between TLR3 and IRAK2 and hence the inhibition of TLR3 signalling by Mal was due to its ability to sequester IRAK2 from TRAF3. The interaction of TRAF3 and IRAK2 may be central to the degradation of TRAF3 by TRAF6 as TRAF6 is known to be recruited to IRAK2 in TLR3 signalling (Keating et al., 2007). As I had shown that Mal and IRAK2 interacted I wanted to examine if IRAK2 could interact with TRAF3 and, as a control, TRAF6 as this interaction has already been shown (Wesche et al., 1999).

As described in Figure 4.25 when cell lysates containing both TRAF6 and IRAK2 were incubated with the flag antibody and Western blotted for the presence of Myc-IRAK2 a band showing a TRAF6 and IRAK2 interaction was seen (lane 3, bottom panel). The flag antibody successfully pulled down the flag-TRAF6 in the same sample (lane 3, top panel).

All positive and negative controls (lanes 1, 2, 4 and 5, both panels) and whole cell lysates were as expected (lanes 6 to 10, both panels).

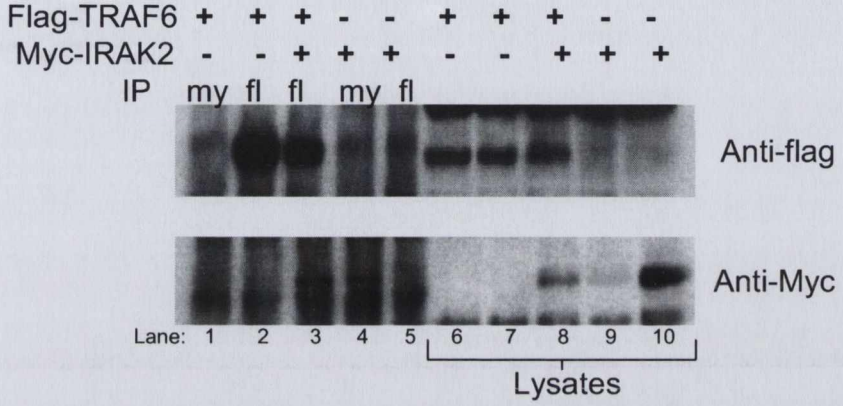

Figure 4.25. **TRAF6 and IRAK2 interact in a co-immunoprecipitation assay** HEK-293T cells were transiently transfected with 3 μg flag-TRAF6, Myc-IRAK2 and EV for 24 h, lysed in low stringency lysis buffer and co-immunoprecipitated with either Flag or Myc antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-flag or anti-Myc antibodies. These data are representative of three experiments.

TRAF3 and IRAK2 also interacted in a co-immunoprecipitation assay as shown in Figure 4.26. The cell lysates containing both proteins of interest were incubated with the flag antibody and Western blotted for the presence of Myc-IRAK2 and lane 1 (bottom panel) revealed a band showing there was an interaction between TRAF3 and IRAK2. The flag antibody pulled down flag-TRAF3 in the same sample (lane 1, top panel), all controls were as anticipated (lanes 2 to 5, top and bottom panels) and the whole cell lysates showed that the proteins were expressed at comparable levels (lanes 6 to 10, both panels).

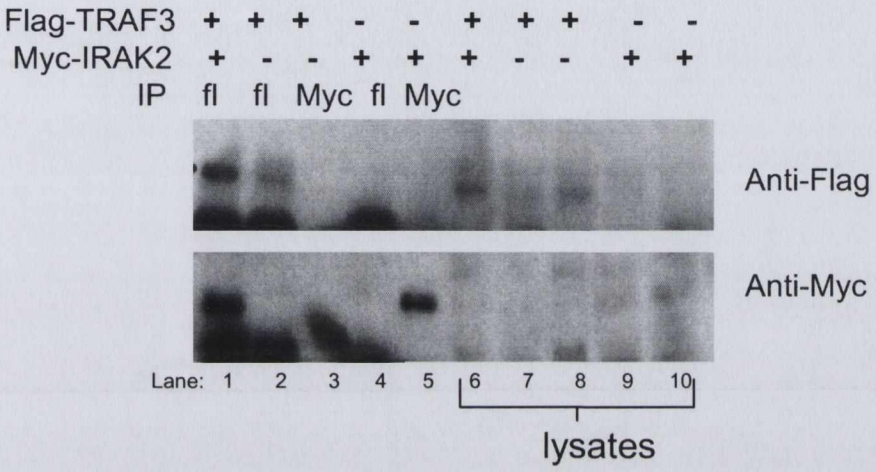

Figure 4.26 **TRAF3 and IRAK2 interact in a co-immunoprecipitation assay** HEK-293T cells were transiently transfected with 3 μg flag-TRAF3, Myc-IRAK2, and EV for 24 h, lysed in low stringency lysis buffer and co-immunoprecipitated with either Flag or Myc antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-flag or anti-Myc antibodies. These data are representative of three experiments.

4.3.9 TRAF6 interacts with Mal but TRAF3 does not

Since IRAK2 and TRAF3 were shown to directly interact I next wanted to examine if Mal and TRAF3 could interact as it has previously been shown that MyD88 and TRIF interact with TRAF3 (Hacker et al., 2006). I used TRAF6 as a positive control as it has previously been to directly interact with Mal (Mansell et al., 2004).

The flag antibody was incubated with cell lysates containing both flag-TRAF6 and Ha-Mal and the samples were Western blotted for the presence of Mal. As demonstrated in Figure 4.27 TRAF6 and Mal did interact (lane 3, bottom panel). Flag-TRAF6 was pulled down by the flag antibody in the same sample (lane 3, top panel). The controls (lanes 1, 2, 4 and 5, both panels) and lysates were as expected (lanes 6 to 10, both panels).

As seen in Figure 4.28A when the co-immunoprecipitation assay was carried out using flag-TRAF3 and Ha-Mal no interacting band was seen in the sample incubated with the flag antibody and Western blotted for the presence of Mal (lane 1, bottom panel). The flag antibody did precipitate the flag-TRAF3 (lane 1, top panel), the controls were correct (lanes 2 to 5, both panels) and the lysates were expressed evenly (lanes 6 to 10, both panels).

The co-immunoprecipitation assay was then carried out in the other direction with the sample containing both proteins incubated with the Ha antibody and Western blotted for the presence of flag-TRAF3. As shown is Figure 4.28B no interaction was seen (lane 1, top panel). The Ha antibody did precipitate Ha-Mal (lane 1, bottom panel), the controls were as anticipated (lanes 2 to 5, both panels) and the whole cell lysates were even (lane 6 to 10, both panels).

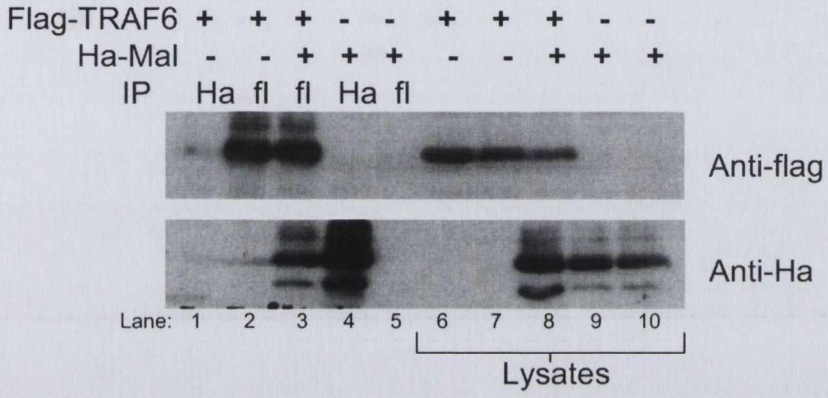

Figure 4.27. **TRAF6 and Mal interact in a co-immunoprecipitation assay**

HEK-293T cells were transiently transfected with 3 μg flag-TRAF6, Ha-Mal and EV for 24 h, lysed in low stringency lysis buffer and co-immunoprecipitated with either Flag or Ha antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-flag or anti-Ha antibodies. These data are representative of three experiments.

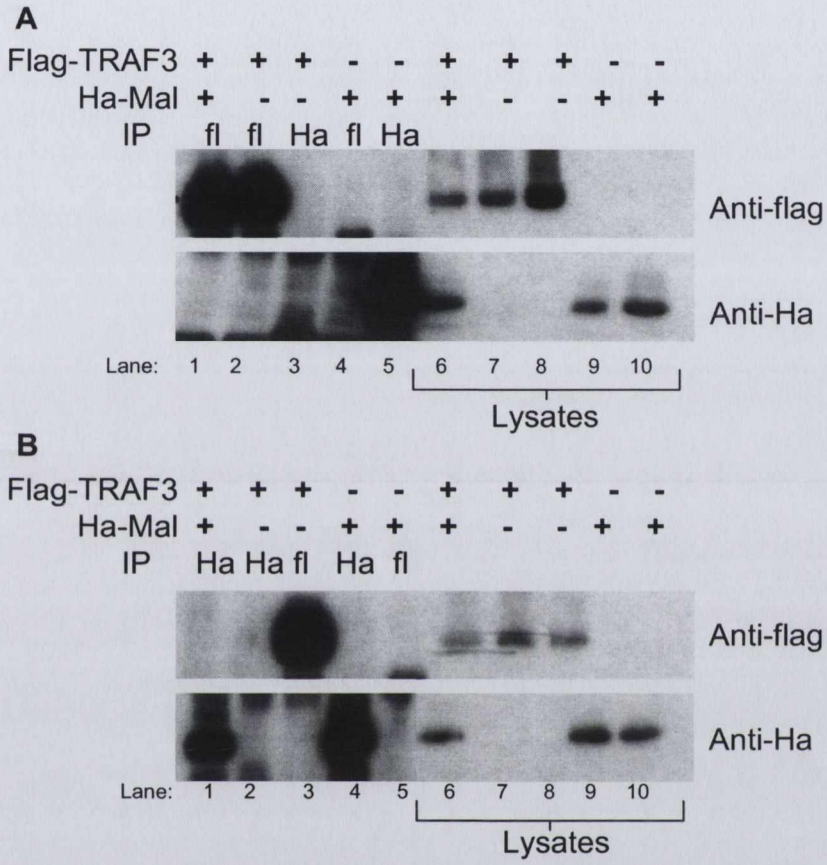

Figure 4.28. **TRAF3 and Mal do not interact in a co-immunoprecipitation assay**

HEK-293T cells were transiently transfected with 3 μg flag-TRAF3, Ha-Mal and EV for 24 h, lysed in low stringency lysis buffer and co-immunoprecipitated with either Flag or Ha antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-flag or anti-Ha antibodies. These data are representative of three experiments.

4.3.10 TRAF3 and IRAK2 can interact in the presence of Mal

The co-immunoprecipitation assays described above revealed that TRAF3 could interact with IRAK2 but not with Mal. I therefore decided to carry out the TRAF3 and IRAK2 co-immunoprecipitation assay in presence of Mal as I theorised that Mal may sequester the IRAK2 from TRAF3 and thus inhibit TLR3.

Samples containing TRAF3, IRAK2 and Mal were incubated with the flag antibody and Western blotted for the presence of Myc-IRAK2 as shown in Figure 29A. TRAF3 and IRAK2 interacted (lane 3, bottom panel) and in the presence of 1, 3 and 5 μg Ha-Mal (lanes 4 to 6, bottom panel) the interaction remained intact. The flag antibody precipitated flag-TRAF3 (lanes 3 to 6, top panel) and all the control samples were as expected (lanes 1, 2, 7 and 8, both panels). TRAF3 and IRAK2 were expressed at similar levels in the lysates and increasing amounts of Mal were seen in the lysates as shown in Figure 4.29B.

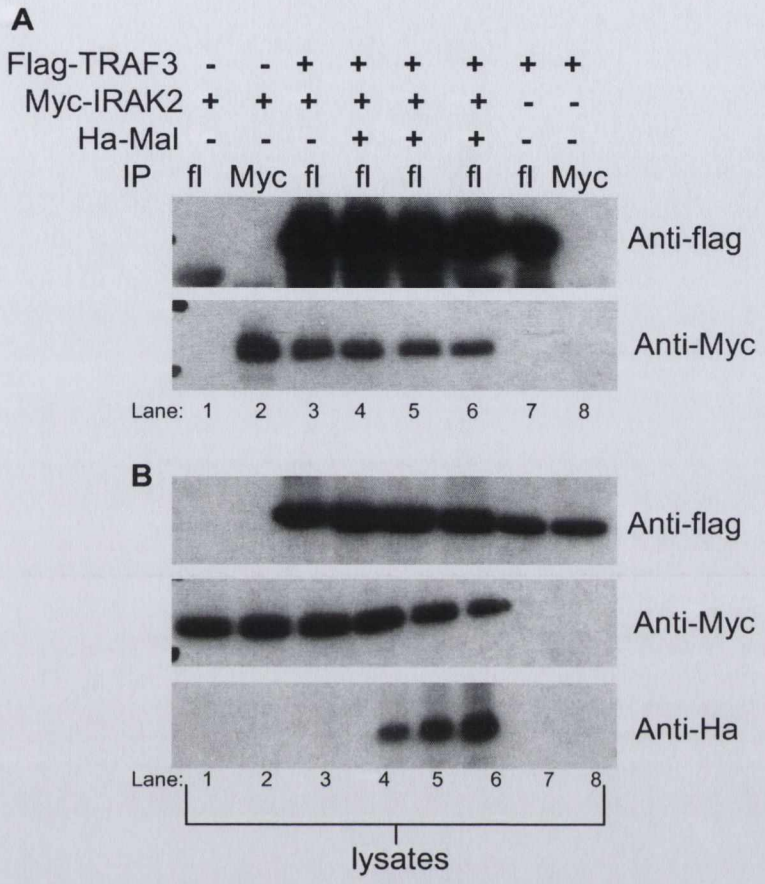

Figure 4.29. **TRAF3 and IRAK2 interact in the presence of Mal in a co-immunoprecipitation assay**

HEK-293T cells were transiently transfected with 3 μg flag-TRAF3 and Myc-IRAK2 and EV and 1, 3 and 5 μg Ha-Mal (lanes 4 to 6) for 24 h, lysed in low stringency lysis buffer and co-immunoprecipitated with either Flag or Myc antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-flag, anti-Ha or anti-Myc antibodies. These data are representative of three experiments.

4.3.11 TRAF3 and Mal only interact in the presence of IRAK2

Seeing that Mal could not interfere with the TRAF3 and IRAK2 interaction I next attempted to co-immunoprecipitate TRAF3 and Mal in the presence of IRAK2. Mal and TRAF3 did not interact in the experiment outlined above in Figure 4.28, however, the presence of IRAK2 may result in the formation of a trimer with Mal keeping TRAF3 and IRAK2 locked in an inactive state and therefore inhibiting TLR3 signalling. I also co-immunoprecipitated TRAF6 and Mal in the presence of IRAK2 as a control.

The incubation of samples containing TRAF6, Mal and IRAK2 with the flag antibody resulted in the presence of bands in the Myc Western blot confirming that TRAF6 and Mal could interact in the presence of 1, 3 and 5 μg IRAK2 (lanes 4 to 6, bottom panel) as seen in Figure 4.30A. TRAF6 and Mal also interacted in the absence of IRAK2 (lane 3, bottom panel). Flag-TRAF6 was precipitated by the flag antibody (lanes 3 to 6, top panel) and the control samples were as expected (lanes 1, 2, 7 and 8, both panels). TRAF6 and Mal were expressed at the same level in the lysates and increasing amounts of IRAK2 were seen in the lysates as shown in Figure 4.30B.

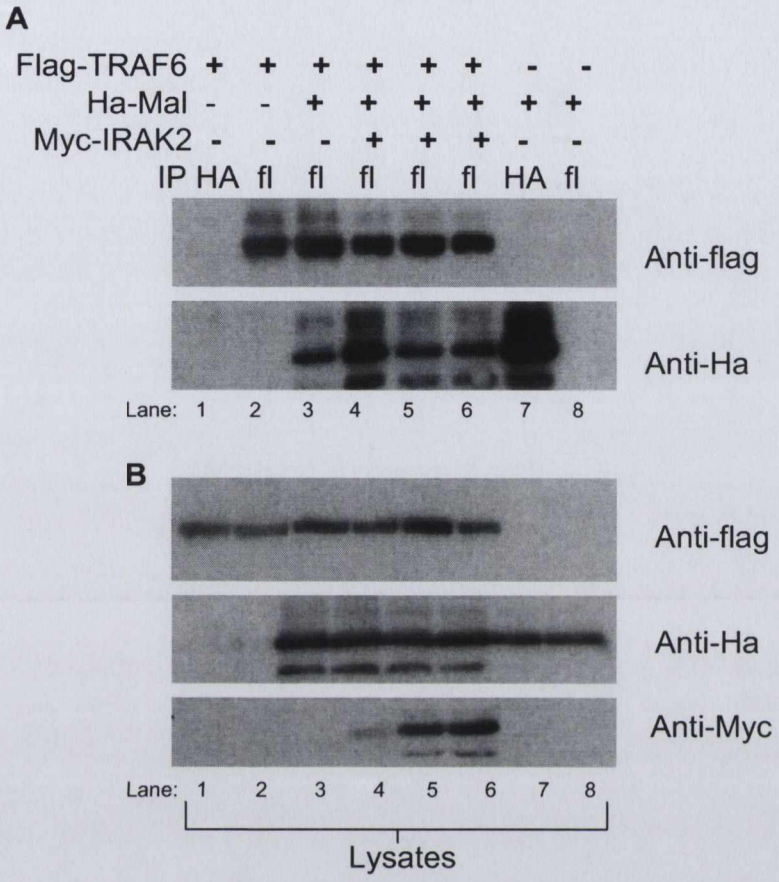

Figure 4.30. **TRAF6 and Mal interact in the presence of IRAK2 in a co-immunoprecipitation assay**

HEK-293T cells were transiently transfected with 3 μg flag-TRAF6, Ha-Mal and EV and 1, 3 and 5 μg Myc-IRAK2 (lanes 4 to 6) for 24 h, lysed in low stringency lysis buffer and co-immunoprecipitated with either Flag or Ha antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-flag, anti-Ha or anti-Myc antibodies. These data are representative of three experiments.

An interesting result was revealed when the experiment was repeated using TRAF3, Mal and IRAK2. As outlined in Figure 4.31A the incubation of cell lysates containing TRAF3 and Mal with the flag antibody followed by Western blotting for the presence of Ha-Mal revealed no interaction (lane 3, bottom panel). However, in the presence of 1, 3 and 5 μg IRAK2, TRAF3 and Mal did interact in a concentration-dependent manner (lanes 4 to 6, bottom panel). TRAF3 was pulled down with the flag antibody in the same samples (lanes 3 to 6, top panel) and the positive and negative control sample were correct (lanes 1, 2, 7 and 8, both panels). TRAF3 and Mal were similarly expressed and IRAK2 was expressed in increasing amounts in the whole cell lysates as described in Figure 4.31B.

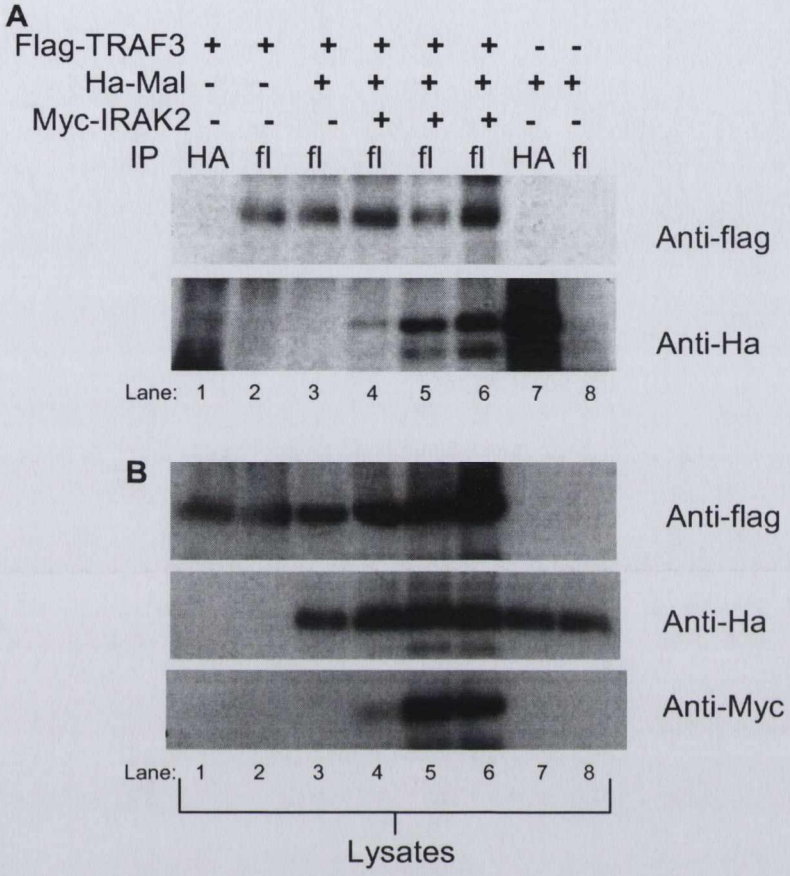

Figure 4.31. **TRAF3 and Mal only interact in the presence of IRAK2 in a co-immunoprecipitation assay**

HEK-293T cells were transiently transfected with 3 μg flag-TRAF3, Ha-Mal and EV and 1, 3 and 5 μg Myc-IRAK2 (lanes 4 to 6) for 24 h, lysed in low stringency lysis buffer and co-immunoprecipitated with either Flag or Ha antibodies for 3 h at 4^0C. The beads were washed three times and analysed by Western blotting using the anti-flag, anti-Ha or anti-Myc antibodies. These data are representative of three experiments.

4.4 Discussion

During my investigations into the role of Mal in TLR2 and TLR4 signalling I also revealed a previously unreported inhibitory role for Mal in TLR3 signal transduction. Initially, as a control, I stimulated the wild type, MyD88- and Mal-deficient macrophages with PolyIC along with the TLR2 and TLR4 ligands. This yielded unexpected results, with a lack of Mal resulting in the enhancement of TLR3 signalling. Therefore further investigations were undertaken using the TRIF-deficient macrophages as a control to ensure the activation of the system by PolyIC was through TLR3.

The initial signalling experiments using the immortalised macrophages to examine the induction of IL6 demonstrated that the absence of MyD88 or Mal allowed for enhanced IL6 production after PolyIC stimulation. This was proven to be TLR3-dependent PolyIC stimulation as no IL6 production was seen in the TRIF-deficient macrophages.

I next pre-treated the wild type macrophages with a Mal inhibitor peptide to ensure the enhancement of IL6 production seen was not an artefact of gene deletion. Stimulation with PolyIC in the presence of the Mal inhibitor peptide resulted in boosted IL6 production over a control peptide in the wild type macrophages confirming the inhibitory role of Mal in TLR3 signalling. The Mal inhibitor peptide also inhibited TLR4 induced IL6 production as expected.

To further verify this I next generated primary wild type and Mal-deficient macrophages and examined IL6 production in response to PolyIC stimulation. Similar results to the immortalised cells were seen with enhanced IL6 production in the Mal-deficient macrophages.

To assess the global nature of TLR3 inhibition by Mal I next generated primary wild type and Mal-deficient dendritic cells and tested their ability to produce IL6 in response to PolyIC stimulation. These experiments yielded similar results to those seen in the macrophages with increased levels of IL6 produced in the Mal-deficient dendritic cells when compared to the wild type cells when stimulated with PolyIC. In all the primary cells tested the boosting of IL6 in the absence of Mal due to PolyIC stimulation was somewhat subtle but statistically significant.

I finally examined the production of IL6 and RANTES in a human system using the human astrocytoma cell line, U373. As these cells contain Mal I used a novel Mal inhibitor peptide (VIPER) (Lysakova-Devine et al., 2010) to inhibit Mal and stimulated the cells with a range of TLR ligands. As was seen for all other cell types tested, by inhibiting Mal enhanced levels of IL6 were produced in response to PolyIC stimulation. A boost in RANTES production was also seen in response to PolyIC stimulation. All other ligands tested behaved as anticipated in the samples pre-treated with VIPER with no inhibition of TLR2 signalling and impaired cytokine production in response to LPS stimulation. VIPER specifically targets TLR4 and not TLR2 therefore these results were not unexpected.

As I had confirmed that Mal was an inhibitor of IL6 and RANTES production in TLR3 signalling I next examined the activation of the downstream signalling molecules p38, JNK and IκB-α in the Mal-deficient macrophages in response to PolyIC stimulation. These experiments revealed normal p38 phosphorylation and IκB-α degradation but enhanced phosphorylation of JNK in the absence of MyD88 or Mal. This inhibition had been previously reported for MyD88 (Johnson et al., 2008) but not Mal. In this paper IRF3

and NFκB activation were examined and seen to be normal in the MyD88-deficient cells therefore suggesting this inhibition to be JNK-specific.

The fact that JNK phosphorylation alone was enhanced in the Mal-deficient macrophages led me to examine the MAP-kinases upstream of JNK in the hope of locating the MAP kinase kinase responsible for phosphorylating JNK in TLR3 signalling. Two candidate kinases were identified within the literature, MAP-kinase kinase 4 (MKK4) and MAP-kinase kinase 7 (MKK7) (Derijard et al., 1995; Ho et al., 2006; Lawler et al., 1998). Commercially available antibodies against the phosphorylated forms of MKK4 and MKK7 were not capable of detecting these proteins in the cell lysates of the macrophages; therefore, a JNK2 kinase assay was next carried out using antibodies generated in the Saklatvala lab (Imperial College, London, UK).

The JNK2 K/R (kinase dead form) protein was generated and its concentration calculated through a Bradford assay. As an initial experiment to ensure the JNK2 protein was correctly folded I tested the ability of MKK4 and MKK7 to phosphorylate it in response to IL1-α stimulation as this had been previously shown (Finch et al., 2001). I was able to repeat this experiment by firstly immunoprecipitating MKK4 or MKK7 from wild type macrophages stimulated with IL1-α. These samples were then incubated with the JNK2 K/R protein and incorporation of radio-labelled phosphate into JNK was seen. This indicated that the MKKs had been phosphorylated in response to IL1-α stimulation.

I next tested if the stimulation of the wild type macrophages with a variety of TLR ligands would result in the phosphorylation of MKK4 and MKK7 using the JNK2 kinase assay to ensure the MAP-kinases could be activated in my PolyIC experiments. Stimulation with Pam_3CSK4, PolyIC, LPS, R848 and mCpGB resulted in phosphorylation of MKK4

and to a lesser extent MKK7 in the wild type macrophages. There was some non-specific phosphorylation in the MKK7 experiments making those results less clear.

As I had verified that stimulation of the wild type macrophages would result in the phosphorylation of MKK4 and MKK7 to detectable levels in response to TLR stimulation I next carried out the JNK2 kinase assay in response to PolyIC stimulation using the wild type and Mal-deficient macrophages. Unfortunately in these experiments no clear enhancement of MKK4 or MKK7 phosphorylation was evident and hence no further clue as to the activator of JNK in response to PolyIC was revealed.

At this time I also attempted to assay the levels of cell death in the wild type and Mal-deficient cells in response to PolyIC stimulation using propidium iodide staining. As both TLR3 and JNK are linked to the activation of apoptosis I wanted to test if the absence of Mal resulted in increased cell death upon PolyIC stimulation. I examined IL6 production in these cells to ensure the Mal-deficient cells were producing enhanced levels of IL6 as a control for the propidium iodide staining. However, the Mal-deficient cells never showed enhanced IL6 production over the wild type cells in these experiments and hence the levels of cell death were similar in both cell types.

Due to the fact that the Mal-deficient macrophages lacked any enhancement in PolyIC-dependent MKK4 or MKK7 phosphorylation or differences in dead cell numbers with propidium iodide staining I again tested their ability to produce IL6 in response to PolyIC stimulation. A new batch of PolyIC had been generated during the course of my experiments and purchased from Sigma as Amersham no longer stocked PolyIC.

Using the wild type and Mal-deficient macrophages I tested the ability of the new PolyIC to produce IL6. These experiments revealed a very different trend to those carried

out with the PolyIC purchased from Amersham. An inhibition of IL6 production was seen in the absence of Mal when compared to the wild type cells. The question of LPS contamination arose as this would explain the greater ability of the wild type cells to produce IL6 than the Mal-deficient cells. I therefore pre-treated the Sigma PolyIC with Polymyxin B for 24 h before stimulation of the macrophages and examined the production of IL6. Polymyxin B is an antibiotic that is also used to remove endotoxin from compounds as it binds negatively charges molecules such as LPS (Cardoso et al., 2007).

Upon treatment with Polymyxin B the levels of IL6 produced in the wild type cells in response to the Sigma PolyIC dropped greatly and the boosted IL6 production in the Mal-deficient cells was revealed. This confirmed that the PolyIC from Sigma was in fact contaminated with LPS. This resulted in the wild type cells being doubly activated through TLR3 and TLR4 whereas the Mal-deficient cells did not have the ability to signal through TLR4 hence the reduced levels of IL6 produced by them.

To ensure previous data generated with the Amersham PolyIC was correct it was also pre-treated with Polymyxin B and used to stimulate the wild type and Mal-deficient macrophages. When IL6 production was examined similar results to those previously shown were seen in the presence and absence of Polymyxin B. Increased levels of IL6 were produced by the Mal-deficient cells in comparison to the wild type cells.

LPS was used as a control and IL6 production by the wild type cells was completely inhibited by the presence of Polymyxin B. R848 was also used and its ability to produce IL6 in the wild type and Mal-deficient macrophages was not affected by the addition of Polymyxin B.

Ideally the Sigma PolyIC could have been used for the JNK2 kinase assays after pre-treatment with Polymyxin B to remove the LPS contamination. However, upon examination of the levels of IL6 produced by the wild type cells in response to the Sigma PolyIC treated with Polymyxin B this was not feasible. The level of IL6 production was very low with the wild type cells producing less than a 2 fold induction in response to 10 and 25 μg/ml Sigma PolyIC and the Mal-deficient cells could only enhance this to 5 fold induction of IL6. When this was compared with the Amersham PolyIC the stark contrast in the ability of the Sigma PolyIC to induce the production of IL6 became very evident. Stimulation of the wild type macrophages with the Amersham PolyIC resulted in 7 to 10 fold induction of IL6 and this was enhanced to between 15 and 25 fold induction of IL6 in the Mal-deficient cells.

Therefore the ability to detect the phosphorylation of MKK4 and MKK7 in the wild type and Mal-deficient macrophages was deemed too difficult to optimise due to the knowledge that the macrophages contained low levels of these kinases (as they were undetectable by Western blotting with commercially available antibodies) and the poor activation of the TLR3 signalling cascade leading to the phosphorylation of MKK4 and MKK7.

Several other PolyIC reagents (from Imgenex and Invitrogen) were tested in the macrophages and they also yielded low levels on IL6 production. A new batch of PolyIC had been generated at this time by these suppliers and was found to have a lower molecular weight than the previous batch used from Amersham. This may explain the lack of ability of the new PolyIC ligand to activate TLR3 signalling in murine cells. A higher molecular weight version of PolyIC was generated by Invitrogen upon request but this also failed to

activate TLR3 signalling in the macrophages. The lower molecular weight PolyIC was capable of activating human cells as demonstrated by my use of U373 cells. Therefore, there is a difference in the ability of human and murine TLR3 to bind dsRNA with hTLR3 responding to the presence of a lower molecular weight dsRNA to a greater extent than the mTLR3. As VIPER was capable of inhibiting Mal in the U373 cells this was also an alternative method for carrying out the kinase assays. Unfortunately VIPER is not yet commercially available and I was unable to obtain enough VIPER and control peptide from the Bowie lab to carry out these experiments. The commercially available Mal inhibitor was far less potent than VIPER and as such was deemed unsuitable for use in the kinase assays.

Due to a lack of PolyIC that could consistently activate the macrophages as expected I next moved on to studying the interaction of TLR3 with its downstream signalling molecules. As it had been previously shown that TLR3 directly interacts with IRAK2 (Keating et al., 2007) and that MyD88 and Mal can interact directly with IRAK2 (Fitzgerald et al., 2001; Muzio et al., 1997) I theorised that the inhibitory role of Mal on TLR3 signalling may be due to its ability to sequester IRAK2 away from TLR3.

The sequestration of IRAK2 from TLR3 seemed a likely candidate for TLR3 inhibition due to another recent study that revealed IRAK2 to be important for TLR4-mediated mRNA stabilisation and post-translational control in macrophages (Wan et al., 2009). A lack of IRAK2 resulted in a decrease in IL6 and TNF-α production in response to LPS stimulation but not in reduced mRNA levels for these cytokines. Therefore it was concluded that IRAK2 is needed for mRNA stabilisation. It was also shown that IRAK2 can form complexes with several MAP kinases including MKK3, MKK6 and MK2 and

thus activate the p38/MK2 signalling cascade. IRAK2 also bound TRAF6 upon TLR4 activation. A lack of IRAK2 did not prevent phosphorylation of p38 by the MKK3/6 signal pathway, in response to TLR4 activation, to a great extent. This suggested another MAP kinase as the target of the complex formed with IRAK2.

The phosphorylation of JNK was also examined in this study and a lack of IRAK2 had no effect on its activation. However, in this paper only short time points of 10 and 30 min were examined. It has recently been shown that IRAK2 is important for late NFκB signalling by TLRs (Kawagoe et al., 2008) and therefore further examination of the activation patterns in the IRAK2-deficient macrophages at later time points would be helpful in clarifying which down stream molecules are IRAK2-dependent.

In the work by Kawagoe et al. TLR3 signalling was said not to be affected by the absence of IRAK2, however, of the cytokines examined only TNF-α was measured to a detectable level and there was no difference in the levels of TNF-α produced by wild type and IRAK2-deficient cells. IL6 and KC (the mouse homologue of CXCL1) levels were undetectable in both wild type and IRAK2-deficient mice and as such it is difficult to confirm the lack of role IRAK2 plays in TLR3 signalling.

As no clear MAP kinase target was identified as a target for IRAK2 it is possible that JNK may be regulated by IRAK2 at later time points than those examined in the Wan et al. paper. Consequently, it seemed a likely target for the inhibition of JNK-specific signalling by Mal. The interaction of Mal and IRAK2 may also prevent IRAK2 forming the complexes it requires to stabilise mRNA expression and hence in the absence of Mal there is more mRNA stabilisation and increased cytokine production inTLR3 signalling.

With this theory in mind I began by repeating the co-immunoprecipitation of Mal and IRAK2 as done by Fitzgerald et al. (Fitzgerald et al., 2001) and confirmed the interaction. I next attempted to co-immunoprecipitate TLR3 and IRAK2 as done by Keating et al.(Keating et al., 2007) with the hope of inhibiting this interaction with the addition of Mal. Unfortunately I was unsuccessful at repeating the co-immunoprecipitation assay either with or without PolyIC stimulation.

As TRIF is an interacting partner of TLR3 I next tested if it could in fact interact with IRAK2 and hence act as a bridge between IRAK2 and TLR3. I was unable to show an interaction between IRAK2 and TRIF, however, and therefore moved on to GST-pulldown assays.

As with the co-immunoprecipitation assays used in chapter 3 I found it difficult to repeatedly isolate large amounts of TLR3 from the membrane of the HEK-293T cells. I therefore generated a GST-TLR3-TIR domain containing fusion protein by cloning the TIR domain of TLR3 from a flag-TLR3 vector and ligating it into the pGEX-4T2 vector. The plasmid was then sequenced and the recombinant protein was made. I then used the GST-TLR3-TIR protein carry out GST-pulldown assays with TRIF and IRAK2. An interaction was seen with TRIF and TLR3 but again no interaction was seen between IRAK2 and TLR3.

To confirm the interaction between TLR3 and TRIF was specific I also tested the ability of GST-TLR3 to pull down MyD88, Mal and Caspase1. Somewhat surprisingly TLR3 did pull down MyD88 although this had been shown once before (Doyle et al., 2003). This interaction seemed specific as Mal, another TIR domain containing protein, did not pull down with TLR3. Caspase1 also did not pull down with TLR3.

The ability of MyD88 to interact with TLR3 in this assay reveals another potential site for the inhibition of TLR3 signalling. In the Johnson et al. paper (Johnson et al., 2008) MyD88 was seen to be inhibitory to TLR3 signalling to JNK phosphorylation and this could occur through MyD88 interfering with the TLR3 and TRIF interaction. Mal may also be involved in this interference through its ability to bind MyD88. However, how this could be linked to JNK-specific inhibition of TLR3 signalling is difficult to imagine as blocking the TLR3 interaction with TRIF would most likely inhibit all down stream signal activation as TRIF is the sole adaptor protein used in TLR3 signalling.

Since I was unable to show any interaction between IRAK2 and TLR3 and therefore could not attempt to prove Mal's interference with this interaction in TLR3 signalling I next examined the role TRAF3 may play in this. In 2006 a study showed that TRAF3 interacts with TRIF and is vital for IFN production in TLR3 and TLR4 signalling. A lack of TRAF3 had no effect on NFκB activation in this paper (Oganesyan et al., 2006). At the same time a second paper revealed that TRAF3 also bound MyD88 and a lack of TRAF3 resulted in enhanced pro-inflammatory cytokine production due to decreased IL10 production (Hacker et al., 2006).

As TRAF3 could bind TRIF and MyD88 I wanted to establish its ability to interact with Mal. Since IRAK2 had also been linked to TLR3 signalling and can interact with TRAF6 I hypothesised that it may also interact with TRAF3 and thus TRAF3 may be the bridge between TRIF and IRAK2. Mal may then inhibit these interactions to down regulate TLR3 signalling. Therefore the interactions between TRAF3, IRAK2 and Mal could be central to the inhibition of TLR3 signalling.

With these theories in mind I began by co-immunoprecipitating IRAK2 and TRAF3 and an interaction was seen. I used the already known interaction between TRAF6 and IRAK2 as a control. I next investigated if TRAF3 and Mal could interact as TRAF3 does interact with other TIR containing adaptor proteins. In this co-immunoprecipitation assay I was unable to detect any interaction between Mal and TRAF3 but could see an interaction between Mal and TRAF6, as previously described.

Given that I had seen an interaction between IRAK2 and TRAF3 but not between Mal and TRAF3 I next hypothesised that IRAK2 was linked to the role of TRAF3 in TLR3 signalling to allow the activation of JNK and that Mal may sequester it away from TRAF3 thus down regulating the TLR3 response. To test this theory I repeated the IRAK2 and TRAF3 co-immunoprecipitation assay in the presence of Mal. I hoped to break or reduce the IRAK2 and TRAF3 interaction by co-transfecting Mal. However, the presence of Mal had no affect on the ability of IRAK2 and TRAF3 to interact.

I finally repeated the Mal and TRAF3 co-immunoprecipitation assay, in which I saw no interaction, in the presence of IRAK2. In this experiment the presence of IRAK2 resulted in a concentration-dependent interaction between Mal and TRAF3. I also repeated the TRAF6 and Mal co-immunoprecipitation assay in the presence of IRAK2, as a control, and as anticipated IRAK2 had no affect on the interaction between TRAF6 and Mal.

The ability of IRAK2 to form a trimer with Mal and TRAF3 is an interesting result and could explain the mechanism of Mal inhibition of TLR3 signalling. A study by Tseng et al. found that lysine (K)-48 linked poly-ubiquitination of TRAF3 led to its degradation. This was seen to be crucial for the activation of the MyD88-dependent signalling pathway resulting in MAP-kinase phosphorylation and pro-inflammatory cytokine production in

TLR4 signalling. Lysine (K)-63 linked poly-ubiquitination of TRAF3 led to the activation of TRIF-dependent signal transduction resulting in the phosphorylation of IRF3 and the production of IFNs. Blocking the degradation of TRAF3 prevented pro-inflammatory cytokine production but had no effect on IFN production (Tseng et al., 2010).

A Mal and TRAF3 interaction via IRAK2 therefore, may prevent the degradation of TRAF3 in TLR3 signalling and hence dampen down the MAP-kinase activity of JNK. This would not be evident in TLR4 signalling as the MyD88-dependent pathway can also activate MAP-kinases through the degradation of TRAF3 via TRAF6 and the K-48 linked ubiquitin ligases cIAP1/2. In TLR3 signalling, however, the MyD88-dependent pathway is not available to form the multi-protein complexes that are used to degrade TRAF3. Consequently, in TLR3 signalling another mechanism for TRAF3 degradation may be required that is reliant on the TRIF and TRAF3 interaction. This degradation would be independent of cIAP1/2 as blocking their activity in TLR3 signalling does not affect pro-inflammatory cytokine production and they are not found in the signalling complexes containing TRAF3 and TRIF (Tseng et al., 2010).

The Tseng et al. study also found that the degradation of TRAF3 was required for the phosphorylation of TAK1 and subsequent activation of the MAP-kinase cascade. However, un-phosphorylated TAK1 was capable of activating the IκB kinase complex in the presence of TRAF3 suggesting separate mechanisms of activating the MAP-kinase signalling cascade and the NFκB pathway via TAK1. This was interesting as it provides a link between the enhancement of JNK phosphorylation in the Mal-deficient cells to TRAF3 degradation without having an effect on NFκB activation. Therefore, the removal of Mal

would allow TRAF3 degradation and an enhancement of JNK phosphorylation and IL6 production without affecting NFκB activity as was seen in the PolyIC time courses.

The presence of a Mal, TRAF3 and IRAK2 trimer may therefore sequester TRAF3 and IRAK2 away from the machinery required to degrade TRAF3. TRAF6 has been shown to be crucial for TRAF3 degradation and hence activation MAP-kinases and pro-inflammatory signalling. As IRAK2 can recruit TRAF6 it may aid in the degradation of TRAF3. IRAK2 could therefore be the kinase responsible for the formation of the multi-protein complexes that allow K48-linked poly-ubiquitination and degradation of TRAF3 in TLR3 signalling. As IRAK2 is also linked to mRNA stabilisation the sequestering of it in a trimer with TRAF3 and Mal may also result in a decrease in cytokine production in TLR3 signalling.

The co-immunoprecipitation assays outlined above provide some clues as to the mechanism behind the inhibition of TLR3 signalling by Mal but it must be stated that all these experiments were carried out through over expression of the proteins of interest. This can result in false positives and the use of antibodies raised against the proteins of interest to carry out endogenous co-immunoprecipitation assays could verify these interactions. Unfortunately due to the lack of Mal and TRAF3 antibodies that can consistently immunoprecipitate their proteins this proved to be too difficult to do.

Due to the difficulties that arose during this project, including a lack of PolyIC and good endogenous antibodies, a clear picture of Mal inhibition of TLR3 is not available. Undoubtedly the absence of Mal results in the over production of IL6 and RANTES and the enhanced phosphorylation of the MAP-kinase JNK. However, which MAP kinase

kinase is responsible for this remains unclear. As MKK7 is the only MAP kinase kinase specific to JNK phosphorylation it seems the more likely candidate.

Potential kinases involved upstream of either MKK4 or MKK7 must also be identified and tested in Mal-deficient cells. One example of a MAP3-kinase of interest is MLK3, a member of the mixed lineage kinase family, which has been shown to specifically target JNK phosphorylation in MEFs stimulated with TNF-α (Brancho et al., 2005). Other examples include MEKK1 and MEKK3 but it is unclear if they solely target JNK due to conflicting evidence (Symons et al., 2006). TAK1 would also be of interest as outlined above.

As Mal functions early in the signal transduction pathway of the TLRs the use of co-immunoprecipitation assays helped to identify possible targets for its inhibition of TLR3. From these assays I have concluded that Mal, IRAK2 and TRAF3 form a trimer. The presence of Mal in this trimer could block the ability of IRAK2 to stabilise mRNA and prevent the degradation of TRAF3 and thus prevent over activation of TLR3 signalling.

TLR3 signalling is linked to the activation of apoptosis, due to the ability of TRIF to bind RIP1 and hence recruit FADD, as is JNK activation therefore a mechanism to down regulate these pathways may prevent apoptosis in response to TLR3 activation (Dhanasekaran and Reddy, 2008; Kaiser and Offermann, 2005). TLR3 is the only TLR with a clear role in the induction of apoptosis and therefore a method to regulate this would be specific to TLR3. I suggest a model in which Mal, by the sequestration of IRAK2 and TRAF3, down regulates the activation of JNK and the levels of cytokines produced by cells in response to TLR3 activation as outlined in Figure 4.32.

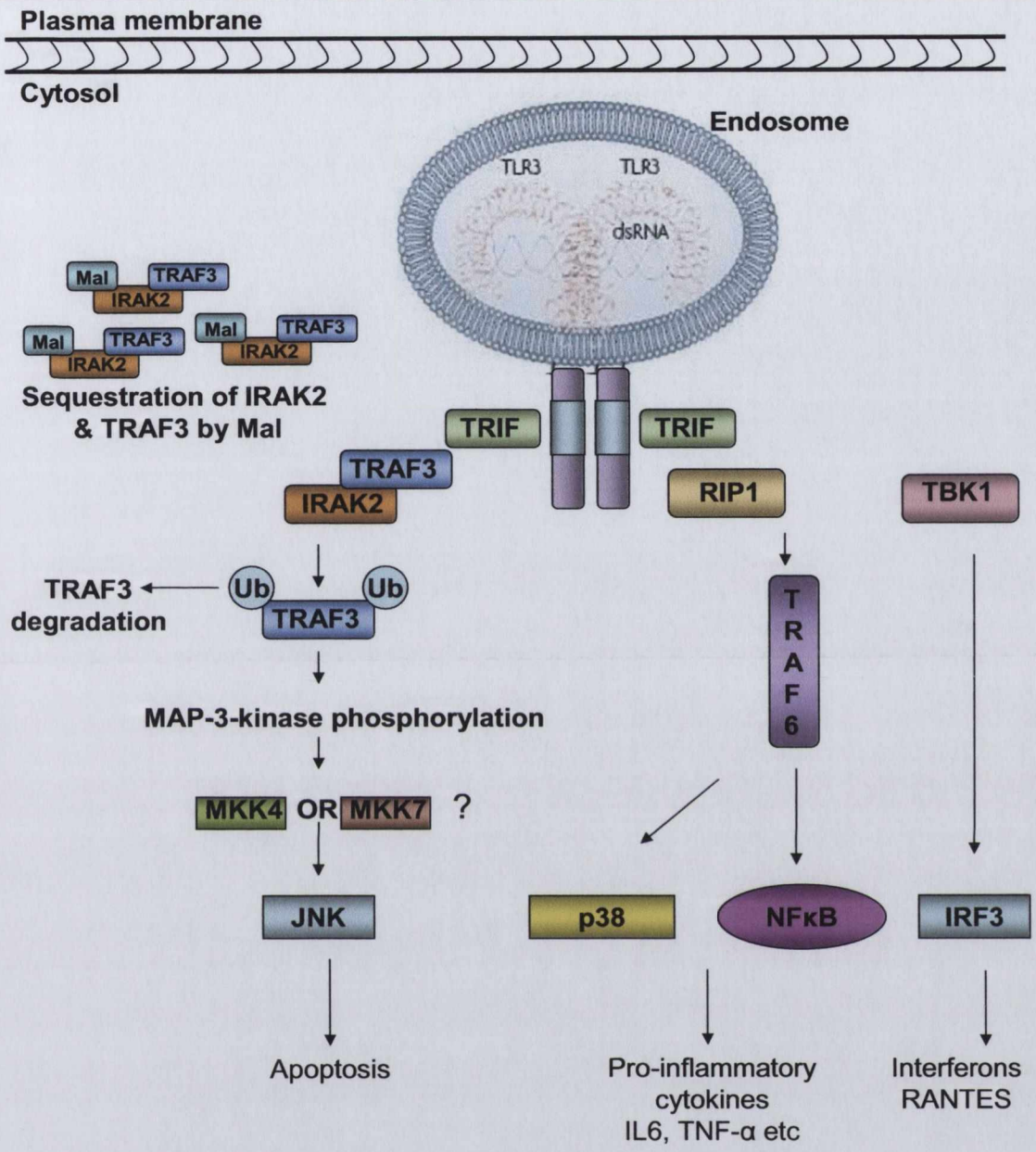

Figure 4.32 **Model of the inhibitory role of Mal in TLR3 signalling**

Mal sequesters IRAK2 and TRAF3 away from TRIF in TLR3 signalling to prevent the abundant degradation of TRAF3 and blocking IRAK2-dependent mRNA stabilisation. This results in down regulation of MAP-3-kinase phosphorylation, MKK4 or MKK7 phosphorylation and ultimately JNK phosphorylation, thus preventing the over-activation of apoptosis in response to TLR3 stimulation. TLR3 also activates p38 and NFκB through RIP1 and TRAF6 and IRF3 through TBK1. Adapted from (Hennessy et al., 2010).

Chapter Five

Final Discussion and Future Perspectives

Chapter 5

5.1 Final Discussion and Future Perspectives

Previous work into the role of Mal in TLR signalling has suggested its bridging role in TLR2 and TLR4 signalling to the MyD88-dependent pathway as reviewed in Sheedy et al. (Sheedy and O'Neill, 2007). To date it was thought that Mal was crucial for both TLR2 and TLR4 signalling; however, this study reveals that Mal is not essential for TLR2 signalling and has an absolute requirement for TLR4 signalling. Furthermore, a novel inhibitory role for Mal in TLR3 signalling was revealed.

Much work has been undertaken to identify the important areas of Mal that are involved in its signalling to NFκB. This has led to the identification of many key regions of Mal that are utilised to activate the molecules associated with the MyD88-dependent signalling pathway. However, the exact interacting partnerships that occur in TLR1/2, TLR2/6 and TLR4 signalling had not thoroughly been investigated to date and as such I began by examining these interactions

In these experiments the interactions between TLR1/2, TLR2/6, TLR3 and TLR4 and the adaptor proteins MyD88, Mal and TRIF were clarified. MyD88 interacted with TLR1, TLR2, TLR3, TLR4 and TLR6 in GST-pulldown assays whereas Mal interacted with TLR1, TLR2 and TLR4 only. TRIF was used as the control and was only seen to interact with TLR3. The interactions between TLR2 and TLR4 and Mal were not unexpected, however, the interactions between TLR1, MyD88 and Mal and between TLR6 and MyD88 are novel observations and warrant further investigation. The fact that TLR3

could interact with MyD88 as well as TRIF is also an interesting result which may explain its ability to inhibit TLR3 signalling.

The results from these experiments suggest that Mal is not actually required for the interactions between MyD88 and TLR2 or TLR4. Therefore Mal appears not to have a bridging role in signalling via these TLRs but is in fact itself crucial for signal activation in response to the TLR4 ligand LPS but not TLR2 ligands.

The generation of the GST-TLR-TIR domain containing fusion proteins in this project has much potential for further investigation into these interactions. The fusion proteins could be used to clarify which regions of the TLR1, 2, 3, 4 and 6-TIR domains are vital for their ability to interact with the adaptor proteins and initiate signal transduction. The use of mutagenesis studies would provide a clearer picture of the amino acids vital for the interaction between the TLRs and the adaptor proteins.

There are already several candidate amino acids that could be targeted by mutagenesis such as the proline 712 to histidine mutation seen in the TIR domain of TLR4 in C3H/HeJ mice (Poltorak et al., 1998). These mice were shown to be resistant to LPS but highly susceptible to gram-negative bacterial infection. This mutation, when introduced into TLR2 at proline 681, renders TLR2 incapable of responding to gram-positive bacteria (Underhill et al., 1999a). An analogous mutation when generated in the TIR domain of Mal at proline 125 resulted in a lack of NFκB activation (Fitzgerald et al., 2001). In this study several residues in the TIR domain of Mal, TLR2, and TLR4 were described as being important for signalling via these proteins. These amino acids were located in TIR domain box 1, box 2 and box 3 of the TIR domains. The TIR domain boxes contain residues known to be important for TIR domain containing protein interactions.

Figure 5.1 shows the sequence alignment of the TIR domains of TLR1, 2, 3, 4 and 6, MyD88, Mal and TRIF with TIR domain boxes 1, 2 and 3 highlighted. The sequence similarity between the TLRs and the adaptor proteins at these regions make them ideal candidates for mutagenesis. The fact that the proline (marked in red) found to be crucial for TLR4 and Mal signalling is conserved in all TLRs (bar TLR3) and MyD88, Mal and TRIF reveals its importance and would also be a good residue for mutagenesis.

The mutagenesis studies would provide a very useful insight for the modelling of the TIR domains of these TLRs and the surfaces used to bind MyD88, Mal and TRIF. The TIR domains of TLR1 and TLR2 have been modelled (Xu et al., 2000). The interface between Mal, TRAM and TLR4 has also been revealed through modelling (Nunez Miguel et al., 2007). Hence those studies would provide the perfect template for these investigations.

Prior to this project the lack of dependence on Mal in TLR2 signalling had not been revealed. The initial signalling experiments using immortalised macrophages revealed that Mal is not crucial to TLR2 signalling as at all concentrations of Pam_3CSK4 and Malp-2 tested the Mal-deficient macrophages were capable of producing IL6. When Mal-deficient cells were stimulated with *Salmonella typhimurium,* a known TLR2 activator (Weiss et al., 2004), they also produced IL6 at levels comparable to that of the wild type cells again showing a lack of requirement for Mal in TLR2 signalling.

The lack of requirement of Mal was also seen when the down stream molecules p38, JNK and IκB-α were examined in the Mal-deficient macrophages. A somewhat concentration-dependence on Mal was seen in these experiments were at lower concentrations of TLR2 ligand or *S. typhimurium* the absence of Mal appeared to result in

a more dramatic reduction in IL6 production and less activation of down stream signalling molecules. Perhaps, therefore, Mal is to some extent required in TLR2 signalling to sensitise the system to the presence of low concentrations of pathogen.

In response to all the concentrations of the TLR2 ligands tested MyD88 revealed its central role in TLR2 signalling with absolutely no IL6 production in the MyD88-deficient macrophages. As MyD88 was shown to interact with TLR1, TLR2 and TLR6 it is not surprising that it plays such a crucial role in TLR2 signal activation.

Primary macrophages and dendritic cells derived from Mal-deficient mice also demonstrated the lack of requirement for Mal for the production of IL6 and TNF-α in response to TLR2 stimulation.

The lack of requirement of Mal and the crucial role MyD88 plays in TLR2 signalling was also shown in the study by Cole et al in which TLR2 translocated to the phagosome to signal in response to *F. tularensis* infection. This occurred in the absence of Mal but was totally MyD88-dependent (Cole et al., 2010).

On the other hand TLR4 induced IL6 and TNF-α production was shown to be totally dependent on both Mal and MyD88 but the activation of down stream signalling molecules, curiously, were not. This has been seen in previous studies and is attributed to the activation of the TRIF-dependent signalling pathway (Kawai et al., 1999). However, the fact that phosphorylation of p38 and JNK and the degradation of IκB-α can occur in the absence of Mal or MyD88 but the production of IL6 cannot remains unclear.

The non-essential nature of Mal in TLR2 signalling is clearly shown in this project with the mechanism explained by the interaction studies. For TLR2 signal activation MyD88 is the crucial adaptor protein and is recruited to TLR1, TLR2 and TLR6 hence

overcoming the need for Mal to interact with TLR1 and TLR2. In TLR4 signalling the lack of heterodimer formation with another TLR in response to LPS prevents sufficient MyD88 recruitment in the absence of Mal hence the absolute requirement of both adaptors for signal transduction.

During these experiments, an unexpected but interesting inhibitory role for Mal in TLR3 signalling was revealed in which IL6 and RANTES production and JNK phosphorylation were enhanced in the absence of Mal. The use of two Mal inhibitor peptides further confirmed this inhibitory role. MyD88 was also shown to be inhibitory toward TLR3, however, as the inhibition of TLR3 by MyD88 had previously been shown I focused on the ability of Mal to inhibit TLR3 in a similar manner to that seen for MyD88.

The mechanism behind the inhibition of TLR3 by Mal remains elusive to this point. It is clear that the inhibition is specific to the phosphorylation of JNK and this is identical to the Johnson et al. paper in which MyD88-dependent inhibition of TLR3 was JNK-specific.

Through the interaction experiments several potential methods of Mal-dependent inhibition have been revealed. The interactions between TRAF3 and IRAK2 and the formation of the trimer with IRAK2 bridging Mal and TRAF3 are potentially crucial interactions for TLR3 signalling. As TRAF3 degradation is vital for MAP-kinase activity in TLR signalling the formation of a Mal, TRAF3 and IRAK2 trimer may block this degradation and hence prevent over activation of JNK. A lack of Mal should therefore result in increased degradation of TRAF3. The fact that TRAF3 degradation is required for the phosphorylation of TAK1 and the subsequent MAP-kinase signalling pathway

activation but that un-phosphorylated TAK1 can activate the NFκB signalling pathway may explain the very specific nature of this Mal inhibition.

In TLR3 signalling the prevention of JNK phosphorylation may be a method to down regulate apoptosis. TLR3 is the only TLR linked to the induction of apoptosis due to its interaction with TRIF and hence FADD activation. Therefore Mal, by preventing the degradation of TRAF3 and subsequently phosphorylation of JNK, may regulate the level of apoptosis in the cell due to TLR3 activation.

As IRAK2 has been linked to mRNA stabilisation in response to LPS it may also be held in an inactive form by the formation of the trimer thus preventing it from activating the molecules required for mRNA stability in TLR signalling. Late NFκB signalling is also linked to IRAK2 activity. In the time course experiments with PolyIC stimulation it was only at the later times of 60 and 90 min that the inhibitory nature of Mal became apparent. This may be due to the over-activation of IRAK2 in the Mal-deficient cells. IRAK2 may also be required for the degradation of TRAF3 in TLR3 signalling as there is no method of activating the MyD88-dependent multi-protein complex formation linked with TRAF3 degradation.

Further work is needed to verify these hypotheses and the use of the Mal-deficient macrophages and functional PolyIC are central to this. Examination of apoptosis in these cells in response to PolyIC stimulation and the activation of the upstream kinases, such as TAK1, are vital. A TRAF3 antibody suitable for examining its degradation in the Mal-deficient cells in response to PolyIC stimulation would also provide much information on the role of TRAF3 in the inhibitory nature of Mal. Examination of the phosphorylation of IRAK2 in response to PolyIC stimulation in the Mal-deficient cells would also clarify the

role of IRAK2 in the inhibition of TLR3 signalling. However, due to the lack of functional PolyIC at this time these experiments remain difficult to optimise.

The findings in this project reveal that Mal is crucial for TLR4 signalling, non-essential for TLR2 signalling and inhibitory for TLR3 signalling. The differential role of Mal in these three TLR signalling complexes has much potential in the area of therapeutics. The over- and under-activation of the TLR signal cascades is known to be a factor in many infectious and inflammatory diseases (Loiarro et al., 2010; O'Neill et al., 2009).

Mal is also targeted by pathogens to allow their evasion of the immune system as revealed in the Sengupta et al study on *Brucella* (Sengupta et al., 2010). *Brucella* is a gram-negative strain of bacteria that is recognised by TLR2 and TLR4. It encodes a protein, TcpB, that can interact directly with Mal and target it for degradation and thus allows *Brucella* to block TLR4 signal transduction. The fact that vaccinia virus encodes a protein that prevents TLR4 signalling via its ability to interact with Mal and TRAM as outlined in the Lysakova-Devine paper (Lysakova-Devine et al., 2010) also reveals that the ability to interfere with Mal allows immune evasion by pathogens. Thus, Mal is a good target for the design of inhibitory molecules to down regulate TLR signal transduction in inflammatory and autoimmune disease.

As Mal is not essential for TLR2 signal activation it provides a highly specific target for TLR4 signal modulation. TLR4 activation is linked to the progression of many inflammatory conditions including sepsis, rheumatoid arthritis (RA), reperfusion injury, and allergy (Brandl et al., 2005; Dunne et al., 2010b; Hennessy et al., 2010). The proof for the role of TLR4 in the progression of these inflammatory conditions comes from investigations using TLR4-deficient mice which are protected against disease as well as the

knowledge that the expression of TLR4 is increased in patients with these aliments. It has also been shown that the activation of TLR4 in patients was detrimental to recovery from these inflammatory states (O'Neill et al., 2009).

A good target for the down regulation of TLR4 signalling in sepsis has been identified and is currently undergoing drug trials. Eritoran (E5564) functions as an antagonist of TLR4 signalling by interacting with MD2 and TLR4. This interaction prevents the conformational change seen in MD2 upon interaction with LPS, and as such is being used to aid in the recovery from severe sepsis. Mouse models have revealed this decrease in sepsis (Mullarkey et al., 2003; Savov et al., 2005). In severe sepsis the blocking of TLR4 signal transduction is required to ensure recovery and therefore preventing the activation of the MyD88-dependent signal cascade via inhibition of Mal would be a good target for investigations into inhibitory peptide design.

In rheumatoid arthritis elevated levels of TNF-α and IL6 are produced by dendritic cells upon LPS and heat shock protein 22 (HSP22, an endogenous ligand for TLR4) stimulation (Roelofs et al., 2006). As I have shown that Mal specifically inhibits LPS induced IL6 and TNF-α production without having a huge affect on TLR2 signalling it could provide a good target for the down regulation the MyD88-dependent signalling pathway of TLR4.

As TLR2 is also involved in disease progression in RA the inhibition of Mal may not completely block inflammation. The TRIF-dependent pathway would also remain intact therefore preventing complete down regulation of TLR4 signalling. However, although TLR4 is known to be involved in disease progression it is also required for fighting infection and as such by allowing some TLR4 activity a balance between the

detrimental and beneficial nature of TLR4 may be achieved (O'Neill et al., 2009). An issue in using Mal to inhibit TLR4 signalling specifically on the MyD88-dependent pathway is that the inhibition of Mal would also lead to the activation of TLR3.

TLR3 activation has, however, been linked to the treatment of several diseases. PolyIC is currently being investigated as a TLR3 agonist with the potential for the treatment of HIV, influenza and chronic fatigue syndrome (CFS) (Ribes et al., 2010). However, as PolyIC uptake has been shown to have toxic side effects modified versions are currently being designed that can maintain the protective activation of TLR3 without such toxic side effects (Jasani et al., 2009). TLR3 activation has also been linked to the destruction of cancer cells in melanoma and breast cancer through the generation of a novel TLR3 ligand mimic (IPH-3102) (Hennessy et al., 2010).

Inhibiting Mal could provide a good alternative to the use of PolyIC as a TLR3 agonist. I have shown that the inhibition of Mal allows for enhancement of TLR3 signal transduction with regards IL6 production and phosphorylation of JNK. Therefore a Mal inhibitor peptide, such as VIPER, may be useful for the treatment of the diseases and in killing of cancer cells as outlined above. Again the issue of TLR4 inhibition due to the loss of Mal as the same time remains a problem.

TLR2 has also been linked to disease progression and a role for it has been seen in rheumatoid arthritis, type I diabetes and atherosclerosis (Edfeldt et al., 2002; Kim et al., 2007b; Seibl et al., 2003). Therefore the ability to specifically inhibit TLR2 signalling without having an effect on the activity on TLR4 would provide possible targets for drug design. The knowledge that TLR2 utilises TLR1 and TLR6 to be functional coupled to the fact that the interfaces between these TLRs and their ligands has been solved makes these

areas ideal for the targeting of TLR2 signalling. Targeting of TLR2 signalling at the adaptor protein level is not ideal due to the crucial role played by MyD88 as shown in this project. As MyD88 is also utilised by all the other TLRs (bar TLR3) to initiate signal transduction it is not a specific enough target for TLR2 signal inhibition.

Genetic analysis of the TLRs and adaptor proteins in population studies has also revealed that the TLR signal cascades are vital for maintaining the balance between combating pathogens and prevention of over activation of inflammation. Many of these studies have revealed single nucleotide polymorphisms (SNPs) that when found in a individual result in altered susceptibility to certain diseases as reviewed by Corr et al (Corr and O'Neill, 2009).

Single nucleotide polymorphisms (SNPs) in TLR4 are associated with decreased LPS responsiveness. The conversion of an aspartic acid at position 299 to a glycine and of a threonine at position 399 to an isoleucine results in an increased incidence of gram-negative infection and sepsis in individuals that are either homo- or heterozygous for these SNPs (Arbour et al., 2000; Lorenz et al., 2002). Both these SNPs occur in the ecto-domain of TLR4 and further investigations have revealed that the residues lie in a region important for ligand docking (Rallabhandi et al., 2006). These two SNPs have also been implicated in the progression of inflammatory bowel disease (IBD) (Corr and O'Neill, 2009).

Several SNPs in TLR2 have also revealed its role in the onset of disease. The P631H or R753G polymorphisms in TLR2 result in reduced development of inflammatory bowel disease (IBD) therefore showing TLR2 is required for the development of IBD (Pierik et al., 2006). The R753G polymorphism may also reduce the risk of atherosclerosis

although further investigations are required (Lepper et al., 2007). TLR2 is activated in synovial fibroblasts during the progression of this disease however.

Another example of the ability of SNPs to alter immune signalling is found in Mal when the serine at position 180 is converted to a leucine. A heterozygous state for this SNP has been shown to be protective against the development of pneumococcal disease, malaria and Systemic Lupus Erythematosus (SLE) (Castiblanco et al., 2008; Khor et al., 2007). A homozygous state for serine at this position results in hyper-responsiveness to these inflammatory diseases due to over activation of Mal. Individuals homozygous for leucine at this position are unable to mount an immune response that is Mal-dependent due to non-functional Mal. Hence, in these individuals TLR4 activation is down regulated. As Mal is inhibitory in TLR3 signalling, as revealed by my investigations, the non-functional variant of Mal would also result in the over activation of TLR3. This may also be detrimental for the host due to excessive inflammation and apoptosis.

In conclusion and as outlined in Figure 5.2 this project reveals that Mal is not essential for TLR2 signalling as Mal-independent recruitment of MyD88 to the TLR2 complex allows for signalling without Mal. Mal may have a sensitising role in TLR2 signalling with a greater dependency on Mal being revealed at lower ligand concentrations. Mal is crucial for TLR4 responses as there is no alternative method for MyD88 recruitment and is inhibitory in TLR3 signalling to JNK.

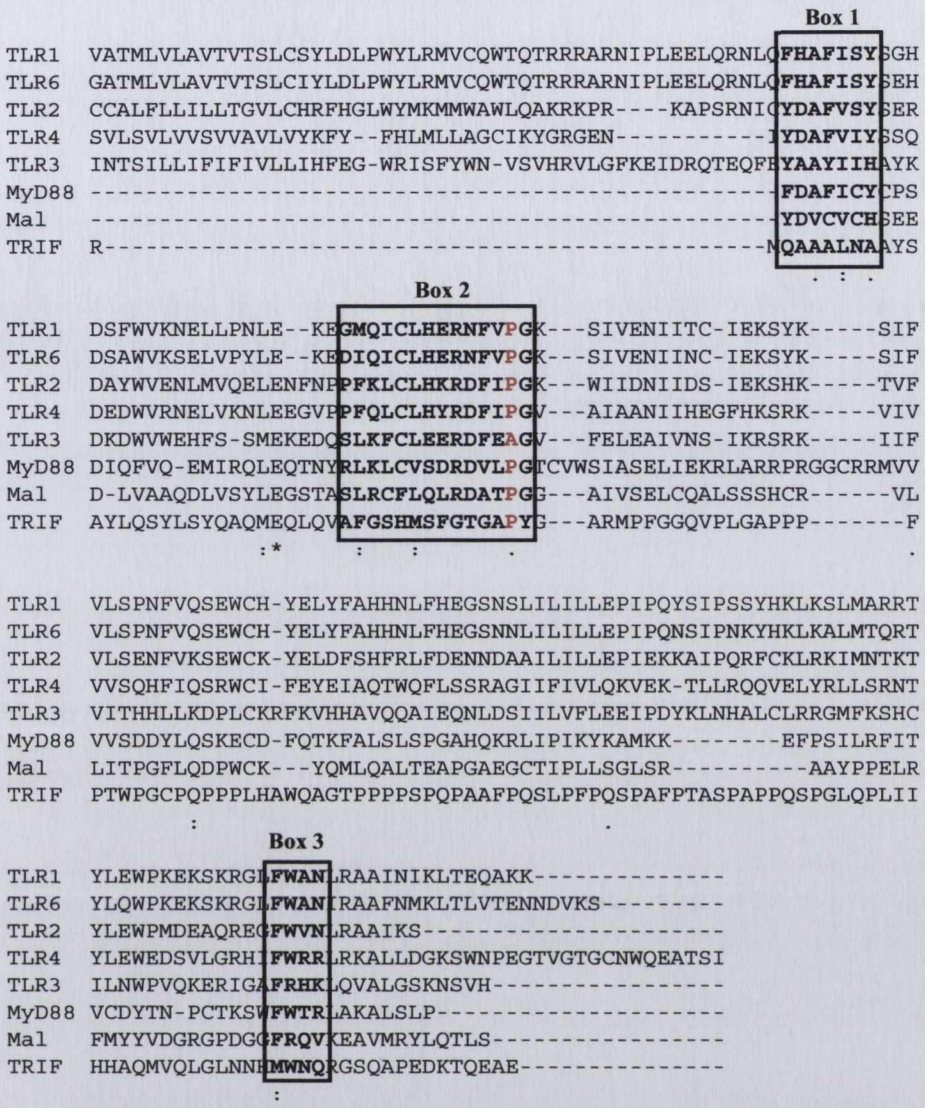

Figure 5.1 **Potential sites for mutagenesis studies in the TIR domains of the TLRs of interest**

Previous studies have highlighted the proline marked in red as important for TLR2, TLR4 and Mal signalling. It is therefore a good candidate for mutagenesis in TLR1 and 6 as it is also found in these TLRs as well as in MyD88 and TRIF. TLR3 contains an alanine in this position and as such this site may only be important for plasma membrane TLR signalling. Box 1, 2 and 3 in the TIR domains are known to contain residues important for signalling and would therefore provide good target amino acids for mutagenesis. Generated using ClustalW2 (Thompson et al., 1994) and (Fitzgerald et al., 2001).

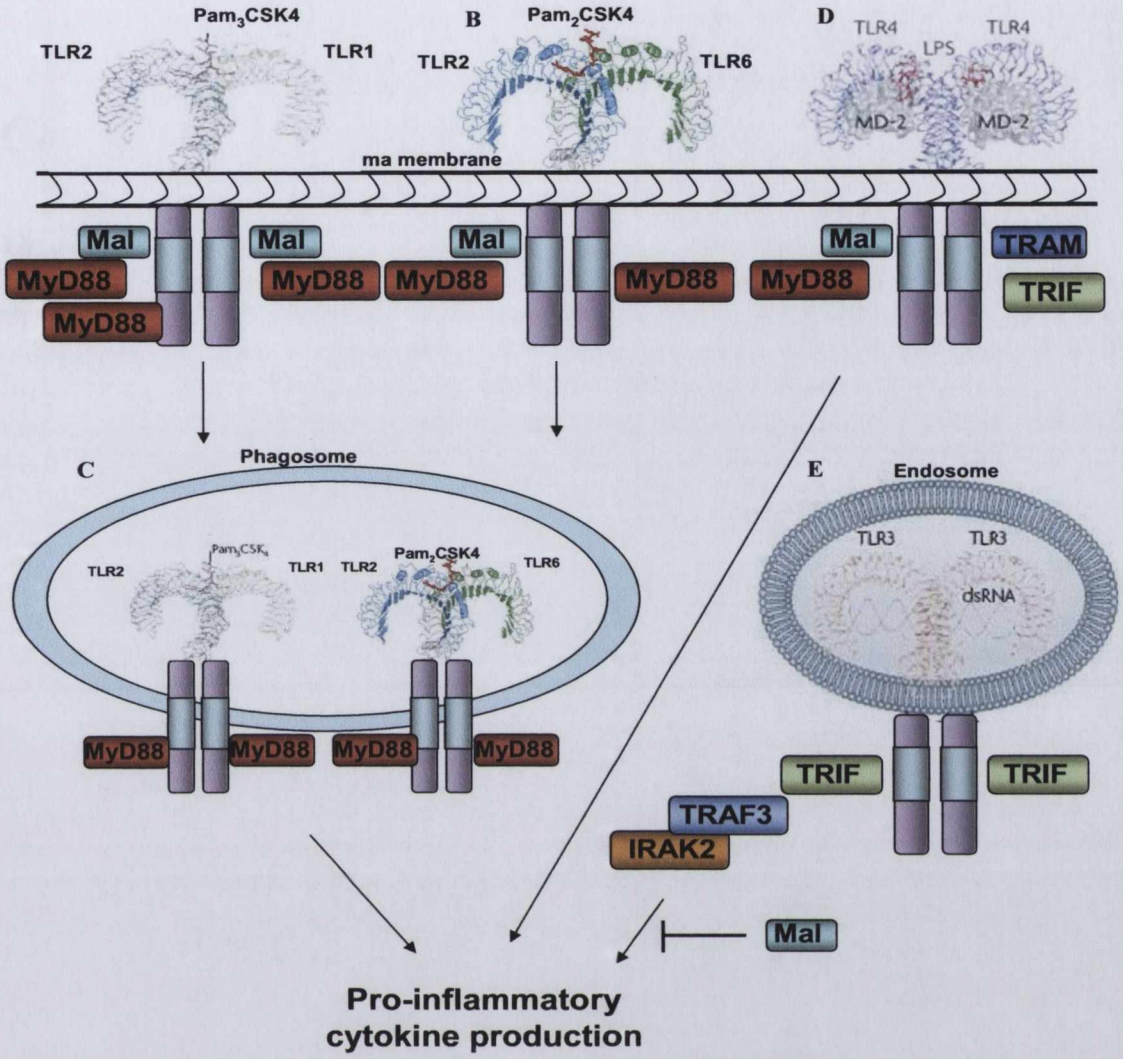

Figure 5.2 **Mal is not essential for TLR2 signalling and is an inhibitor of TLR3 signalling**

Mal and MyD88 are recruited to TLR1 and TLR2 resulting in signal activation in response to Pam_3CSK4 (A). TLR2 recruits Mal and MyD88 and TLR6 binds MyD88 to signal in response to Pam_2CSK4 (B). The TLR2 complexes can move to the phagosome to initiate Mal-independent signal transduction (C). The ability of TLR1 and TLR6 to bind MyD88 explains the non-essential nature of Mal in TLR2 signalling. In TLR4 signalling however Mal and MyD88 are both absolutely required for signal activation at the plasma membrane (D). Mal also acts as an inhibitor of TLR3 signalling (E). Adapted from (Hennessy et al., 2010; Kang et al., 2009).

Chapter Six

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6.1 References

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Chapter Seven

Appendices

Chapter 7

7.1 Record of Publication

Tanya M. Watters, Elaine F. Kenny, Luke A.J. O'Neill (2007). Structure, function and regulation of the Toll/IL-1 receptor adaptor proteins. Immunol Cell Biol. *85*(6):411-9.

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