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The Relative Role of IRAK-2 in TLR Signalling

Thesis submitted to the University of Dublin

For the Degree of Doctor of Philosophy

2012

By Sinéad Flannery,

Dept of Biochemistry and Immunology,

Trinity College Dublin,

Ireland

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Abstract

Toll-like receptors (TLRs) are pattern recognition receptors that recognise microbial ligands and subsequently trigger intracellular signalling pathways involving transcription factors such as NFκB and MAPKs such as p38. TLR signalling can regulate both transcriptional and post-transcriptional events leading to altered gene expression, and thus appropriate immune responses. The interleukin-1 receptor associated kinase (IRAK) family comprises four kinases that regulate TLR signalling. However, the role of IRAK-2 has remained unclear, especially in human cells. Recent studies using cells from inbred IRAK-$2^{-/-}$ mice showed that murine IRAK-2 was not required for early TLR signalling events, but had a role in delayed NFκB activation and in cytokine production. IRAK-2 in mice has four splice variants, two of which are inhibitory, while human IRAK-2 has no splice variants.

Thus IRAK-2 in mice and humans may function differently, and therefore we analyzed of the role of IRAK-2 in TLR responses in primary human cells. siRNA knockdown of IRAK-2 expression in human PBMCs showed a role for human IRAK-2 in both TLR4 and TLR8-mediated early NFκB and p38 MAP kinase activation, and in induction of TNF mRNA. Moreover, human IRAK-2 was required for regulating MyD88-dependent TNFα mRNA stability via the TNF 3'UTR.

Mechanistically, the involvement of IRAK-2 in TLR signalling to activate NFκB is thought to be via TRAF6. Here, IRAK-2 was shown to trigger the E3 ligase activity of TRAF6 to catalyse the formation of physiologically relevant free K63-linked polyubiquitin chains. These polyubiquitin chains were shown to be necessary but not sufficient for IRAK-2 function.

Collectively these data demonstrates for the first time an essential role for IRAK-2 in both transcriptional and post-transcriptional TLR events.

Chapter 1: Introduction

1. Introduction

The immune response of vertebrates can be classified into two categories; innate and adaptive immunity. The innate immune system is not only critical in the initial detection of microorganisms but also is essential for the activation of adaptive immunity. This initial detection of infection is mediated by PRRs (pattern recognition receptors). The best characterised PRRs are the TLRs (Toll-like receptors) which recognise PAMPs (pathogen-associated molecular patterns) on microorganisms [1]. Detection of PAMPs by PRRs induces the activation of signalling cascades that lead to an inflammatory response which involves the upregulation of genes including proinflammatory cytokines such as TNF (Tumour Necrosis Factor), IL-1 (Interleukin-1) and IL-6, type I IFN (Interferons) and chemokines [2]. Recent evidence suggests that PRRs can also recognise endogenous ligands that are generated during tissue injury. The innate immune system can also contribute to many inflammatory diseases such as autoimmunity if inflammation persists and thus must be tightly regulated [3].

1.1 Pattern Recognition Receptors (PRRs)

PRRs are a key component of the innate immune system, and have been well established as being the first line of defence against invading pathogens [1]. PRRs recognise PAMPs which are highly conserved motifs in various microbes including bacteria, viruses, fungi and protozoans [4]. These PAMPs are essential for the survival of the microorganism thus they are not likely to be subject to mutation leading to escape of immune detection. They are also expressed on a broad class of

pathogens, for example, a well known PAMP is lipopolysaccharide which is located in the cell wall of gram negative bacteria [5]. PRRs are germline-encoded, nonclonal, expressed on all cells of a given type and independent of immunological memory. Thus, the expression of these PRRs by immune and tissue cells allows the host to detect and respond to infection by invading pathogens [6]. The PRR-mediated recognition of these PAMPs leads to the subsequent activation of signalling pathways that are critical for the regulation of inflammation, the antiviral response, the subsequent activation of the adaptive immune response and the control of autoimmune and inflammatory disease [1].

At least five families of PRRs have been now been described. They are the Toll-Like Receptors (TLRs), RIG-(Retinoic acid Inducible Gene-1)-Like Receptors (RLRs), the Nod-Like Receptors (NLRs), the C-type lectin receptors (CLRs) and the AIM-2 (absent in melanoma)-like receptors (ALRs) [7-9] .

1.1.1 RIG-like Receptors (RLRs)

The RLRs are a group of cytosolic PRRs. They are involved in cell-intrinsic recognition of viruses. The RLR family is composed of three members to date; RIG-I, MDA5 (Melanoma differentiation associated gene 5) and LGP-2 (Laboratory of Genetics and Physiology 2) [10, 11]. Both RIG-I and MDA-5 are ubiquitously expressed [6].

RIG-I contains two N-terminal CARDs (Caspase Activation and Recruitment Domain), a C-terminal regulatory domain (CTD) and a DEAD box RNA helicase domain [10]. The C-terminal domain within RIG-I acts as an internal repressor of activation. This

repressor domain inhibits the multimerisation of RIG-I and its binding to its downstream adaptor. RIG-I agonists induce a conformational change that relieves auto-repression and exposes the CARDs.

The ligand for RIG-I was initially thought to be dsRNA since the synthetic analogue of dsRNA, Poly I:C (polyinosine-polycytidylic acid) was able to activate RIG-I [12]. It is now known that a critical determinant of RIG-I activation is via recognition of a 5'triphosphate (5'ppp) moiety on RNA [13] . Studies from knockout mice revealed that RIG-I is indispensable for IFN responses for negative strand viruses that contain a 5'ppp such as Sendai Virus and Influenza [12]. It has also been shown that cleaved self RNA, short replication intermediates and non-genomic viral transcripts can all activate RIG-I. A recent study from Rehwinkel *et al.* has demonstrated that transfection of naked viral RNA does not necessarily mimic a viral infection [14]. Thus, through a virus infection model, the physiological RIG-I agonist was shown to be progeny viral genomes with a 5'ppp [14].

MDA-5 also contains two CARDs and an RNA helicase domain but lacks the CTD. Thus as MDA-5 is similar in sequence to RIG-I, it was originally presumed that MDA-5 recognised similar types of viral dsRNA [6]. Indeed, some viruses such as Dengue Virus and West Nile Virus are sensed by both RIG-I and MDA-5, such that the loss of either RLR is redundant for IFN production[15]. However, through knockout mice studies it was revealed that both RIG-I and MDA-5 are activated by distinct RNA species. MDA-5 was shown to recognise viruses that produce large amounts of dsRNA such as picornaviruses [16]. Thus, the ligand for MDA-5 was initially assumed

to be long linear dsRNA. However, a recent study by Pichlmair *et al.* has shown that the ligand MDA-5 recognises is higher order structured RNA that contains ssRNA and dsRNA [17].

LGP-2 lacks a CARD and is thought to act as a negative regulator since mice lacking LGP-2 displayed enhanced production of IFNα and IFNβ in response to Poly I:C treatment [18]. However, a positive role for LGP-2 has also been demonstrated as recently it has been shown that LGP-2 supports both RIG-I and MDA-5-mediated antiviral responses [19].

Of note, endogenous host RNAs do not activate RIG-I or MDA-5 as their 5' ends are protected by a methylguanosine cap, or else cellular RNA is generally modified with unusual bases which prevent activation of RIG-I or MDA-5 [6].

1.1.1.2 RIG-I Signalling

Upon viral infection, RIG-I binds to viral 5'ppp RNA, through the CTD. Binding of RNA causes dimerisation of RIG-I and activates its ATPase activity. The RIG-I dimer has an open conformation that exposes the N-terminal CARDs. It has recently been shown that the CARDs bind K63-linked polyubiquitin chains and the binding of these chains causes additional conformational changes and oligomerisation of RIG-I [20]. Thus, the binding of polyubiquitin chains allows RIG-I to become fully activated. Furthermore, RIG-I is also known to be polyubiquitinated by the ubiquitin ligase TRIM25 and this is important for activation of RIG-I [21]. Thus a new signalling

platform is formed which activates the mitochondrial localised protein IPS-1 (IFNβ promoter stimulator 1) also known as MAVS (Mitochondrial Antiviral Signalling Protein), VISA (Virus-Induced Signalling Adaptor) and CARDIF (CARD adaptor inducing IFN-β)) [22-25]. IPS-1 can activate downstream signalling pathways that result in the activation of transcription factors including IRF3 (Interferon Regulatory Factor) 3, IRF7 and NFκB (Nuclear Factor kappa B) (see Figure 1.1). STING (stimulator of interferon genes) has also been implicated in RIG-I signalling [26]. MDA-5 signals similarly to RIG-I although MDA-5 is not thought to signal through STING [26]. However, the role of STING in RNA sensing is still controversial.

1.1.2 NOD-like receptors (NLRs)

The NLR family consist of 22 human members and 34 members in mice. The NLR family members are characterised by an N-terminal PYD (pyrin domain) or a CARD domain, a central NACHT (domain present in NAIP, CIITA, HET-E and TP-1) domain and a C-terminal LRR (leucine rich repeat) domain [27]. The PYD and CARD domains are involved in protein-protein interactions. The NACHT domain is required for ATP-dependent oligomerisation and the LRR domain is thought to be involved in ligand binding [28].

There are three distinct sub-families within the NLR family; The NODs, the NLRPs and the IPAF subfamily. Within the NOD family, NOD1 and NOD2 both recognise breakdown products of bacterial cell walls. NOD1 recognises mesodiaminopelic acid which is commonly found in Gram-negative bacteria [29, 30]. NOD2 recognises muramyl dipeptide which is common to Gram-negative and Gram-positive bacteria

[31]. Upon ligand sensing, both NOD1 and NOD2 oligomerise and recruit RIP2 via CARD/CARD interactions, ultimately leading to NFκB activation (see Figure 1.1).

Other NLR family members have a role in the regulation of caspase-1 activity through inflammasome formation [32]. Inflammasome complexes assemble upon activation by an appropriate stimulus, leading to the cleavage of pro-caspase-1 to active caspase-1. Activation of caspase-1 is mediated via the signalling molecule ASC (apoptosis-associated speck-like protein containing a CARD) [33]. Pro-caspase-1 is recruited to ASC by a CARD/CARD domain interaction (of ASC and caspase-1). This leads to auto-cleavage of caspase-1. Active caspase-1 processes cytokines such as IL-1β and IL-18 into active IL-1β and IL-18 respectively. IL-1β and IL-18 are synthesised as pro-isoforms that are not biologically active [34].

The NLRP3 inflammasome is the best characterised inflammasome to date. A diverse range of stimuli activate the NALP3 inflammasome including microbes, end or by-products of stress and danger signals or exogenous compounds such as aluminium, asbestos and silica [35].

In addition to regulating IL-1β and IL-18, inflammasome complexes also regulate an inflammatory form of cell death called pyroptosis [36]. Pyroptosis occurs most frequently upon infection with intracellular pathogens. It is known to be caspase-1-dependent and independent of pro-apoptotic caspases such as caspase-3 or caspase-8.

1.1.3 C-type lectin receptors (CLRs)

CLRs are a large superfamily of proteins characterised by the presence of one or more C-type lectin domains (CTLDs) [9]. Although some of the CLRs carry out non-immune functions, some of the CLRs are PRR that can trigger both innate and adaptive immune responses [9]. Syk (spleen tyrosine kinase)-coupled CLRs are critical for the initiation of inflammatory responses and also function in adaptive immunity [9]. Syk-coupled CLRs signal via immunoreceptor tyrosine activation (ITAM)-like motifs in their cytoplasmic tails to activate NFκB, MAPK and NFAT (nuclear factor of activated T cells) pathways [9].

A well-characterised CLR is Dectin-1 [37]. Dectin-1 recognises β-glucan carbohydrates in the cell wall of some fungal species [37]. Recently, a Dectin-1 selective agonist has been described. While fungal particles were shown to trigger multiple innate receptors, curdlan, a β-glucan derived from *Alcaligenes faecalis,* was shown to be Dectin-1 specific [38]. Curdlan was shown to stimulate dendritic cells (DCs) to activate ERK, JNK, p38 MAPK and NFκB pathways. Furthermore, curdlan-stimulated DCs were also shown to trigger adaptive immunity including Th17 responses [38].

1.1.4 Cytosolic DNA sensors

Another rapidly expanding group of PRRs are intracellular DNA receptors. Initial studies revealed that sensing of viral DNA by the immune system occurred via TLR9 in the endosomes of plasmacytoid DC (pDCs) [39]. It was known that cytosolic DNA could trigger type I IFN, however, it was unclear how exogenous DNA released into the cytoplasm could activate the immune response. Cytoplasmic DNA sensing

pathways which recognise exogenous DNA have now been described. These pathways are known to signal via signalling mediators such as STING, TBK-1 and IRF3 (see section 1.2.2.4 also) (See Figure 1.1).

1.1.4.1 DAI

DNA-dependent activator of IRF (DAI, also known as Z-DNA-binding protein 1 and DLM-1) has been discovered to be one such cytoplasmic DNA receptor [40]. DAI was initially thought to have a vital role in innate immune signalling [40]. In L929 cells, upon HSV infection, DAI was shown to be required for type I IFN responses. In addition, it was demonstrated that DAI could interact with TBK-1 and IRF3 and this interaction was enhanced upon DNA stimulation. Subsequent reports have shown that DAI plays a redundant role in other cell types [41]. In MEFS treated with DAI siRNA, IFNβ mRNA induction was reduced only marginally [42]. DAI-deficient mice responses to dsDNA occurred normally, thus suggesting a role for other cytoplasmic receptors [41].

1.1.4.2 RNA Polymerase III

It has previously been reported that in certain human lines, transfection of dsDNA could induce IFN-β via the RNA sensor RIG-I [13]. This was somewhat surprising as it was unclear how dsDNA could be detected by an RNA sensor such as RIG-I.

Chiu *et al.* demonstrated a novel DNA sensing pathway which involved RNA polymerase III (RNA pol III) [43]. RNA pol III is a ubiquitous enzyme normally found in the nucleus, where it is involved in the synthesis of tRNA, 5S rRNA and other small

RNAs. It is also found in the cytosol but its cytosolic function remained unclear until recently [44]. It was revealed that in certain cell types, AT-rich dsDNA was transcribed by RNA pol III into dsRNA that has a 5'triphosphate and thus could be detected by RIG-I [43]. Several DNA viruses were shown to be detected by RNA pol III including Adenovirus, HSV-1 and EBV (Epstein-Barr Virus). Inhibition of RNA pol III was shown to block type I IFN induction by the intracellular bacteria *Legionella pneumophila*.

1.1.4.3 AIM-2

AIM-2 is a member of the PYHIN (pyrin and HIN domain-containing protein) family. There are four members in humans: IFIX (pyhin -1), IFI16, MNDA and AIM-2. This family of proteins contain at least one DNA binding HIN domain and one PYD domain. In contrast to the other PYHIN-family members, AIM-2 is located primarily in the cytosol [45].

AIM-2 was shown to be a receptor for sensing cytosolic dsDNA [45-48]. AIM-2 binds DNA through its HIN domain and signals via its PYD domain. AIM-2 forms an inflammasome complex with ASC to activate caspase-1 and NFκB. Knockdown of AIM-2 resulted in impaired poly (dA-dT)-induced IL-1β release and caspase-1 cleavage both in macrophages and THP-1 cells [45].

Recently, AIM-2 $^{-/-}$ mice were generated [49]. AIM-2 was shown to be required for inflammasome activation in response to various pathogens including *Francisella tuleranesis,* VACV, mCMV (murine cytomegalovirus) and *Listeria monocytogenes*. In

addition, there was a critical role for AIM-2 in the regulation of IL-18 production and NK-cell-dependent production of IFN-γ in mCMV infection [49].

Interestingly, AIM-2 is not involved in type-1-IFN production by cytosolic DNA but rather has a negative role in IFN induction.

1.1.4.4 IFI16

Given that the role of DAI appeared to be redundant and cell-type specific and that RNA Pol III specifically recognised only AT-rich DNA, it was clear that there were other cytoplasmic DNA sensors that remained to be discovered. In addition, AIM-2 could sense cytosolic DNA but did not induce type I IFNs.

Unterholzner *et al.* reported that the PYHIN protein IFI16 (Interferon-inducible protein 16) was a critical sensor for cytosolic DNA [8]. IFI16 is unique among the human pyhin family members in that it contains 2 HIN domains as well as a one pyrin domain. Unterholzner *et al.* showed that a 70-base pair repeated motif from VACV (VACV 70 mer) could induce IFN-β when transfected into RAW264.7 or THP-1 cells but not HEK 293 cells. Furthermore IFI16 could bind directly to this VACV 70mer and a 60mer from Herpes Simplex Virus (HSV) to induce IFNβ [8]. The IFN induction by IFI16 was shown to be dependent on STING, TBK-1 and IRF3 (see Figure 1.1). In addition, HSV activation of the NFκB subunit, p65, and IRF3 was shown to be p204-dependent (p204 is the murine ortholog of IFI16). Interestingly, IFI16 was located primarily in the nucleus, although a small fraction of IFI16 was shown to be present within the cytoplasm where it could be co-localised with transfected DNA.

1.1.4.5 Emerging DNA sensors

The discovery of new DNA sensors is a rapidly expanding area of immunology. Other DNA sensors that have recently been described include DHX9 and DHX36 [50]. DHX9 and DHX36 are part of the RHA (RNA helicase A) subfamily of DExD/H helicases. DHX9 and DHX36, located in the cytosol of human pDCs, were shown to recognise microbial CpG-B and CpG-A DNA respectively. This recognition was shown to be MyD88-dependent [50].

It is apparent that there are still other DNA sensors that have yet to be discovered. For example, a recent paper has described an AT-rich (e.g. ATTTTTAC) motif in malaria DNA that induces the type-I-IFN pathway but is independent of any currently known DNA sensors, including TLR9 which has been previously reported to recognise malaria DNA [51]. This pathway was shown to be dependent on STING, TBK-1 and IRF3 and IRF7.

1.1.5 Toll-Like Receptors (TLRs)

The Toll Receptor was first identified in Drosophila. It was initially identified as having a role in the development of Drosophila embryos and later its role in immunity was discovered [52]. Toll Receptor in Drosophila is involved in anti-fungal responses [52]. It was subsequently shown that the cytoplasmic domain of the Toll protein shared structural similarities to the IL-1R, thus implying that Toll and IL-1R may signal in a similar manner [53]. There are, in fact, 10 TLRs in the fly but only one has been implicated so far in host defence [54]. In 1997, the first human TLR was discovered which has a TIR (Toll IL-1 receptor) domain and, similar to the fly toll, it

also contains LRRs extracellularly [55]. The TIR domain is defined by a motif of ~160 amino acids composed of five β-sheets surrounded by α-helices and connected together by flexible loops [55]. TLRs can be described as transmembrane proteins with LRRs located extracellularly and a cytoplasmic TIR domain. The TLR family has 10 members in human and 13 in mice and has a vital role in detecting PAMPs. They belong to a now classified family termed the IL-1R/TLR superfamily and they all contain a TIR domain. The IL-1 receptor does not have LRRs but 3 immunoglobulin domains extracellularly. The IL-1R/TLR superfamily can be divided into three subgroups:

a) The immunoglobulin domain sub group: (IL-1R-like)

b) Leucine-rich repeats sub-groups: (TLRs)

c) Adaptor sub-group

TLRs are expressed on various immune cells such as macrophages, DCs, B cells and neutrophils as well as non-immune cells such as fibroblasts, epithelial cells and keratinocytes [1]. The TLRs differ from one another in the cell types in which they are expressed, their ligand specificity, the different signalling adaptors used and also the different types of cellular responses they induce [56]. When activated, they are known to lead to the activation of transcription factors such as NFκB, IRF3 and MAP kinases, ultimately leading to the induction of pro-inflammatory cytokines and type I IFNs. TLRs are described in further detail in section 1.3

1.2 Transcription Factors

1.2.1 Nuclear Factor Kappa B

NFκB is a family of dimeric "Rel family" transcription factors [57]. In mammalian cells, five Rel proteins belonging to two classes have been identified [58]. One class includes Rel A (p65), Rel B and c-Rel and these are synthesized as mature products. The second group consists of p50 and p52, which are synthesized as the large precursors p105 and p100 [58]. The NFκB family have an N-terminal Rel homology domain (RHDs). The RHD is required for dimerisation and binding to DNA sequences that are present in promoter and enhancer regions of genes [57]. The most common form of NFκB in TLR signalling is p50-p65 (RelA) . This dimer is sequestered in the cytoplasm as an inactive form by the inhibitory IκB protein [59]. Upon stimulation with IL-1 or TLR ligand, the multi-subunit IKK (IκB kinase) complex phosphorylates specific serine residues of IκB proteins. The IKK complex contains two catalytic subunits which can form homo- or hetero-dimers (IKKα or IKKβ) and a regulatory subunit NFκB-essential modifier (NEMO), also known as IKKγ [59]. Both IKKα and IKKβ are structurally similar and have a kinase domain, a leucine zipper domain, helix-loop-helix structures and a NEMO-binding domain [60]. NEMO functions as a docking platform recruiting upstream IKK activating molecules. In the case of IκBα, IKK-mediated phosphorylation occurs at Ser 32 and Ser36 in the N-terminal portion of the molecule [61]. After the IκBα molecule is phosphorylated, it then gets

ubiquitinated leading to its proteasomal degradation [62]. Thus, NFκB is released, it translocates into the nucleus and binds to DNA consensus sequences known as κB sites [61]. This pathway is termed the canonical pathway. There is another NFκB-activating pathway termed the alternative pathway. This pathway is triggered by CD40, LT (lymphotoxin β receptor) and the BAFF receptor (B-cell activating factor belonging to the TNF family) [63]. In this pathway, the two subunits p105 and p100 are phosphorylated and ubiquitinated leading to the degradation of the C-terminal region of p105 and p100 resulting in the generation of mature p50 and p52 [63]. The maturation of the two subunits relies on the NFκB-inducing kinase (NIK). Upon NIK activation, p100 gets phosphorylated through an IKKα-dependent pathway [64].

1.2.2 Interferon Regulatory Factors (IRFs)

The IRFs are a family of transcription factors with diverse roles in the regulation of the immune system. The family has nine members [65]. They have a role in type I IFN induction [65]. Each IRF is composed of a well-conserved N-terminal helix-turn-helix motif which contains a DNA-binding domain of ~ 120 amino acids [66] . This region recognises the IFN-stimulated response element (ISRE) which is a DNA sequence found in the promoters of many different genes involved in immunity [67]. IRF3 and IRF7 are the main regulators of type I IFN gene expression [65, 66].

1.2.2.1 IRF3

IRF3 is constitutively expressed [67]. It resides in the cytosol in an inactive form [66]. Upon stimulation, specific serine residues in the C-terminal regulatory region are

phosphorylated. Phosphorylated IRF3 forms either a homo-dimer or a hetero-dimer with IRF7 [65, 66]. This results in the nuclear translocation of the dimer. This dimer then binds to co-activators CBP and p300 and this complex binds to DNA sequences [66].

1.2.2.2 IRF7

IRF7 is only expressed in small amounts in most cells [68]. It is strongly induced by type I IFN-mediated signalling. It is involved in a positive feedback regulation of type I IFN genes [68]. Upon viral infection, IRF7, like IRF3, is phosphorylated resulting in the formation of homo-dimers and hetero-dimers with IRF3. These dimers translocate into the nucleus and induce the expression of chemokines and IFNβ. This IFNβ then binds to and activates IFN receptor which leads to the activation of IFN stimulated gene factor 3, a complex of STAT-1 (Signal Transducers and Activators of Transcription), STAT-2 and IRF9, which translocates into the nucleus and induces the transcription of IRF7 gene [66].

1.2.2.3 IRF5

In contrast to IRF3 or IRF7, IRF5 expression is much more restricted. Unlike IRF3, which is expressed constitutively in all cell types, the expression of IRF5 and IRF7 is restricted to B cells and DCs (although their expression is inducible in other cell types by type I IFNs) [69]. Specific IRF5 haplotypes are associated with dysregulated expression of type I IFNs and, thus, are associated with being a risk factor for SLE [70]. Only certain viruses, such as NDV (Newcastle Disease Virus), VSV (Vesicular

Stomatitis Virus) and HSV-1 have been shown to activate IRF5 [69]. Activation of IRF5 is restricted to TLRs that signal via MyD88 (Myeloid differentiation primary-response gene 88) [69]. A study from the Pitha group has shown that IRF5 interacts with MyD88 and also TRAF6 (Tumour-Necrosis Factor (TNF) Receptor Associated Factor) [71]. TRAF6 has been shown to polyubiquitinate IRF5 and this ubiquitination is vital for IRF5 translocation into the nucleus [71]. IRAK-1 has been shown to interact with IRF5 also and this reaction precedes and is required for IRF5 ubiquitination and activation [71].

1.2.2.4 Mechanism of IRF Activation

IRFs are activated by certain TLRs (TLR 3, 4, 7, 8, 9) as well as cytosolic PRRs such as RIG-I and IFI16. In contrast to NFκB which in order to be activated must be released from a cytoplasmic inhibitory protein, IRF3 and IRF7 have no inhibitory protein sequestering them in the cytoplasm. For their activation they require phosphorylation of their C-terminus by two kinases: TBK-1 and IKKε [72].

While the NFκB pathway has NEMO as its scaffolding protein strong evidence suggests a role for three proteins TANK, NAP-1 (NFκB-activating kinase-associated protein) and SINTBAD (similar to NAP-1 TBK-1 adaptor) as scaffolding proteins for the assembly of TBK-1 and IKKε kinase complexes [73-75]. TANK, NAP-1 and SINTBAD are required for TBK-1 and IKKε assembly but it is unclear whether distinct scaffold proteins are required for assembly of TBK-1 and IKKε in a pathway specific manner (see Figure 1.2) [72]. Apart from the TLRs, other PRRs activate IRF3 through TBK-1

and IKKε. RIG-1 and MDA-5, the cytosolic receptors that recognise RNA from viruses, signal through TRAF3 to activate TBK-1/IKKε, thus activating IRF3 [76].

1.2.3 MAP Kinase Activation

MAPKs (Mitogen-activated protein kinases) are protein Ser/Thr kinases that convert extracellular stimuli into a wide range of cellular responses [77]. The best characterised MAPK include p38 isoforms (α, β, γ and δ), JNK 1, 2, 3 (c-Jun N-terminal kinases) and ERK 1, 2 (extracellular signal-regulated kinase 1, 2) [77]. MAPK cascades are composed of consecutively activating protein kinases, which are known as MKK (MAPK, MAPK kinase) and MKKK (MAPKK kinase). An activated MKKK phosphorylates MKK, which then activates a specific MAPK [78]. For p38 activation, TAK-1 activates MKK3 and MKK6 which, in turn, activate p38 MAPKs. JNK MAPKs are activated by TAK-1 activation of MKK4/ MKK7 and ERK1 and ERK2 are activated by MKK1 and MKK2 (See Figure 1.3). JNK and p38, in turn, activate transcription factors such as CREB and AP-1.

In addition to transcription factor activation, p38 has a critical role in post-transcriptional regulation of mRNA [79]. p38 can upregulate cytokine production via MK-2-(p38/MAPK-activated protein kinase 2)-dependent stabilisation and increase translation of mRNA containing 3'UTR AU-rich elements (see section 1.6) [79].

Another MAP kinase pathway that is activated in TLR signalling is the ERK pathway. While TAK-1 activates JNK and p38 via MKK3 and MKK6 respectively, the ERK

pathway is activated via Tpl2 (tumour progression locus-2) phosphorylation of MEK1 and MEK2 [80]. Regulation of tpl2 involves an NFκB1 precursor protein, p105. p105 is essential for stabilising Tpl-2 and keeping it in inactive state [81]. Following TLR stimulation, Tpl2 is released from p105 and activated Tpl-2 can phosphorylate MEK-1 and MEK-2, the kinases upstream of ERK-1 and ERK-2 [82]. It has also been demonstrated that IKKβ phosphoryates p105 which is required for Tpl-2 release. Another protein which is known to be involved in the regulation of ERK activation is ABIN-2. ABIN-2 is a Tpl-2-interacting protein and abin-2 $^{-/-}$ mice have lower levels of Tpl-2 and are deficient in ERK activation [83]. However, the role of ABIN-2 in ERK activation is thought to be cell-type specific [83].

1.3 Toll-Like Receptor Family

The TLR family members can be divided in two sub-populations depending on their localisation within the cell. TLR1, TLR2, TLR4, TLR5, TLR6 and murine TLR11 are expressed on the cell surface and TLR3, TLR7, TLR8 and TLR9 are known to be localised in intracellular vesicles such as the endosome and the endoplasmic reticulum (ER). TLR2 and TLR4 can translocate to the endosome to signal (See Figure 1.2). The TLRs signal through five adaptors; Myeloid differentiation primary response gene 88 (MyD88) [84], MyD88 Adaptor-Like protein (MAL) [85], TIR domain containing adaptor protein inducing IFN-β (TRIF) [86], TRIF-Related Adaptor Molecule (TRAM) [87] and Sterile α and ARMadillo motif-containing protein (SARM) [88] (see Figure 1.3).

1.3.1 TLR1, TLR2 and TLR6

The PAMP TLR1 recognises is triacyl lipopeptides. TLR1 is functionally associated with TLR2. TLR1 and TLR2 interact via hydrogen bonds and also hydrophobic interactions which have a stabilisation role for the dimer [89]. The crystal structure of the TLR1/TLR2 hetero-dimer in complex with the tri-acylated lipopeptide Pam_3CSK_4 is said to take the formation of an 'm' shape which resembles the crystal structure of TLR4/MD-2 with its ligand, LPS [89, 90]. TLR1 is located on the cell surface. A single nucleotide polymorphism within TLR1 at the junction of the transmembrane and the intracellular domain results in irregular trafficking of the receptor to the cell surface making TLR1 unresponsive to its ligands [91]. Interestingly this polymorphism in TLR1 results in a decreased incidence of leprosy suggesting that the pathogen that causes leprosy, *Mycobacterium leprae*, uses TLR1 to facilitate infection [92].

As mentioned above TLR2 occurs as a dimer with TLR1 but also with TLR6. TLR6 with TLR2 recognises di-acylated lipopeptides [93]. TLR2 has been shown to recognise a broad variety of microbial components due to its association with TLR1 and TLR6. TLR2 must heterodimerise with either TLR1 or TLR6 to recognise tri- and di-acylated lipopeptides [89].

A novel role for TLR2 in antiviral responses has recently been described [94]. It was demonstrated that mCMV and VACV could induce typeI IFN via TLR2. This pathway was shown to function only in two distinct cell types; IMs (inflammatory monocytes) and BMDCs (bone marrow-derived DCs) [94].

1.3.2 TLR3

TLR3 recognises dsRNA and its synthetic analogue poly I:C [95]. TLR3 is expressed on conventional DCs and on a variety of epithelial cells and also very robustly in the brain [1]. Upon recognition of dsRNA TLR3 triggers an immune response to induce type I IFN and inflammatory cytokines [95].TLR3 signals through a sole TIR adaptor, TRIF [96]. The crystal structure of the TLR3 ECD (ectodomain) has been solved. The human TLR3 ECD revealed a large horse-shoe shaped solenoid assembled from 23 LRRs [97]. The TLR3 ECD only binds dsRNA at an acidic pH (pH <6.5) in accordance with its endosomal location in most cell types [98, 99]. The crystal structure of the TLR3 ECD binding dsRNA has been solved [100]. It was shown that TLR3 ECDs bind as dimers to dsRNA and the structure does not change upon binding of the ligand. The dsRNA:TLR3 signalling complex is similar to the complex of TLR1:TLR2 ECDs binding the ligand Pam_3Csk_4 in that in both complexes the ligand bridges two TLR molecules and both form dimers with a similar m-shaped structure. TLR3 lacks a conserved proline residue in the cytoplasmic region which is crucial for TLR4 to signal, thus TLR3 signals through a unique TRIF-dependent pathway [97].

1.3.3 TLR4

TLR4 in association with MD-2 (myeloid differentiation factor 2) recognises LPS (lipopolysaccharide) [101]. LPS is a major component of the outer membrane of gram-negative bacteria and has potent immunostimulatory activity. TLR4 also recognises envelope proteins from viruses such as RSV (Respiratory Syncytical Virus) and MMTV (Mouse Mammary Tumour Virus) [102]. LPS from gram-negative bacteria

associates with LPS binding protein (LBP) and then binds to CD14 [103]. CD14 is a GPI-(glycosylphosphatidylinositol)-linked protein expressed on the cell surface of phagocytes. LPS is then transferred to MD2 which associates with the extracellular portion of TLR4. TLR4 then forms a homo-dimer [101]. The crystal structure of the TLR4/MD-2/LPS complex has recently been solved [90]. Binding of LPS induces the formation of an m-shaped receptor complex containing two copies of the TLR4-MD-2-LPS complex. TLR4 holds a unique status in that it employs all four adaptors (see Figure 1.3) [56]. TLR4 activates MyD88-MAL and TRIF-TRAM signalling pathways sequentially [104]. First TLR4 recruits MAL-MyD88 to the plasma membrane. TRAM-TRIF signalling does not occur at the plasma membrane but rather from early endosomes [104]. TLR4 has been shown to be endocytosed and recruits the TRAM and TRIF adaptors to induce a type I IFN response [104].

For TLR4 signalling, stimulation of TLR4 with LPS or endogenous ligand results in the recruitment of MAL and MyD88 to the plasma membrane. IRAK-4 (interleukin-1 receptor-associated kinase-4) interacts with MyD88 through DD (death domain) interactions [105]. IRAK-4 can then phosphorylate IRAK-2 and/or IRAK-1 (see section 1.4.1.1). Hyperphosphorylated IRAK-1 (and possibly IRAK-2) is released and then IRAK-1 and/or IRAK-2 may associate with TRAF6. IRAK-2 can trigger the polyubiquitination associated with TRAF6 [106] which results in the recruitment of TAK-1/TAB-2 complex and the activation of TAK-1. TAK-1 can activate both NFκB and ERK via the IKK complex. TAK-1 also activates p38 and JNK MAP kinases via MKK3/6 and MKK4/7 respectively (see Figure 1.4)

1.3.4 TLR5

TLR5 recognises bacterial flagellin. Flagellin is the major protein constituent of bacteria flagella which are the motility mechanisms used by many microbial pathogens [107]. TLR5 is expressed on intestinal cells, and epithelial cells thus highlighting the important role for TLR5 in microbial recognition in the gut and at mucosal surfaces [107]. A susceptibility to pneumonia caused by the flagellated bacteria *Legionella pneumophila,* has been linked to a common stop codon polymorphism in the ligand binding domain of TLR5 [108]. *Helicobacter pylori* is an example of a bacterial species that can evade the flagellin-specific immune response as their flagellin lacks pro-inflammatory properties [109].

1.3.5 TLR7, TLR8 and TLR9

TLR7, TLR8 and TLR9 form an evolutionarily-related sub-group within the TLR super-family [110]. These three TLRs, along with TLR3, all recognise oligonucleotide (RNA and DNA)–based molecular patterns from both bacteria and viruses. TLR7, TLR8 and TLR9 signal through similar signalling mechanisms although they are expressed in different cell types and are known to induce different cytokine responses [110].

1.3.5.1 TLR7

TLR7 recognises guanosine- or uridine-rich single stranded RNA (ssRNA) [111]. TLR7 is expressed on the endosome. TLR7 binds to both self and viral RNA, but TLR7 is able to discriminate between self and viral RNA by recognising the endocytic location of

viral RNA [112]. Human PBMCs have been shown to preferentially produce IFN-α, IP-10 (Interferon gamma inducible protein 10) and I-TAC (Inducible T-cell α chemoattractant) in response to a TLR7-selective agonist [113]. pDCs are the primary cells that are stimulated by this TLR7-selective agonist. TLR7 is predominantly expressed in lung, placenta and spleen. B-cells are also known to express TLR7 [114].

1.3.5.2 TLR8

While other TLRs, such as TLR3 and TLR4, have been studied extensively, less is known about TLR8. The reason for this is that the murine form of TLR8 is not responsive to agonists otherwise known to activate human TLR8 [115].

TLR8 specifically recognises ssRNA and synthetically-derived small molecules such as imiquimod and resiquimod [116]. Both of these derivates elicit robust antiviral and anti-tumour immune responses *in vivo* [117]. TLR7 and TLR8 have a key antiviral role and upon recognition of viral nucleic acids they induce type I IFNs. Using two different ssRNA TLR8 agonists, it was shown that TLR8-activated PBMCs induce high levels of pro-inflammatory cytokines including TNFα, IL-12, IL-6 and IL-8 [113]. In this study it was also demonstrated that monocytes are the primary cells activated by TLR8. Monocytes produce cytokines in response to both TLR7 and TLR8 agonists but the cytokine levels induced by TLR8 agonists are approximately 100 times greater than those induced by TLR7 agonists [113]. Another study has shown that TLR8 is predominantly expressed in lung and peripheral blood leukocytes, in particular monocytes [115]. A recent report has demonstrated that TLR8, but not TLR7 is expressed on neutrophils [118]. A low pH has been shown to be required for TLR8

function [119]. TLR8 has been shown to dimerise with TLR7 and TLR9 [120]. In HEK293 cells co-expression of TLR8 with either TLR7 or TLR9 inhibits the capacity of the latter two to respond to their agonists [120].

1.3.5.3 TLR7 and TLR8 Signalling

Both TLR7 and TLR8 signal through a single TIR adaptor, MyD88. TLR7 and TLR8 have a unique signalling pathway that allows induction of IFN-α through MyD88. The transcription factors IRF5 and IRF7 form hetero-dimer, and are subsequently phosphorylated by IRAK-1 in TLR7 and TLR8 signalling [66, 69]. IRF5 and IRF7 interact with both MyD88 and TRAF6 also [66, 69]. TRAF6 polyubiquitinates IRF5 and this ubiquitination is vital for IRF5 translocation into the nucleus. The interaction between IRAK-1 and IRF5 precedes this and is required for IRF5 ubiquitination and activation [see Figure 1.3] [71]. IRAK-4 has also been shown to be essential for this pathway [121]. TBK-1 and IKKε are not involved in this pathway but rather IKKα is essential for TLR7 and TLR8-induced IRF7 phosphoryation [66].

TLR8 has been shown to have 13 tyrosine residues in its cytoplasmic domain and these tyrosine residues are critical for its signalling activity [120]. TLR8 has also been shown to be modified following stimulation. As expected, due to its tyrosine residues, TLR8 undergoes tyrosine phosphorylation but also undergoes monoubiquitination after 2 hours, which has been suggested may be involved in the intracellular trafficking of TLR8 [120]. A study of TLR8 signalling showed that TLR8 signalling is quite different in comparison to IL-1 or other TLRs [122]. While both TLR8 and IL-1

signalling to NFκB is IRAK-1-dependent, IRAK-1 does not get modified after TLR8 stimulation [122]. Hallmarks for IRAK-1 activation upon IL-1 stimulation include IRAK-1 phosphorylation, ubiquitination followed by its degradation. Qin *et al.* showed that these modifications are not detected in response to TLR8 stimulation, thus suggesting a different signalling role for IRAK-1 in the TLR8 pathway [122]. Furthermore, while TAK-1 is required for IL-1 signalling to NFκB and JNK, results from TAK-1 -/- MEFs clearly show that TAK-1 is not required for TLR8-mediated NFκB activation [122]. TLR8 signalling was shown to be MEKK3-dependent. Thus TLR8 likely uses MEKK3 rather than TAK-1 to mediate NFκB and JNK activation [122] (Figure 1.5). This study also reported that the inhibitory sub-unit of NFκB, IκBα, did not get degraded. However, another study has shown conflicting data demonstrating that IκBα does indeed get degraded upon stimulation with a TLR8 ligand, although it occurs after 30 mins [115].

1.3.5.4 TLR9

As mentioned previously, TLR9, along with TLR3, TLR7 and TLR8 are localised intracellularly. TLR9 recognises different types of ODNs (oligodeoxyucleotides) depending on their sequence motifs and secondary structures [123]. Class A CpG ODNs form a higher order structure and are known to activate pDCs. Class B are known to be of a linear structure and activate B cells [124]. It was always believed that TLR9 recognises only microbial DNA, however it is now known that TLR9 also

binds self DNA [123]. Generally, only microbial DNA elicits an inflammatory response [123]. It has been hypothesised that the mislocalisation of TLR9 to the cell surface results in the recognition of self-DNA by TLR9 [124]. Two studies have investigated how TLR9 discriminates between self and non-self DNA [125, 126]. TLR9 has previously been shown to reside mainly in the ER in resting cells but recognition of DNA by TLR9 occurs in endolysosomes [125, 126]. A study from the Leifer group revealed how TLR9 traffics from the ER through the golgi complex and resides in endolysosomes [125, 127]. It has been suggested that control of TLR9 movement along these pathways most likely allows discrimination between self and foreign DNA [128]. What regulates the translocation of TLR9 from the ER through the golgi complex to endolysosomes is still uncertain. It is known that the ER membrane protein UNC93B interacts with TLR9 and is crucial for its signalling [129]. This protein is also essential for TLR3 and TLR7 signalling. A study from Anthony Coyle's group has revealed that HMGB1 (High Mobility Group Box-1), which is a nuclear DNA-binding protein that is released from necrotic cells may also be important for the regulation of TLR9 signalling [130]. It does this by binding directly to TLR9 [130]. HMGB1 associates with the HMGB1 receptor RAGE (receptor for advanced glycation end-products) and this association regulates the TLR9 response [130]. HMGB1 has also been shown to bind to immune complexes containing DNA. These immune complexes have been associated with Systemic Lupus Erythematosus (SLE). It has also been suggested that some of these pathways may be shared with other TLRs, such as TLR7 and TLR8 [130]. TLR 8 has been shown to be expressed in abundance on

the skin of patients with SLE. Thus, regulation of TLR9 and its associated proteins may be important in the treatment of auto-immune inflammatory disease.

1.3.6. TLRs and human disease

The role for TLRs in initiating an inflammatory response has been well established. If an inflammatory response persists this results in inflammatory diseases and auto-immunity. Many TLRs and their signalling mediators have been implicated in a wide range of diseases including SLE, RA (Rheumatoid Arthritis), Athersclerosis, Type I diabetes and IBD (inflammatory bowel disease) [3].

SLE is a chronic inflammatory disorder that can affect multiple parts of the body including heart, lungs, liver, kidneys, blood vessels and nervous system. It is thought to result from a break down in tolerance to ubiquitous self-antigens, including DNA and RNA [3]. Increased serum levels of IFNα and activated pDCs are characteristic of SLE patients. TLR 7, 8 and 9 are implicated in SLE as pDCs express high levels of TLR7 and TLR9 are efficient inducers of IFN. In addition administration of endogenous RNA and DNA can activate TLR7, 8 and 9 and induce an auto-immune reaction similar to SLE [131]. RA is another chronic inflammatory auto-immune disease which is marked by persistent inflammation at joints. Both TLR2 and TLR4 have been linked to contributing to RA [132]. TLR2 and TLR4 expression was increased in synovial tissue of RA patients compared to healthy controls.

1.4 The TIR- domain containing adaptor proteins

1.4.1 MyD88

MyD88 was discovered in 1990 [133] and by 1998 the role of MyD88 was shown to be mainly involved in IL-1 and TLR signalling [134]. It contains a TIR domain, an intermediary domain (ID) and a DD. Studies with MyD88-knockout mice generated from the Akira group showed that these mice lacked responsiveness to LPS [134]. They were also unresponsive to ligands for TLR2, TLR4, TLR5, TLR7 and TLR9 [134]. MyD88 has been shown to be crucial for signalling to NFκB, JNK and p38 for all TLRs, with the exception of TLR3 and TLR4 which can use an alternative adaptor TRIF (described below). A recent study showed that in MyD88-deficient cells, the time course of activation of NFκB is delayed in TLR4 signalling. NFκB activation was shown to occur in two waves in response to LPS [135]. The first wave is activated by MyD88 directly engaging with the pathway that leads to IKK complex activation. The MyD88 mechanism for signalling involves its TIR domain interacting directly with the TIR domain of TLR5 ,TLR7, TLR8 and TLR9. TLR2 and TLR4 require another adaptor, MAL, to act as a bridging adaptor for MyD88 recruitment [85]. The death domain of MyD88 allows it to interact with the IRAKs which are essential kinases in IL-1/TLR signalling to form the Myddosome complex (see section 1.4.1.1). Recruitment of the IRAKs to MyD88 allows for signalling to a key downstream target, TRAF6, which ultimately leads to NFκB activation (see section 1.8.1).

As well as having a crucial role in NFκB signalling, MyD88 is important for the activation of several of the IRF transcription factors [136]. IRF1 requires MyD88 in order to translocate into the nucleus in Hela cells [136]. IRF7 has been shown to require MyD88 for its activation by TLR7, 8 and 9 in pDCs [66].

Since MyD88 is a critical TLR/IL-1 signalling molecule to NFκB, JNK, p38 and the IRFs, its regulation is vitally important. For example, IRAK-M negatively regulates TLR pathways through its interaction with MyD88 [137]. In addition MyD88s, a short splice variant of MyD88 has also been shown to inhibit MyD88 by preventing MyD88 from binding to TLRs [138, 139]. MyD88 itself has recently been shown to be a negative regulator of TLR3 signalling since MyD88-deficient corneal epithelial cells were shown to have an enhanced response to Poly(I:C) [140]. In addition, MyD88 has been shown to negatively regulate TLR3-induction of IFN-β and RANTES [141]. This study also showed that MyD88 interacts with IRF3 and inhibits IKK-ε-mediated phosphorylation of IRF3 [141].

In addition to its role in NFκB and IRF activation, MyD88 has been recently described to be required for IFNγ-dependent pro-inflammatory gene induction. IFN-γ utilises a signalling pathway involving Janus kinases and STATs in which MyD88 had previously no known function [142]. However, MyD88 was shown to be required for the post-transcriptional stabilisation of IP-10 and TNFα mRNA after IFN-γ stimulation [142].

1.4.1.1 The Myddosome

Recently, the crystal structure of the MyD88-IRAK-4-IRAK-2 DD complex has been solved [105]. It was shown that MyD88, IRAK-4 and IRAK-2 interact in a left-handed helical oligomer formation consisting of between 6 and 8 MyD88s, 4 IRAK-4s and 4 IRAK-2s in a complex termed the Myddosome [105, 143]. In contrast to TIR:TIR interactions which are known to be quite weak, the DD interactions were shown to be very stable [105]. The sequential assembly of the complex is also known. MyD88 is recruited first to the TLR receptor and then oligomerisation of MyD88 occurs allowing for the recruitment of IRAK-4. The first IRAK-4 DD interacts with three MyD88 DDs. It was shown that the interactions between MyD88, IRAK-4 and IRAK-2 are very specific and certain key residues of IRAK-2 DD and IRAK-4 DD that are required for these interactions were revealed (Figure 1.6). Even though IRAK-1 was not shown in the solved Myddosome structure, critical residues in IRAK-2 DD were shown to be conserved in the IRAK-1 DD also [105]. Interestingly, IRAK-2 DD did not form a stable complex with either MyD88 or IRAK-4 alone. Thus, the MyD88 and IRAK-4 DD complex must form first and then IRAK-2 is recruited. The Myddosome structure is formed in such a way that the kinase domains of IRAK-4 are in close proximity to the kinase domains of IRAK-2/IRAK-1, thus allowing efficient phosphorylation of IRAK-2 or IRAK-1 [105]. It has also been reported that non-DD interactions between MyD88 and IRAK-2 are also important for Myddosome formation. The Myddosome structure was shown to be conserved in Drosophila. Drosophila Toll receptor signals via dMyD88, Tube and Pelle (a homologue or IRAK-4).

The Drosophila Myddosome was shown to have a simpler stoichiometry of 1:1:1 but a similar conserved three tiered system [105].

Children who suffered from life-threatening pyogenic bacterial infections were discovered to have a deletion of residue E52 or a mutation at residue L93 (L93P) in MyD88. When these residues are mutated in MyD88, the formation of the Myddosome complex is disrupted [105]. Two natural occurring MyD88 variants S34Y and R98C have been shown to have reduced activation of NFκB due to interference of assembly of the Myddosome structure [144] .

1.4.2 MAL

MAL, also known as TIRAP (TIR-adaptor protein), was the second TIR domain-containing adaptor protein to be discovered [85, 145]. It contains a TIR domain, a phosphatidylinositol-4,5 biphosphate (PtdIns(4,5)P2)-binding motif (PIP2) at amino acids 15-35 and a TRAF6-binding motif at amino acids 188-193. MAL associates constitutively with the plasma membrane through its PIP2-binding motif. MAL acts as a bridging adaptor for MyD88 for TLR2 and TLR4 [85, 145]. For the other TLRs, MyD88 can directly bind to the TIR domain. However, like MyD88, TLR2 and TLR4 are largely electropositive, therefore MyD88 is unable to bind to these TLRs. As MAL is largely electronegative it acts as a bridging adaptor for MyD88 with TLR2 and TLR4 [146]. A study of MAL-deficient mice showed that these mice were defective in TLR4 signalling in terms of cytokine induction. IL-1R and TLR9 signalling was normal but TLR2 was also impaired [147]. Recently it has been shown that MAL plays a greater

role in TLR4 signalling than in TLR2 pathways [148]. MAL was not necessary for TLR2 signalling at high concentrations of the ligands Pam_3Csk_4 and MALP2 but was required for TLR4 signalling at all ligand concentrations tested [148]. MAL was shown to interact with TLR1, TLR2 and TLR4 but not TLR6 while MyD88 could interact with TLR1, TLR2, TLR4 and TLR6 [148].

A SNP (single nucleotide polymorphism) that was discovered in the MAL gene at position 180, converting a serine to a leucine, revealed an interesting link between MAL and protection against a wide spectrum of diseases including malaria, TB, bacteraemia and IPD (invasive pneumonoccal disease) [149]. Heterozygotes for the gene showed a near halving of disease risk [149]. A novel role for MAL in the inhibition of TLR3-induced JNK activation and IL-6 induction has recently been demonstrated. Thus, MAL and MyD88 both have a role in negatively regulating the TLR3 pathway [140, 148].

The crystal structure of the MAL TIR domain has recently been solved [150]. MAL TIR domain has a low level of amino acid sequence similarity to the other human TIR domains, its closest homologue being the MyD88-TIR domain with a 24% identity. MAL contains an AB loop rather than a BB loop, which is a signature motif located in the other TIR domains. The AB loop contains a long loop that connects the first α helix (αA) to the β-strand. The AB loop sequence was shown to be significantly similar to the BB loop sequence found in other TIR domains [150]. This configuration may be an important structural feature which distinguishes the role of MAL from other TIR domains.

1.4.3 TRIF

TRIF, also known as TICAM-1 (TIR-containing adaptor molecule-1) was the third TIR adaptor protein involved in TLR signalling to be discovered [86, 96]. As its name suggests it contains a TIR domain. It also contains a TRAF6-binding domain and a receptor-interacting protein (RIP) homotypic interaction motif (RHIM). It was also identified through database analysis of TIR domain-containing proteins [86]. It was shown that this novel adaptor could activate the IFNβ promoter [86, 96]. It is now known that TRIF accounts for the MyD88-independent aspect of TLR4 signalling [151]. It is also the sole adaptor involved in TLR3 signalling. TRIF directly binds to TLR3, whereas for TLR4 TRIF uses a bridging adaptor, TRAM. TRIF and TRAM are responsible for the late activation of TLR4-induced NFκB. TRIF has also been implicated in mediating an apoptotic signalling pathway [152].

1.4.4 TRAM

TRAM, also known as TICAM2 (TIR-containing adaptor molecule-2) was the fourth TIR domain-containing adaptor protein identified [87, 153]. Through interaction studies with TRIF and using TRAM-deficient cells, it was shown that TRAM interacts with TRIF and also that TRAM functions exclusively in the TLR4 pathway [87, 153]. TRAM was shown to specifically mediate the MyD88-independent TLR4 signalling pathway. Both TRIF and TRAM are required for the late activation of NFκB activation [87]. TRAM and TLR4 are also known to translocate to the endosome to activate IRF3 in a TRIF-dependent manner [104]. How TRAM signals at both the plasma membrane and endosome was unclear until a study from Kagan *et al.* identified a bipartite

localisation motif which would allow TRAM to signal both at the endosome and plasma membrane [104]. Thus, TRAM functions as a bridging adaptor like MAL, but signals from endosomal compartments leading to type I IFN induction [104].

A splice variant of TRAM with a negative regulatory function, TAG (TRAM adaptor with Golgi Dynamics (GOLD) domain), has recently been described [154]. The GOLD domain is a membrane-trafficking domain that localises proteins to membrane vesicles. TRAM was shown to interact with TAG in endosomes and could inhibit TRAM-dependent signalling to IRF3 [154].

1.4.5 SARM

SARM, (also known as Myd88-5) was the fifth characterised TIR domain-containing adaptor protein. It contains an N-terminal HEAT/Armadillo motif and two SAM (sterile α motif) domains. It is highly conserved in *Caenorhabditis elegans* (C. elegans), *Drosophila* and mammals. It is of interest to note that the *C. elegans* ortholog of SARM, TIR-1 has a function in worm immunity and in development, thus showing a positive role for the SARM ortholog in the worm. Carty *et al.* showed a negative role for human SARM [88]. It functions to inhibit TRIF-dependent but not MyD88-dependent signalling [88]. Significantly, in human PBMCs, TLR3 and TLR4-induced cytokine production was shown to be increased when SARM expression was targeted by siRNA. An alternative role for murine SARM has also been shown. Kim *et al.* have shown a role for SARM in stress-induced neuronal toxicity and that SARM is preferentially expressed in neurons [155]. Kim *et al.* showed no role for SARM in TLR signalling in mouse macrophages, thus perhaps showing a difference between the

functions of murine and human SARM [155]. A role for SARM in restricting pathogenesis of WNV (West Nile Virus) has recently been described [156]. WNV is a neurotropic flavivirus and data from SARM $^{-/-}$ mice infected with WNV revealed that SARM functions to restrict WNV infection and subsequent neuronal injury in a brain-specific manner [156].

1.5 Interleukin-1 Receptor-Associated Kinases (IRAKs)

The IRAKs are a family whose members contain an N-terminal DD and are categorised as serine/threonine protein kinases. They play a critical role in IL-1R and TLR signalling to various transcription factors such as NFκB, JNK, p38 and IRFs [157]. To date, there are four members in the IRAK family. Human IRAK-1, IRAK-2 and IRAK-4 are expressed ubiquitously in cells whereas human IRAK-M is only detectable in monocytes and macrophages in an inducible manner [157]. Although they are categorised as serine/threonine protein kinases, IRAK-M does not exhibit kinase activity and the kinase activity of IRAK-2 is still controversial [157].

Structurally, IRAK family members share similar domains (see Figure 1.6). They contain an N-terminal DD, a proST domain, a central conserved kinase domain and a C-terminal domain (except for IRAK-4 which lacks a C-terminal domain) [158, 159]. The DD is vital for signalling since it interacts with other signalling molecules such as MyD88 and IRAK members that lack the death domain act in a dominant negative manner [160, 161]. The proST region is rich in serines,

prolines and threonines. IRAK-1 is reported to undergo hyperphosphorylation in this region [159]. This domain for IRAK-1 is said to contain two potential PEST sequences which may facilitate its degradation. IRAK-2 does not have these sequences and it is not degraded. The central kinase domain contains an activation loop which is important for kinase activity. Each IRAK kinase domain also contains an invariant lysine residue in its ATP binding site which is also critical for the catalytic activity [162]. The crystal structure of the kinase domain of IRAK-4 has been reported by two separate groups [163, 164]. IRAK-4 contains characteristic structural features of both Ser/Thr and also tyrosine kinases. The IRAK family have a tyrosine gatekeeper residue at the centre of the ATP-binding site [163]. The gatekeeper residue refers to the residue upstream of the hinge that controls access to a pre-existing internal hydrophobic pocket at the back of the ATP-binding site [163]. The tyrosine residue as a gatekeeper is exclusive to the IRAK family making them a unique family of kinases [164]. The different IRAK proteins have different residues that undergo phosphorylation (see Figure 1.6). Lastly, the C-terminal domain contains TRAF6 interaction motifs [165]. IRAK-1 contains three TRAF6 interaction motifs, IRAK-2 is reported to have two TRAF6 interaction motifs and IRAK-M contains one TRAF6 interaction motif [165]. It has recently been shown that IRAK-2 (and not IRAK-1) can interact with TRAF6 [166].

Although it was initially thought that the IRAK proteins acted in a somewhat redundant manner in signalling to NFκB, molecular and cellular analysis revealed that

distinct IRAK proteins are differentially regulated and play unique roles mediating downstream signalling processes [106, 167]. As the field evolves it is becoming clearer that each member has a distinct role not only in signalling to NFκB but also to other transcription factors such as the IRFs. Furthermore, studies have shown roles for the IRAKs in the adaptive immune system and in apoptosis [168, 169].

1.5.1 IRAK-1

IRAK-1 was the first member of the IRAK family to be discovered and was initially shown to have a role in IL-1 signalling [170]. It is a protein of 712 aa in length giving it a molecular mass of ~85kDa (see Figure 1.6). Human IRAK-1 is ubiquitously expressed while interestingly murine IRAK-1 has a more restricted expression being primarily expressed in liver, kidneys and testis [170, 171]. Human IRAK-1 has three splice variants [172]. Since the TLRs share the TIR domain with the IL-1 receptor it was hypothesized that IRAK-1 might also participate in TLR-mediated signalling and many studies have now shown that various TLR pathways utilise IRAK-1. Many roles for IRAK-1 have been proposed including roles in NFκB activation, IRF activation and STAT3 activation [167, 173, 174].

1.5.1.1 IRAK-1 post-translational modification during IL-1/TLR signal transduction

Upon ligand binding to IL-1R/TLRs, MyD88 is rapidly recruited to the receptor via interaction of its TIR domain [84]. IRAK-1 interacts with MyD88 through DD interactions (see Figure 1.6). Thr66 in the DD has been shown to be vital for the formation of homo-dimers of IRAK-1 but mutation of this residue did not prevent

IRAK-1 interaction with IRAK-2 or IRAK-M [175]. Mutation of this residue prevented activation of NFκB [175].

IRAK-1 has also been shown to undergo phosphorylation upon TLR stimulation and some residues on IRAK-1 that have been suggested as phosphorylation targets by IRAK-4 (see Figure 1.6). The phosphorylation of IRAK-1 occurs in a number of steps: It has been shown, *in vitro*, that IRAK-1 is initially phosphorylated at Thr 209 [159]. This is a critical residue in IRAK-1 as mutation of this residue completely disrupts its kinase activity [159]. Phosphorylation of Thr 209 results in a conformational change in the kinase domain of IRAK-1 allowing subsequent phosphorylations to then take place including phosphorylation of Thr 387, a critical residue in the activation loop in the kinase domain of IRAK-1 (see Figure 1.6) [159]. This residue has also been suggested to be a potential target for phosphorylation by IRAK-4. The final step of these sequential phosphorylations occurs in the ProST region of the protein (also previously referred to as the undetermined domain), which is subject to hyper-autophosphosphorylation. MyD88 only binds non-phosphorylated IRAK-1 [176], thus upon phosphorylation, IRAK-1 is released from the receptor complex and may bind to TRAF6, ultimately leading to NFκB activation. However, if and how IRAK-1 "activates" TRAF6 is still unclear. IRAK-1 is subject to other modifications besides phosphorylation. It also undergoes ubiquitination and sumoylation [173]. After phosphorylation and activation of IRAK-1, it has been reported that IRAK-1 undergoes K48-linked polyubiquitination leading to its rapid degradation [176]. Until recently it was assumed that IRAK-1 was only targeted by K48 ubiquitination, however studies have now shown that IRAK-1 undergoes K63-linked

polyubiquitination [173, 177, 178]. K63-linked polyubiquitination of a protein is normally required for signal transduction rather than degradation and upon its ubiquitination IRAK-1 can interact with NEMO [178]. Mutation of the ubiquitination sites on IRAK-1 prevents NEMO binding and subsequent IL-1R/TLR-induced NFκB activation. Thus, IRAK-1 is ubiquitinated, although what protein ubiquitinates it is still uncertain. Separate groups have proposed TRAF6 and the Pellino proteins as the E3 ligase for IRAK-1 ubiquitination [173, 177, 178]. The Pellino proteins are a family of E3 ubiquitin ligases that play an important role in IL-1R/TLR signalling (see section 1.5.2). One report has shown that IRAK-1 undergoes ubiquitin editing and, thus, is subjected to both K63- and K48-linked ubiquitination [179].

1.5.1.2 NFκB activation and IRAK-1 kinase activity

IRAK-1-deficient mice were used to examine the role of IRAK-1 in IL-1R/TLR-induced activation of NFκB and MAPK pathways [180-182] (see Table 1.1). IRAK-1 $^{-/-}$ macrophages showed a partial decrease in LPS-induced IKKβ activation and IL-1- and LPS-induced NFκB DNA binding were also affected [180, 181]. Further, IL-1-induced p38 and JNK activation were shown to be reduced in IRAK-1 $^{-/-}$ mouse primary embryonic fibroblasts (EF) cells [182]. Interestingly at low concentrations of IL-1, IκB degradation was affected in IRAK-1 $^{-/-}$ fibroblasts, however at higher concentrations IκB was completely degraded in wild type and IRAK-1 $^{-/-}$ cells [182]. Furthermore, an effect of IL-1-and LPS-induced cytokine production was only significant at lower concentrations of stimuli in IRAK-1-deficient cells. Therefore deletion of IRAK-1

attenuates, but does not eliminate, IL-1R/TLR-mediated NFκB, MAPK and gene induction [180-182].

One study of particular interest showed that IRAK-1 was completely dispensable for TLR7/9 mediated NFκB and MAPK cytokine production in pDCs [167]. However this study revealed a novel role for IRAK-1 in IRF activation which will be discussed later (see section 1.5.1.3).

Despite the fact that IRAK-1 is a kinase, the function and relevance of its kinase activity in the activation of NFκB by IL-1R/TLRs is still uncertain. It has been shown that the kinase activity of IRAK-1 is not essential for its function to activate NFκB, as a kinase inactive mutant of IRAK-1 can still activate NFκB [183, 184]. In contrast to this, IRAK-1 has been shown to phosphorylate the pellino proteins upon over-expression in cells while *in vitro* studies have shown that IRAK-1 can directly phosphorylate the pellinos [173, 177, 178].

Overall, whether the kinase activity of IRAK-1 is directly required for NFκB activation is still uncertain and the generation of a knock-in mouse expressing catalytically inactive IRAK-1 will be required to resolve this issue. Given that IRAK-1 is an active kinase and its kinase activity may not be essential for TLR-induced NFκB, IRAK-1 may play other roles in innate signalling where its kinase activity is critical.

1.5.1.3 IRAK-1 and IRF activation

A role for IRAK-1 in IRF activation has emerged in recent years. A study by Uematsu *et al.,* mentioned earlier, showed that TLR7- or TLR9-induced NFκB and MAPK activation was normal in IRAK-1 $^{-/-}$ pDCs [167]. Strikingly, TLR7 or TLR9 induction of

IFNα was completely abolished in these mice. Interestingly, in this study IRAK-1 was shown to directly phosphorylate IRF7 *in vitro* and the kinase activity of IRAK-1 was shown to be critical for IRF7 transcriptional activation. Thus, this study was the first indication that IRAK-1 may have a more essential role in TLR-induced IRF activation than NFκB in certain contexts [167]. In contrast to this, it has also been proposed that IRAK-1 is a negative regulator of the IFN pathway and it is regulated through the phosphatase SHP-1 [185].

IRAK-1 was subsequently shown to be important for TLR7- and TLR8-induced IRF5 activation in human cells [69, 71]. Convincingly, the kinase activity of IRAK-1 was required for ubiquitination of IRF5, as using a kinase inactive mutant of IRAK-1 failed to induce the formation of polyubiquitinated IRF5 [71]. It is thought that the explanation for this is that IRAK-1 regulates the TRAF6-mediated ubiquitination of IRF5, thus showing a novel requirement for IRAK-1 in the antiviral pathway [71]. IRAK-1 and IRF5 have also been implicated in SLE pathogenesis. A SNP in IRF5 has been revealed as a risk factor for SLE and more recently it has also been shown that IRAK-1 has a crucial role in the development of this disease [186, 187]. IRAK-1 has also been shown to interact with TRAF3 [76], which is a key player in TLR-induced type I IFN production, thus highlighting further the role of IRAK-1 in the IRF axis of TLR signalling.

1.5.1.4. IRAK-1 and STAT activation

IRAK-1 has been shown to a have a novel role in STAT3-dependent IL-10 expression by TLR4 [174]. Surprisingly, IRAK-1 $^{-/-}$ mice that were stimulated with LPS in this study were shown to induce multiple NFκB-dependent pro-inflammatory genes to a similar extent as wild-type mice. However, the IRAK-1 $^{-/-}$ mice failed to induce any IL-10 message in comparison to wild-type mice and LPS-induced IL-10 production was severely impaired in IRAK-1 $^{-/-}$ splenocytes [174]. LPS-mediated IL-10 gene expression has been shown to be dependent on STAT3. Phosphoryation of STAT3 on a crucial serine residue was not observed in IRAK-1 $^{-/-}$ splenocytes. Further, it was demonstrated that IRAK-1 and STAT3 co-localise in the nucleus after stimulation. In addition, IRAK-1 interacts with the endogenous IL-10 promoter. A role for IRAK-1 has also been revealed in STAT1 activation since IL-1-mediated phosphorylation of STAT1 requires IRAK-1 [188]. Furthermore IRAK-1 and STAT1 have been shown to interact *in vivo* in the human glioblastoma cells T98G [188].

1.5.2 Pellino proteins

Pellino proteins are IRAK-interacting proteins that possess RING-finger-like domains. They are known to possess E3 ligase activity. There are four members; Pellino 1, 2, 3a and 3b. Pellinos are now known to have an important role in NFκB signalling [189]. Pellino 3b is a splice variant which has been reported to have an inhibitory effect on NFκB activation. It has been shown to regulate IRAK-1 degradation through its E3 ligase activity [190]. It has been reported that there is bi-directional signalling between the IRAK and Pellino family [177, 191]. The Pellino proteins polyubiquitinate

IRAK-1 and there is reciprocal polyubiquitination by IRAK-1 and IRAK-4 of the Pellino proteins, thus leading to the degradation of the pellino proteins [177]. IRAK-1 and IRAK-4 were also shown to phosphorylate the Pellinos and Pellino E3 ligase activity was greatly enhanced after this phosphorylation. IRAK-2 was shown to interact with the Pellino proteins but failed to degrade the Pellinos [177].

Recently it has been proposed that for TLR3 and TLR4 signalling, IKKε and TBK-1 were shown to phosphorylate Pellino 1 in macrophages to increase the E3 ligase activity of Pellino 1. Interestingly, the phosphorylation of Pellino 1 was dependent on the interaction with IKKε and TBK-1 rather than IRAK-1 or IRAK-4 [192]. Further evidence for a role of Pellino 1 in the TLR3 pathway has been described through the use of Pellino1 $^{-/-}$ mice. Pellino 1 was shown to be a critical E3 ligase in regulating RIP-1 ubiquitination in the TRIF-dependent pathway [193].

1.5.3 IRAK-2

In 1997, a protein of 590 amino acids which shared sequence and functional similarity to IRAK-1 was discovered and this molecule was named as IRAK-2 (Figure 1.6) [161]. Initial reports showed that IRAK-2 activated NFκB when over-expressed. This required the IRAK-2 DD since a truncated IRAK-2 (97-590) which lacks the DD failed to activate NFκB and moreover, acted in a dominant negative manner [161]. Further evidence that IRAK-2 played a role in the TLR pathway emerged when it was shown that IRAK-2 interacted with adaptor molecules MyD88 and Mal and also with TRAF6 [85, 161].

1.5.3.1 Role of human IRAK-2 revealed by viral targeting

For 10 years after its discovery the exact function of IRAK-2 remained unclear. IRAK-2 was presumed to have a redundant role with IRAK-1 in the activation of NFκB. However the importance of IRAK-2 in TLR-induced NFκB activation was discovered through studies with VACV. VACV is a member of the poxviridae family, which are large dsDNA viruses and the proteins they express have diverse ways of evading and subverting the innate immune system [194]. A52 is one such protein shown to be important for virus virulence [194]. It was found that A52 interacts with IRAK-2 and TRAF6, but not IRAK-1. Through the use of an A52 truncated protein which could still interact with IRAK-2 and not TRAF6, it was shown that A52 could inhibit all IL-1R/TLR pathways to NFκB solely through its interaction with IRAK-2 [195]. As the virus was specifically targeting IRAK-2 and not IRAK-1 to inhibit TLR-mediated NFκB signalling, this suggested a predominant role for IRAK-2 in NFκB activation.

Indeed, the significance of IRAK-2 in TLR-mediated NFκB activation is now recognised. Knockdown of human IRAK-2 expression by siRNA impaired NFκB activation by TLR3, TLR4 and TLR8 [106]. Of note, knockdown of IRAK-2 in human PBMCs impaired LPS-induced IL-8 production [106]. The role for IRAK-2 in TLR3 signalling is particularly intriguing as no other IRAK family member has been shown to play a role in this pathway [121, 196]. In support of this role for IRAK-2, it has been shown that endogenous IRAK-2 is recruited to the TLR3 receptor [106].

Mechanistically how IRAK-2 functions is also still uncertain. It may function proximal to the receptor as is the case with TLR3. However it has been reported that IRAK-2

can stimulate the formation of polyubiquitin chains associated with TRAF6 (see Figure 1.9) [106]. It had always been assumed that IRAK-1 was the IRAK family member that triggered polyubiquitination of TRAF6. Strikingly, overexpression of IRAK-2 induced polyubiquitination of TRAF6, while overexpression of IRAK-1 did not [106]. This was shown to be independent of IRAK-1 as exogenous IRAK-2 could induce TRAF6 ubiquitination in cells deficient in IRAK-1 [106].

1.5.3.2 Function of murine IRAK-2 revealed by knockout mouse studies

While the importance of IRAK-2 was confirmed in multiple TLR pathways to NFκB in human cell lines, the relative importance of IRAK-2 in the murine system was still uncertain until very recently. In 2008, IRAK-2 knockout mice were generated [197]. These mice were found to be highly resistant to LPS- and CpG-induced septic shock (see Table 1.1). Since previously it had been shown that the difference in mortality between wild type and IRAK-1 knockout mice was only subtle, this indicated a more critical role for IRAK-2 compared to IRAK-1 [180]. IRAK-2 was also shown to be critical for cytokine and chemokine induction by a number of TLRs [197].

The role of IRAK-2 in TLR2 signalling to NFκB and MAPKs was examined using macrophages from IRAK-2 KO mice. This revealed that IRAK-2 functions redundantly with IRAK-1 in early signalling but is important for late and sustained NFκB and MAPK activation [197]. Similar results were shown for TLR7 and TLR9 signalling to NFκB by a separate group using independently generated IRAK-2 $^{-/-}$ mice [198, 199]. Only when

IRAK-2 $^{-/-}$ mice were crossed with IRAK-1 $^{-/-}$ mice was a dramatic impairment of NFκB activation by TLR2 observed.

A negative role for IRAK-2 in TLR9-induced IFN responses in murine pDCs has also recently been reported, revealing a dual function for IRAK-2 in TLR9 responses, being a negative regulator of CpG-induced IFN responses but being required for TLR9-induced proinflammatory cytokines [199]. (Table 1.2)

1.5.3.3 Differential expression of murine IRAK-2 splice variants in inbred compared to wild derived mice

Mouse and human IRAK-2 molecules show 67% sequence identity [200]. They share the same domain structure and are highly conserved in their DDs and kinase domains. Their C-terminal domain differs slightly as the C-terminal domain of mu-IRAK-2 is 35 amino acids longer than human IRAK-2. Interestingly, one study has reported that over-expression of mu-IRAK-2 cannot activate NFκB [200]. Another distinction between human IRAK-2 and murine IRAK-2 is that while no evidence of splice variants exist for human IRAK-2, four splice variants are found in the murine form (IRAK-2a,- 2b, -2c and -2d) [201]. Since over-expression of IRAK-2a and IRAK-2b activated NFκB whereas Irak-2c and IRAK-2d inhibited NFκB activation, IRAK-2c and IRAK-2d have been proposed as having a negative role in TLR signalling [201]. Consistent with this, IRAK-2c completely lacks a DD which may prevent it from interacting with certain signalling molecules [200, 201].

Intriguingly, a recent study examining the innate immune response of wild derived mice versus classical inbred strains revealed differential expression of IRAK-2 splice variants and suggested a more critical role for IRAK-2 in wild-derived mice [202]. A classical inbred strain of mice, C57BL/6J, expressed high levels of the inhibitory isoform of IRAK-2, IRAK-2c. This isoform inhibits the pro-inflammatory isoform IRAK-2a. This may explain the dominant role of IRAK-1, rather than IRAK-2, in early signalling events. However, as time progresses the inhibitory isoform expression is decreased and IRAK-2a, which is no longer being inhibited, is able to function in a pro-inflammatory manner. Thus, this isoform (in inbred mice) is responsible for sustained NFκB activation [202]. Wild derived mice differ significantly from experimental models as the genetic diversity of wild derived mice has arisen in an evolutionary context. These mice undergo a higher degree of polymorphisms. In the wild-derived strain MOLF/Ei, a natural mutation occurs in the promoter of IRAK-2c. This results in this splice variant being expressed significantly less leading to less inhibition of IRAK-2a. Thus, when IRAK-2a expression was suppressed by siRNA, IRAK-2a was found to be indispensable for early activation of NFκB and p38 MAPK. Thus, the murine IRAK-2 from wild derived mice may behave in a similar manner to human IRAK-2, as the human IRAK-2 contains no splice variants and is shown to be important for TLR signalling to NFκB [202].

1.5.3.4 Kinase activity of IRAK-2

It had been historically established that IRAK-1 and IRAK-4 were the only active kinases from the IRAK family based on the fact that an aspartate residue in the IRAK kinase domain is an asparagine residue in IRAK-2 and a serine in IRAK-M. However IRAK-2, like all other family members, contains a functional ATP-binding pocket with an invariant lysine residue in the protein kinase sub-domain [162]. It has been recently proposed that this residue is sufficient for IRAK-2 to act as an active kinase [197]. A kinase assay showed that IRAK-2 is phosphorylated upon stimulation with a TLR2 ligand [197]. It is thought that IRAK-4 phosphorylates IRAK-2 and induces its kinase activity. In addition, a recent study has revealed that the kinase activity of IRAK-2 was shown to be required for optimal induction of TLR-induced cytokines and chemokines [203]. The kinase activity of IRAK-2 was also required for TLR2-, TLR7- and TLR9-induced late NFκB activation in murine cells [203]. Mutation of the invariant lysine residue (at K235 in mice) in murine cells revealed that the kinase activity of IRAK-2 was important for LPS-induced post-transcriptional regulation. IRAK-2 kinase- dead macrophages (KD) showed impaired LPS-induced KC and IL-6 mRNA stabilisation. It was also shown that this residue in IRAK-2 was critical for LPS-induced translation of cytokines and chemokines in murine cells [203].

1.5.3.6 IRAK-2 and apoptosis

A role for IRAK-2 in cellular apoptosis has also been shown. In a study by Ruckdeschel *et al.,* it was found that dominant-negative MyD88 or IRAK-2 diminished LPS-induced apoptosis [168]. The role for IRAK-2 in NFκB was also confirmed in this study as over-

expression of dominant-negative IRAK-2 impeded NFκB translocation into the nucleus and NFκB was predominantly detected in the cytoplasm of infected cells. In contrast to IRAK-2 and MyD88, TRAF6 and IRAK-1 failed to suppress LPS-enhanced apoptosis thus indicating a pro-apoptotic signalling pathway through MyD88 and IRAK-2 [168].

1.5.4 IRAK-M

The third family member to be discovered, IRAK-M, is a protein of 596 amino acids in length and has a molecular mass of 68 kDa [204]. While the other members of the IRAK family share a lot of similar features, IRAK-M is more unique. As mentioned previously, while expression of other human IRAK members are ubiquitous, expression of human IRAK-M is limited to monocytes and macrophages [204]. Murine IRAK-M, which shares a 71% sequence similarity with its human counterpart, has been shown to be expressed in many cell types occurring most predominantly in the liver and thymus [205]. Since the kinase activity of IRAK-2 is still controversial, IRAK-M is now the only member of the family to definitively lack kinase activity. Its most distinct feature is its function as a negative regulator of TLR signalling [137].

Initial reports on IRAK-M showed that upon over-expression it was able to activate NFκB [204]. Furthermore, when IRAK-M was expressed in cells lacking IRAK-1 it was able to restore NFκB activation. Thus, it was initially assumed that like IRAK-1 and IRAK-2, IRAK-M was a positive regulator of TLR-mediated NFκB signalling [204].

However IRAK-M $^{-/-}$ mice showed an increased inflammatory response (but not increased susceptibility) to the bacterium *Salmonella typhimurium* [137]. IRAK-M $^{-/-}$ mice were shown to have reduced survival upon influenza infection *in vivo* [206] Furthermore, macrophages derived from IRAK-M knockout mice displayed enhanced activation of IL-1R/TLR signalling, thus suggesting a negative role for this family member (see Table 1.1). It was initially proposed that IRAK-M may prevent IRAK-1 and/or IRAK-4 from dissociating from the MyD88 complex thus preventing TLR-mediated signalling to NFκB [137]. A novel role for IRAK-M recently has been shown in specifically negatively regulating TLR2-induced p38, but not JNK or ERK, activation [207]. The regulation of p38 by IRAK-M was found to be completely IRAK-1-independent but rather occured through IRAK-M-mediated stabilisation of the phosphatase MKP-1. MKP-1 had been previously shown to negatively regulate p38 phosphorylation [208]. Thus, in contrast to the proposal that IRAK-M may interact with and regulate IRAK-1 [137], this study suggests that IRAK-M does not contribute to IRAK-1 regulation and function.

IRAK-M was shown to negatively regulate the alternative NFκB pathway in a TLR2-specific manner [209]. The alternative or non-canonical pathway of NFκB relies on the activation of NFκB-inducing kinase (NIK) and the subsequent phosphorylation of p100 through an IKKα-dependent pathway. The alternative NFκB pathway is predominantly triggered by CD40, LT (lymphotoxin β receptor) and the BAFF receptor (B-cell activating factor belonging to the TNF family) [63]. However this study shows that upon stimulation with the TLR2 agonist Pam_3Cysk_4, elevated levels

of NIK protein and increased distribution of RelB were observed in IRAK-M $^{-/-}$ cells [209]. This hinted at the possible regulation of the alternative NFκB pathway by IRAK-M. It appeared that IRAK-M did not regulate the TLR-2-induced classical NFκB pathway as p65/RelA phosphorylation and nuclear translocation were unchanged in wild-type and IRAK-M cells [209]. Thus, it seems that the role for IRAK-M may be more vital in the alternative NFκB pathway, and for TLR2-induced p38 regulation, rather than in the classical NFκB pathway as originally thought.

1.5.5 IRAK-4

IRAK-4 is the most recent member of the IRAK family to be discovered [210]. It was discovered through a database search as a human cDNA sequence that encodes a polypeptide sharing significant but previously unrecognised sequence similarity with IRAK-1. It is the closest human homolog to the *Drosophila* pelle protein. Pelle, in the fruit fly, is involved in signalling downstream of the Toll-Dorsal pathway during embryonic development. The human IRAK-4 protein is 460 amino acids long and shares 87% similarity and 84% identity with murine IRAK-4 [210].

1.5.5.1 Role of IRAK-4 in TLR signalling

The essential role of IRAK-4 in TLR signalling was revealed through knockout (KO) mouse studies. In contrast to IRAK-1 KO mice, mice depleted of IRAK-4 were shown to be completely resistant to LPS-induced septic shock and lacked a cytokine response when challenged with various TLR ligands [211]. Furthermore, IL-1-induced

NFκB, JNK and p38 activation were all severely defective in cells lacking IRAK-4. LPS-induced JNK activation was also inhibited and LPS-induced NFκB activation was delayed [211]. These mice also failed to respond to lymphocytic choriomeningitis virus (LCMV). LCMV is dependent on IL-12 and IL-18, and IL-18 binds to a receptor that is homologous to IL-1R [211].

IRAK-4 plays a critical role proximal to the receptor and MyD88 and mediates NFκB activation through initially interacting with MyD88 [84]. It has recently been described that the DDs of IRAK-4 and MyD88 form large oligomeric structures termed the Myddosome (see section 1.4.1).

MyD88 acts as a scaffold protein for the interaction between IRAK-1 and IRAK-4 [105]. IRAK-4 is presumed to phosphorylate IRAK-1 which leads to the autophosphorylation and activation of IRAK-1 itself. It has been shown that IRAK-4 also induces the degradation of IRAK-1 and MAL [212, 213] and thus acts in a negative feedback manner to regulate the MyD88-dependent pathway.

IRAK-4 has also been shown to have an imperative role in type I IFN induction through TLR7/8/9 [121]. IRAK-4 was also shown to interact with IRAK-2 (75 and Sinead Keating & Andrew Bowie unpublished work) but unlike IRAK-2 appears to have no role in the TLR3 pathway. Overall, IRAK-4 is essential for MyD88-dependent pathways but is dispensable for MyD88-independent signalling pathways, such as that used by TLR3 via TRIF [121].

1.5.5.2 Differential requirements for IRAK-4 kinase activity in signalling

While it is apparent from knockout mouse studies that IRAK-4 has a vital role in IL-1R/TLR signalling, whether the kinase activity of IRAK-4 is essential for its function was unknown. Initial studies using IRAK-4-deficient murine embryonic fibroblasts (MEFs) reconstituted with an IRAK-4 kinase inactive mutant showed that the kinase activity of IRAK-4 is required for the optimal induction of IL-1-induced NFκB, JNK activation and proinflammatory cytokines [214]. With the generation of IRAK-4 kinase inactive knock-in mice it was shown that the kinase activity of IRAK-4 is required for its function [215, 216]. *In vivo,* these mice were highly resistant to TLR-induced shock [200, 201]. Macrophages from these mice revealed that the kinase activity of IRAK-4 was dispensable for the activation of IL-1R-, TLR2-, TLR4- and TLR7-induced NFκB [200, 201]. Interestingly, in one particular study it was observed that TLR2-induced NFκB DNA binding still occurred in IRAK-4 KO macrophages, indicating the existence of an IRAK-4-independent TLR2 pathway [200].

While IL-1R/TLR-induced NFκB was not greatly affected in IRAK-4 kinase inactive knock-in mice, there was a dramatic impairment of IL-1R/TLR-induced cytokine and chemokine production, thus revealing the role for the kinase activity of IRAK-4 in IL-1R/TLR-induced gene induction [215-218].

Furthermore, MAP kinase activation appears to have a greater dependency on IRAK-4 kinase activity than NFκB. IL-1R-, TLR2-, TLR4- and TLR7-induced JNK activation was shown to be strongly dependent on IRAK-4 kinase activity [200-203]. Whether the kinase activity of IRAK-4 is required for IL-1R/TLR-induced p38 activation is still controversial [200-203]. Two groups have shown that the kinase activity of IRAK-4 is

required for IL-1R/TLR-induced p38 activation in macrophages and fibroblasts [217, 218]. In contrast to this it has been shown that IRAK-4 kinase activity is not required for TLR4/TLR7-induced p38 activation [216].

While studies from kinase-inactive IRAK-4 knock-in mice have revealed that the kinase activity of IRAK-4 is required for certain functions in IL-1/TLR signalling, studies from human cells have shown that the kinase activity of IRAK-4 is generally redundant [219, 220]. Using human IRAK-4-deficient fibroblasts reconstituted with a kinase-inactive IRAK-4 mutant, it was shown that the kinase-inactive mutant was able to restore IL-1-induced NFκB, JNK activation and IL-8 gene expression to a similar extent as the wild-type IRAK-4 [204]. In addition, a recent study which depleted human endothelial cells of IRAK-4 using IRAK-4 siRNA and reconstituted the cells with a kinase-inactive IRAK-4 revealed that the kinase activity of IRAK-4 was not required for IL-1 induced IL-8 expression [205]. Furthermore in this study, with the use of selective small kinase inhibitors, it was revealed that the kinase activity of IRAK-4 was redundant for the activation of IL-1-induced p38, JNK and IL-6 expresion. Thus, in the human system it appears that the kinase activity of IRAK-4 is redundant with IRAK-1 [205].

1.5.5.3 IRAK-4 and human disease

IRAK-4 is one of the few IL-1R/TLR signalling proteins to be implicated in human disease to date. A cohort of patients who had recurrent infections and a poor inflammatory response were discovered to have an inherited IRAK-4 deficiency [221-

224]. These patients were susceptible to extracellular pyogenic bacteria such as *Streptococcus pneumoniae.* While IRAK4 $^{-/-}$ mice are sensitive to a wide range of microorganisms patients with an IRAK-4 deficiency suffered from a narrow spectrum of infections [221]. Furthermore, for these patients, infections began early in life but decreased with age, presumably with the help of the adaptive immune system [223]. In contrast, the susceptibility of IRAK-4$^{-/-}$ mice to infection does not decrease with age [208]. This suggests a rather specific role for IRAK-4 in terms of which human pathogens the human immune system requires it for.

Since IRAK-4 is required for all MyD88-dependent signalling pathways to NFκB and also for TLR7/8/9 signalling to IFNα, why IRAK-4-deficient patients were only susceptible to a narrow range of bacteria was unclear. However, it has now been revealed that IRAK-4 is critical for human TLR7/8/9 responses to viruses, but that it is largely dispensable for the induction of IFNα/β and IFNλ in response to TLR3 and TLR4. PBMCs that were deficient in IRAK-4 failed to respond to stimulation with TLR7/8 agonists and no IFNα, IFNβ or IFN λ mRNA or protein was detected in these cells [121]. In contrast, normal p38, JNK, IRF3 and NFκB responses to poly (I:C) and LPS were observed in IRAK-4-deficient PBMCs and fibroblasts. The TLR3- and TLR4-MyD88-independent pathway have been shown to be IRAK-4 independent [121]. This suggests that the MyD88-independent TRIF axis, together with the non-TLR PRRs, are sufficient for the recognition of most viruses by IRAK-4-deficient patients [121].

1.6 Post-Transcriptional Regulation

Although much of the research into IL-1R/TLR signalling focuses on transcriptional regulation, many short-lived inflammatory mRNAs induced by TLRs are regulated at the post-transcriptional level. *Cis*-elements are sequences within an mRNA that play a role in determining the basal half-life of a particular mRNA or whether the mRNA will be stabilised or destabilised in response to different stimuli [79]. Numerous *cis*-elements have now been identified and they are most often located in the 3'UTR (untranslated region).

The best characterised *cis*-elements to date are the AU-rich elements, referred to as the AREs. AREs are located in the 3'UTR of a variety of short lived mRNAs, including cytokines. AREs are divided into different classes depending on their sequence. Class I AREs contain one to three scattered AUUUA pentamer motifs and Class II AREs contain multiple overlapping AUUUA motifs. Class III are not as well-defined and lack an AUUUA motif. Both TNFα and the chemokine IL-8 belong to the Class II ARE group [225]. ARE-binding proteins (ARE-BP), such as tristetraprolin (TTP), HuR and KSRP (KH-splicing regulatory protein) can bind to these ARE regions and positively and negatively regulate mRNA decay [226-228]. The MAPK pathways mediate the regulation of these ARE-BPs [79]. Some of the ARE-BPs regulate both mRNA decay and the translational pathway [229].

1.6.1 Post transcriptional regulation of TNFα mRNA

TNFα is a pro-inflammatory cytokine that plays an important role in mediating chronic inflammatory arthritis including RA. One of the key regulatory mechanisms of TNFα mRNA stability is via TTP. TTP, (also known as Nup475, Tis11, Zfp36) belongs to a family of CCCH zinc finger AU-binding proteins [226]. TTP binds to a variety of mRNAs including GM-CSF, IL-2, IL-3 and TNFα [79]. TTP $^{-/-}$ mice develop an inflammatory syndrome with arthritis and auto-immunity mediated by TNFα. Absence of TTP results in increased stability of TNFα mRNA [226].

In unstimulated cells, TTP is expressed at low levels, is unstable and localised to the nucleus [230]. Upon stimulation of the p38 pathway with LPS, phosphorylation of p38 occurs, which in turn triggers the phosphorylation of MK2 (p38/MAPK-activated protein kinase 2). This leads to the stabilisation of TNFα mRNA, which ultimately results in TNFα protein production. Simultaneously to this, TTP is also induced (which destabilises TNFα mRNA). TTP is phosphorylated by MK-2. 14-3-3 proteins are recruited to phosphorylated TTP. The 14-3-3 proteins localise TTP to the cytoplasm and prevents TTP from undergoing dephosphorylation by protein phosphatase 2α (PP2α) [231]. By an unknown mechanism, PP2α dephosphorylates TTP, leads to dissociation of 14-3-3 proteins. TTP then binds to TNFα mRNA leading to TNF mRNA decay. (see Figure 1.7). TTP is known to bind to TNFα mRNA via a specific nonamer sequence in its 3'UTR. (UUAUUUA(A/U)(A/U)) and then delivers TNFα mRNA to the exosome to be degraded [232].

TNFα mRNA is not solely regulated by TTP. Many other ARE BPs can bind to TNFα mRNA including HuR (which inhibits decay of TNF mRNA), TIA and FXRP-1 (which

regulate translation of TNFα mRNA) [227, 233, 234]. TNFα mRNA can also be regulated via the CDE (constitutive decay element). The CDE is located downstream of the ARE in the 3'UTR of TNF transcripts and is an extra regulatory mechanism of TNFα mRNA [235].

1.6.2 Post transcriptional regulation of IL-8 mRNA

IL-8 is a chemokine that is normally induced by a diverse range of cells in response to stresses such as inflammation, infection and injury. A key response by IL-8 is to recruit neutrophils and endothelial cells to initiate a reparative process. Similar to TNFα, IL-8 is also regulated by transcriptional and post-transcriptional events [236]. Post-transcriptional regulation of IL-8 is mediated via the 3'UTR of the transcript. The IL-8 3'UTR is lengthy, (over 1100nt) and contains a number of AREs [237]. The p38 pathway has been shown to contribute to the regulation of IL-8 expression via the AU-binding protein KSRP. This protein has been shown to bind to the IL-8 3'UTR directly *in vitro* [228].

1.6.3 IRAKs and post-transcriptional Regulation

A common theme for the three positive IRAK family members, IRAK-1, IRAK-2 and IRAK-4 is that they have all been shown to have a role in post-transcriptional regulation [198, 216, 238]. IRAK-1 has been shown to be essential for IL-1-induced stabilisation of the mRNAs of two chemokines MIP2 and KC (the murine equivalent of IL-8), via a signalling pathway that did not require TRAF6 [238]. IRAK-2 has been shown to have a role in LPS-induced post transcriptional and translational regulation

of cytokine and chemokine expression [198]. While it was shown using IRAK-2 $^{-/-}$ macrophages, that IRAK-2 has no role in TLR4-induced early or sustained NFκB, TLR4 induced cytokines and chemokine production was greatly affected [198]. TLR4-induced stability of IL-6 and KC mRNA was shown to be regulated by IRAK-2. It was found that the role of IRAK-2 in mRNA stability was ligand-specific as there was no role for IRAK-2 in TLR7-induced post-transcriptional control [198]. In addition to its role in post-transcriptional regulation, IRAK-2 was also shown to regulate LPS-induced TNFα gene translation. A contrasting report with IRAK-2 KO mice showed no effect of IRAK-2 deficiency on mRNA stability of TLR2-induced cytokines and chemokines tested thus suggesting a specific role for IRAK-2 in TLR4-mediated post-transcriptional regulation [197].

It was also reported that IRAK-4 regulates mRNA stability of various cytokines [216]. As with IRAK-2, while the absence of IRAK-4 did not significantly reduce IL-1R/TLR-induced NFκB activation, it did have a significant effect on IL-1-, TLR4- and TLR7-mediated induction of cytokines and chemokines. In addition, the kinase activity of IRAK-4 has been shown to be imperative for IL-1R/TLR-induced mRNA stability [216]. IRAKs themselves are regulated by post-transcriptional mechanisms such as microRNAs (miRNAs). miRNAs are small non-coding RNAs that suppress gene expression through binding the 3'UTR of target mRNAs. mir146a has been shown to regulate IRAK-1 and IRAK-2 [239]

1.7 Ubiquitination

Ubiquitination is a post-translational modification. It is the covalent attachment of the 76 amino acid peptide ubiquitin to a protein substrate and it has an important regulatory role in the innate immune response [240]. It was originally discovered as a non-lysosomal pathway of protein degradation but now it is clear that the ubiquitination of proteins has a wide impact on a variety of cellular processes, including modification of protein function, facilitation of cell surface receptor turnover and control of gene transcription [241]. Unlike phosphorylation of proteins which is defined as the presence of a single phosphate group on selected amino acids of target proteins, ubiquitination is a complex regulated process involving the attachment of ubiquitin chains of different lengths and conformations primarily on lysine residues.

There is a three step reaction that is involved, which results in the formation of ubiquitin conjugates [242].

1. Ubiquitin Activation (with E1 ubiquitin activating enzyme)
2. Ubiquitin Transfer (E2 ubiquitin carrier protein or conjugating enzyme)
3. Ubiquitin Ligation (E3 ubiquitin protein ligase enzyme)

Firstly the C-terminal glycine residue of ubiquitin is activated by the E1 activating enzyme. This results in the formation of a high energy thioester bond between the glycine residue of ubiquitin and the cysteine residue of the active site of the E1 [226]. At present there are two known E1 enzymes and they activate ubiquitin for all

ubiquitination events [243]. The active ubiquitin is then transferred to the cysteine residue of the active site of the E2 ubiquitin-conjugating enzyme. Approximately 38 E2 enzymes are predicted to exist [244]. The E2 enzymes play a major role in determining the length and linkage type of ubiquitin chains that are formed [227]. Ubiquitin-protein ligases (known as E3 ligases) recruit both the ubiquitin-E2 complex and the target protein and they facilitate transfer of the ubiquitin to the substrate (see Figure 1.8). The first ubiquitin is generally attached via an isopeptide bond between its C-terminal glycine residue and the ε-NH_2 group of an internal lysine from the substrate [226]. There are greater than 600 predicted E3 ligases and they regulate substrate specificity. There are two well-characterised types of E3 ligase; a RING (really interesting new gene)-domain-containing and HECT (homologus to E6-associated protein carboxyl terminus)-domain containing protein. The RING E3 ligases do not seem to possess catalytic activity and thus act as a bridge between the E2-Ub and the substrate. The HECT E3 ligases have essential catalytic activity and thus can form a covalent thioester bond intermediate which allows the HECT E3 ligase to transfer the ubiquitin from the E2-Ub to the target protein [243].

The polyubiquitin chain is then synthesized by the successive conjugation of ubiquitin molecules to an internal lysine of the previously conjugated ubiquitin. Polyubiquitin chains of different topologies direct distinct outcomes for target proteins [245]. Currently there are seven types of ubiquitin chains that can be formed to substrate proteins.

1.7.1 Ubiquitin chains

Firstly, if a target protein is tagged with a single ubiquitin molecule, this is referred to as monoubiquitination. Monoubiquitination has been shown to have a role in regulating DNA repair, receptor endocytosis, vesicle sorting and gene sorting [246].

If the ubiquitin chain is elongated, this results in polyubiquitination of the target protein. The two best-characterised ubiquitin chains that are important for TLR signalling are Lys48- and Lys63-linked polyubiquitin chains (See Figure 1.8). Polyubiquitin chains linked through lysine 48 result in the protein being targeted for degradation via the 26S proteasome [247]. Lys-48 linked ubiquitin tetramers are the minimal recognition motif for the proteasome [248]. Polyubiquitin chains linked through lysine 63 results in the proteasome-independent function of ubiquitin i.e. these chains are non-degradative and are required for kinase activation, DNA repair, trafficking and translation [249]. TRAF6 is a well known E3 ligase that forms Lys63-linked polyubiquitin chains required for TLR signalling [245]. Lys63 chains are said to adopt an open conformation [248].

Other types of ubiquitin chain linkages are now known to exist and have distinct functions. Ubiquitin chains can also be formed through lysine 33 and are said to have a role in TCR (T Cell Receptor) signalling [250], while recently two studies on K27-linked ubiquitin chains on NEMO have been reported [251, 252] . Upon viral infection, the E3 ligase TRIM23 has been shown to attach K27-linked chains on NEMO and this is required for IFNβ production [235]. A second study has shown that *Shigella* infection can induce the formation of K27-linked chains on NEMO which

leads to NEMO degradation and ultimately suppression of NFκB activation [236]. Thus these two studies show K27-linked chains on NEMO resulting in two different outcomes.

A new type of ubiquitin chain formation has recently been reported. Linear ubiquitin chains differ from all the other types of ubiquitin chains that are formed in that the ubiquitin chains are not formed on a lysine residue, but rather via the N-terminal amino group of ubiquitin. A peptide bond is formed between the N-terminal methionine (M1) of one ubiquitin and the C-terminal glycine (G76) of the next. Linear ubiquitin chains are assembled by an E3 complex (containing 2 E3 ligases HOIL-1 (longer isoform of hem-oxidised iron-regulatory protein 2 ubiquitin ligase-1) and HOIP (HOIL-1L interacting protein)) called LUBAC (linear ubiquitin chain assembly complex) [253, 254]. Both NEMO and RIP-1 are known to undergo linear ubiquitination [255]. Recently, the crystal structure of the UBAN (ubiquitin binding domain in ABIN and NEMO proteins) region of NEMO was solved and it was revealed that NEMO binds significantly stronger to linear ubiquitin chains compared to Lys63-linked chains. The linear ubiquitination of NEMO is thought to play a crucial role in TNFα-induced IKK complex activation and NFκB activation but not in MAPK activation Recently, a third component of LUBAC has been reported called SHARPIN (SHANK-associated RH domain-interacting protein) [254]. SHARPIN has been implicated in interfering with TNF-induced cell death and therefore prevents inflammation. Thus linear ubiquitin may be required in regulation of inflammation in immune signalling [254]. Other types of ubiquitin chains that exist are forked/branched ubiquitin chains,

which as yet have an unknown function although they are resistant to proteasomal degradation [256].

The final type of ubiquitin chains identified to date are unanchored polyubiquitin chains [257]. These are free chains that are not covalently conjugated to any substrate. These chains have recently been shown to be the physiologically relevant polyubiquitin chains important for TLR signalling [257]. A recent study by Xia *et al.* has shown that free polyubiquitin chains are required to activate TAK-1. It was shown that free K63-ubiquitin chains bind TAB-2 and this binding leads to autophosphorylation and activation of TAK-1. It was revealed that it was free chains that were central to this process through the use of isopeptidase T, a deubiquitinating enzyme that specifically cleaves unanchored chains [257]. This is the first evidence that free polyubiquitin chains can directly activate protein kinases. In addition, unanchored polyubiquitin chains were shown to activate the IKK complex by binding to NEMO.

Interestingly, free polyubiquitin chains have also been shown to be required for complete RIG-I activation [20]. RIG-I, which is known to be a sensor for 5'ppp from viral genomes, was recently reported to bind two ligands [20]. First RIG-I binds viral RNA via its CTD and then sequentially binds K63-polyubiquitin chains via its CARD domain [20]. The binding of these chains were essential for IRF3 phosphorylation by RIG-I. While the activation of TAK-1 and IKK required very long free polyubiquitin chains, RIG-I could be activated by short unanchored chains that consisted of only 3

or 4 ubiquitin molecules. Thus free polyubiquitin chains are not only required for NFκB but also for IRF activation.

There is also a large variety of different ubiquitin-binding domains (UBD) and the function of the polyubiquitin chains is largely dependent on the recognition of these binding domains [258].

1.7.2 Deubiquitinases (DUBs)

It is clearly demonstrated above that ubiquitination is a key post-translational modification of the activation of signalling cascades. Thus, the negative feedback regulation of these proteins relies in part on DUB enzymes. DUBs are negative regulators of immune signalling and there over 100 DUBs that are known to exist. CYLD (The tumour suppressor cylindromatosis protein) and A20 are two DUBs that have important roles in regulating NFκB activity. A20 is an IKK complex inhibitor. It is a cytoplasmic protein that is induced by NFκB [259]. A20 has a dual function as a DUB and as a K48 E3 ligase. RIP-1 and IRAK-1 undergo ubiquitin editing by A20. Initially, these proteins undergo K63-linked polyubiquitination but at later times K48-linked ubiquitination targets them for degradation. A20 also removes Lys63-linked polyubiquitin chains from TRAF6. CYLD binds directly to NEMO promoting is deubiquitination. CYLD is known to regulate RIG-I and inhibits the polyubiquitination of TBK-1/IKKε [260, 261].

1.8 TRAF6

The TRAF family of adaptor proteins are important in many inflammatory and immune signal transduction pathways. Many receptors utilise the TRAF family members including TNFR, IL-1R and TLRs. There are 7 members of the TRAF family. With the exception of TRAF1 the other members of the TRAF family all contain an N-terminal RING domain followed by several zinc finger domains [262]. They also have a C-terminal TRAF domain which is made up of a coiled-coil domain followed by a conserved receptor-interacting domain. The RING finger domain is associated with E3 ubiquitin ligase activity. The RING finger domain has been shown to be essential for TRAF6 to activate NFκB and MAPK signalling pathways [263]. The TRAF domain is critical for its self-association and its association with receptors or signalling proteins. All mammalian TRAFs are located in the cytoplasm with the exception of TRAF4 which is found in the nucleus. TRAF3 is located in the endosome. Of all the TRAF family members, TRAF2 and TRAF6 have been the most extensively studied although TRAF3 is now emerging as a key protein in pattern recognition signalling [76]. While TRAF2 is important for the TNF signalling pathway, TRAF6 has been shown to be a vital signalling molecule for the activation of NFκB and JNK in the IL-1 and TLR pathways. Recently, TRAF3 has been identified as the critical TRAF in the activation of IRF3 and the induction of type I IFNs but not in canonical NFκB activation [76].

1.8.1 TRAF6 Signalling

The majority of, and potentially all IL-1R and TLR signalling pathways to NFκB converge at TRAF6 irrespective of the TIR adaptor used. Upon stimulation of a signalling pathway, TRAF6 associates with IRAK-2 and/or IRAK-1. This association allows this oligomerisation of TRAF6. TRAF6 oligomerisation activates its ligase activity and certain molecules such as TIFA (TRAF-interacting protein with a forkhead-associated domain) are known to induce the oligomerisation [264]. Oligomerisation of TRAF6 allows it to associate with a heterodimeric E2 enzyme signalling complex termed TRAF6-regulated IKK activators (TRIKA) [245]. TRIKA1 is composed of the E2 enzyme Ubc13 (ubiquitin-conjugating enzyme 13) and the Ubc-like protein Uev1a (ubiquitin E2 variant 1a) [265]. This complex was suggested to bind the RING finger domain of TRAF6 and together TRAF6 and TRIKA catalyse the synthesis of Lys63-linked polyubiquitin chains on target proteins in the TLR pathway and also on TRAF6 itself.

The role of Ubc13 has been tested with conditional knockouts of Ubc13 in mice [266]. Ubc13 knockout mice were found to be embryonic lethal. While it was assumed that the conditional knockout of Ubc13 may have a drastic effect on TLR signalling, it was discovered that NFκB activation by many IL-1R/TLR agonists in different cell types was normal [266]. MAP kinase activation was defective and this seemed to be due to the loss of NEMO ubiquitination as NEMO ubiquitination was shown to be necessary for the activation of JNK and p38 [266]. Given that IL-1R/TLR signalling and TRAF6 polyubiquitination occurred as normal in the absence of Ubc13, it is proposed that

TRAF6 E3 ligase activity can be coupled to other E2 enzymes other than Ubc13 to trigger its autoubiquitination [266]. Another study has shown that in cells from mice heterozygous for the Ubc13 gene following LPS stimulation TRAF6 polyubiquitination was prevented in the absence of Ubc13. It has been suggested that other E2 ubiquitin conjugating enzymes such as UbcH7 and Ubc5 are also likely to be involved in TRAF6 signalling events.

Polyubiquitinated TRAF6 then recruits TRIKA2 which is made up of a trimeric complex of TAK-1 (TGF-β-activated kinase), and TAB1 (TAK Binding Protein) and either TAB2 or TAB3. TAK-1 requires its binding proteins TAB1, TAB2/TAB3 for its activation [267]. TAB1 contains an N-terminal pseudophosphatase domain, which is dispensable for TAK-1 activation and a C-terminal domain that binds to and activates TAK-1 [268]. TAB2 and TAB3 contain a novel conserved zinc finger domain which allows it to recognise Lys63 polyubiquitin chains [245]. This allows the recruitment of TRIKA2 to TRAF6. TAB2 and TAB3 also have an essential role in turning on TAK-1 kinase [245]. It has recently been shown that it is unanchored K63-linked polyubiquitin chains that activate TAK-1 via TAB2. TRAF6 is also ubiquitinated itself although its ubiquitination is not required for its recruitment or interaction of the TAK-1 signalling complex. Furthermore, this autoubiquitination was not essential for NFκB activation [263]. When TAK-1 is activated, this allows the subsequent phosphorylation and activation of IKKβ, MKK3-6 and MKK4-7 which leads to the activation of NFκB and AP-1. As mentioned previously, TRAF6-dependent Lys63 polyubiquitination of other signalling molecules such as IRAK-1, NEMO and also RIP1 is required for NFκB activation [263] (see Figure 1.9).

1.9 Aims

The IRAK family are critical mediators of TLR signalling. It was always assumed that IRAK-1 played a central role in TLR signalling, while the role for IRAK-2 was thought to be redundant. Previous work has shown that human IRAK-2 is required for TLR3 and TLR4 signalling to NFκB. It was hypothesised that IRAK-2 may be important for TLR8 signalling as IRAK-1 was shown to be dispensable for TLR7 and TLR9 signalling. Over-expression of human IRAK-2 could trigger the formation of polyubiquitin chains associated with TRAF6 while expression of IRAK-1 failed to do so. Studies from IRAK-2 $^{-/-}$ mice revealed that murine IRAK-2 was not required in early NFκB nor p38 MAPK activation for multiple TLRs. Therefore, the role of IRAK-2 in the human system was not clear, thus the overall aim was to investigate the relative role of IRAK-2 in TLR signalling in the human system and examine the requirement for IRAK-2 in the formation of polyubiquitin chains associated with TRAF6.

The specific aims were as follows:

- To examine the role of IRAK-2 in TLR8-induced NFκB activation in transformed human cells using IRAK-2 siRNA. IRAK-2 gene silencing will also be used to examine what effect targeted knockdown of IRAK-2 has on TLR4 and TLR8 cytokine induction. IRAK-2 $^{-/-}$ MEFs will be used to examine what effect the absence of murine IRAK-2 has on TLR/IL-1-induced cytokine induction.
- The role of IRAK-2 in TLR4 and TLR8 cytokine induction in primary human PBMCs will be examined. Using siRNA gene silencing, the role of IRAK-2 in early signalling

events in primary human cells will be explored. The role for IRAK-2 in post-transcriptional events in primary human cells will also be investigated.

- Mechanistically, the role of IRAK-2 in TRAF6 function will also be examined. The relative contribution of the E3 ligase activity of TRAF6 will be inspected. A ubiquitination assay will be used to examine the nature of the polyubiquitin chains associated with TRAF6 induced by IRAK-2. Finally, the importance of certain residues in IRAK-2 will be examined with regard to their contribution to TRAF6-associated ubiquitination and NFκB activation.

Thus, this study will demonstrate the overall contribution of IRAK-2 to transcriptional and post-transcriptional TLR responses in primary human cells. In addition, this study will reveal the role of IRAK-2 in TRAF6 function; the seminal E3 ligase critical for all TLR pathways to NFκB.

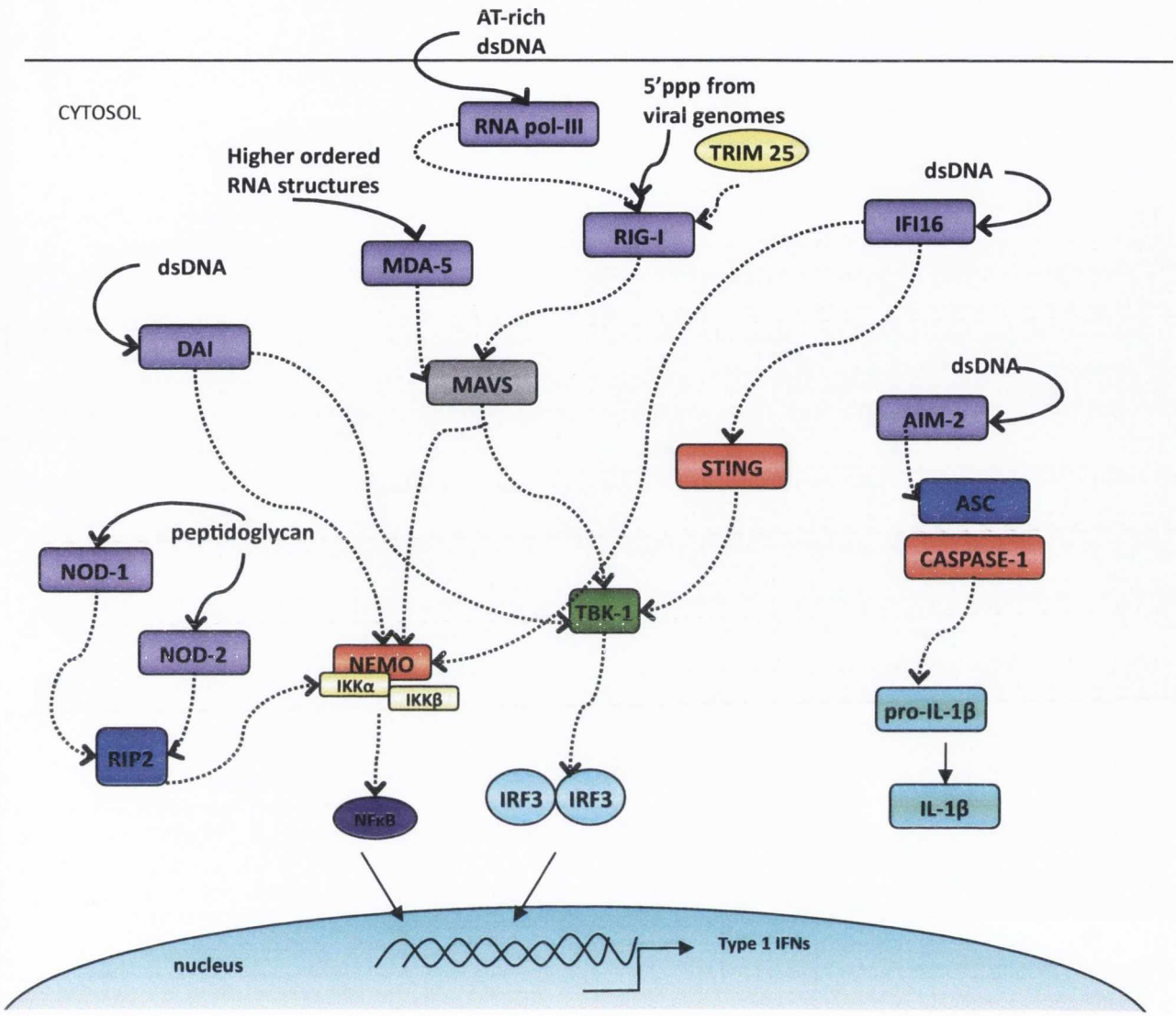


Figure 1.1 Cytosolic PRR signalling. The RLR member RIG-I is known to detect 5'ppp from viral genomes. It is known to require two ligands for activation; 5'ppp RNA and unanchored ubiquitin chains. RIG-I activation also requires ubiquitination by TRIM25. MDA-5 recognises higher ordered RNA viral structures. Both RIG-I and MDA-5 are known to signal via MAVS to induce both NFκB and IRF activation. Infection with viruses or bacteria can trigger the release of DNA into the cytosol which is normally a DNA-free environment. This intracellular DNA can be recognised by various DNA receptors including DAI, IFI16, RNA polymerase III or AIM-2. DAI and IFI16 signals via STING to activate both NFκB and IRF3 to induce type-I IFNs. Cytosolic AT-rich dsDNA is recognised by RNA pol III and transcribed into 5'ppp RNA, which activates RIG-I/ MAVS signalling. dsDNA also activates an AIM-2/ASC/Caspase-1 inflammasome to activate IL-1β. NOD-1 and NOD-2 are cytosolic receptors of peptidoglycan and signal via RIP-2 to activate NFκB.

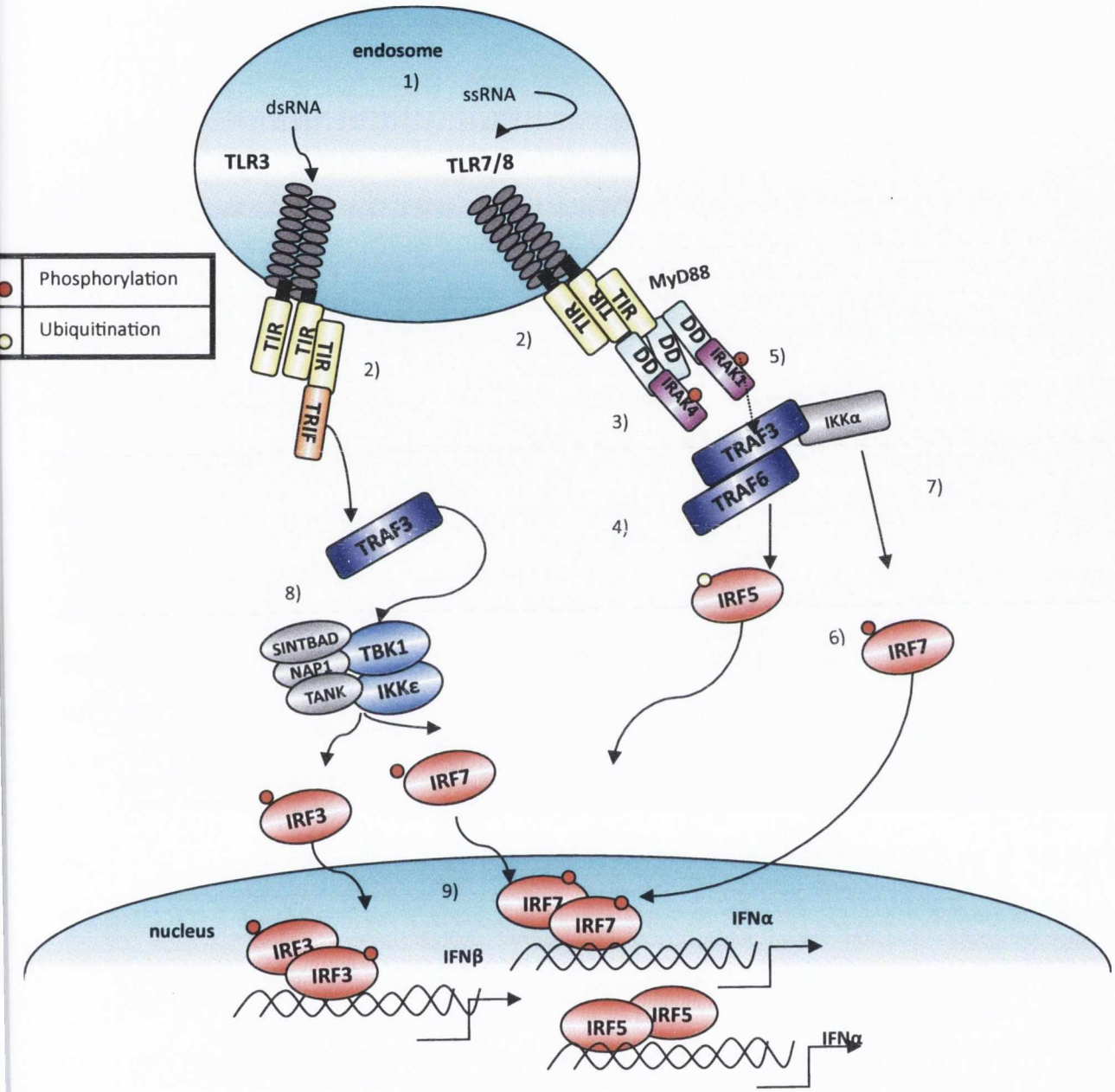

endosome
1)
dsRNA
ssRNA
TLR3
TLR7/8
Phosphorylation
Ubiquitination
TIR
TRIF
2)
MyD88
DD
IRAK4
5)
3)
TRAF3
IKKα
TRAF6
7)
4)
IRF5
6)
IRF7
8)
SINTBAD
NAP1
TANK
TBK1
IKKε
IRF3
IRF7
9)
nucleus
IFNβ
IFNα
IRF5

Figure 1.2. TLR-induced IRF activation. 1) Recognition of dsRNA (by TLR3), ssRNA (by TLR7/8) or CpG motifs (by TLR9) in the endosomes of cells results initially in the dimerisation of the TLRs. **2)** The adaptor molecules TRIF (for TLR3) or MyD88 (for TLR7/8/9) are recruited to the receptor. TLRs, TRIF and MyD88 signal via their TIR domains. **3)** IRAK-1 and IRAK-4 have been shown to play an important role in the TLR7,8,9 MyD88-dependent pathway to IFN but is not thought to have a role in TLR3 activation of IFNs. IRAK-2 is not thought to be involved in the TLR7/8-induced IFN pathway but may have a role in TLR7/8-induced NFκB. **4)** IRAK-1 has been shown to stimulate the ubiquitination of IRF5 via TRAF6. **5)** IRAK-1 has also been shown to interact with TRAF3 which plays an important role in IFN activation. **6)** IRAK-1 is vital for IRF7 phosphosphorylation and its kinase activity is vital for IRF7 transcriptional regulation. **7)** IKKα which was thought to mainly function in NFκB activation has also been shown to play a part in TLR7/9-induced IRF7 activation. **8)** For TLR3, activation of the IRFs occurs via TRAF3 and the kinases TBK-1 and IKKε which phosphorylate IRF3 and IRF7. **9)** Upon activation, the IRFs translocate into the nucleus and induce the transcription of type I IFN (IFNα and IFNβ). (Dashed arrows indicate less defined pathways. TLR9 omitted from picture but is thought to signal similarly to TLR7/8)

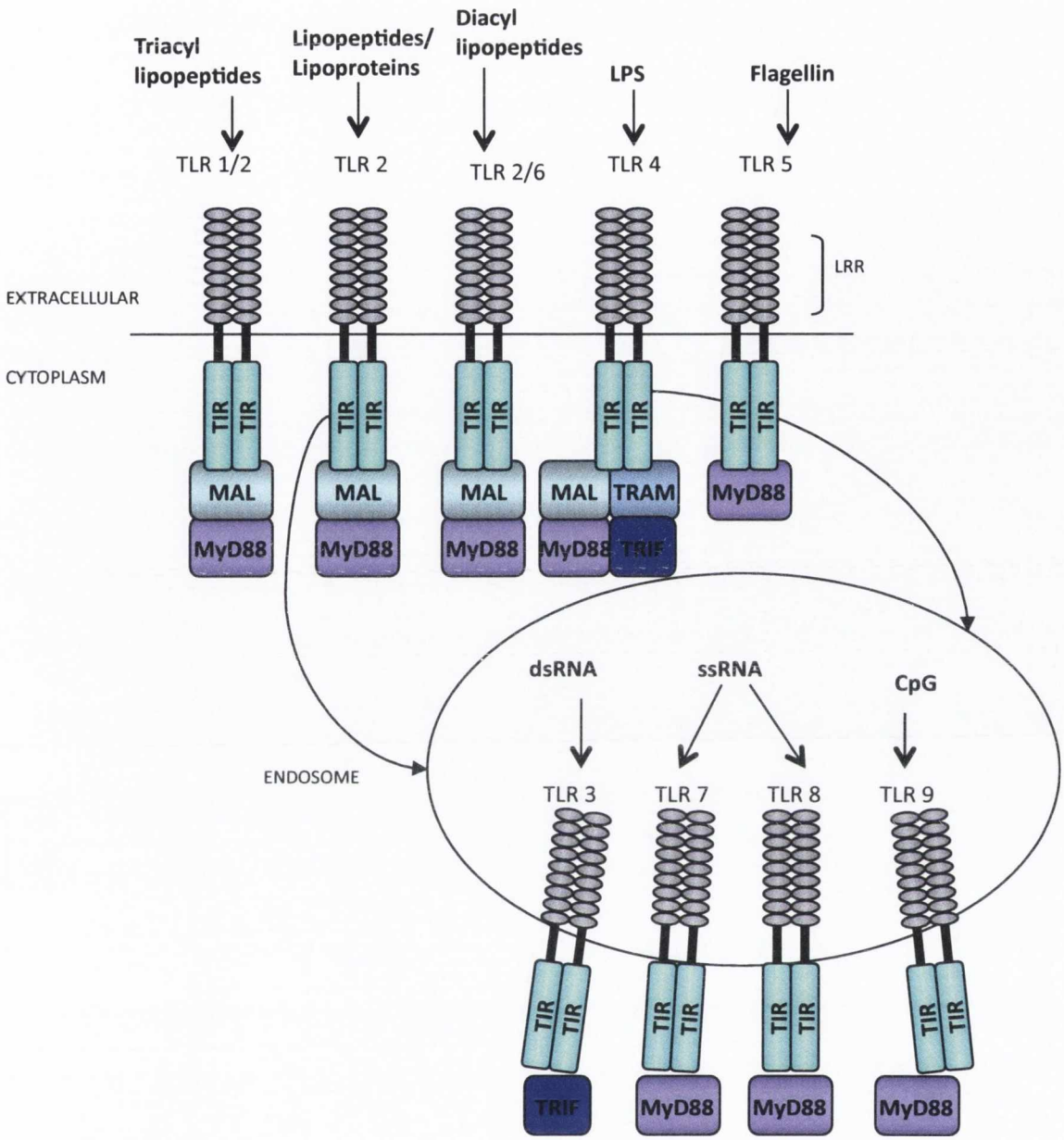


Figure 1.3 The TLR Family and their Adaptor Usage. To date there are 10 human TLRs although only 1-9 are functional (shown above). The TLRs can be divided into two sub-groups depending on their localisation in the cell. TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell surface and TLR3, TLR7, TLR8 and TLR9 are localised in intracellular vesicles such as the endosome or endoplasmic reticulum. TLR2 and TLR4 can also translocate to the endosome and signal there.TLR2 via MyD88 and TLR4 via TRIF (TIR domain-containing adaptor protein inducing IFN-β) and TRAM (TRIF-Related Adaptor Molecule). TLR signalling is initiated by dimerisation of the Tolls and the subsequent recruitment of the TIR domain-containing adaptor proteins MyD88, MAL (MyD88-Adaptor-Like protein), TRIF and TRAM. MyD88 is utilised by all the tolls with the exception of TLR3.

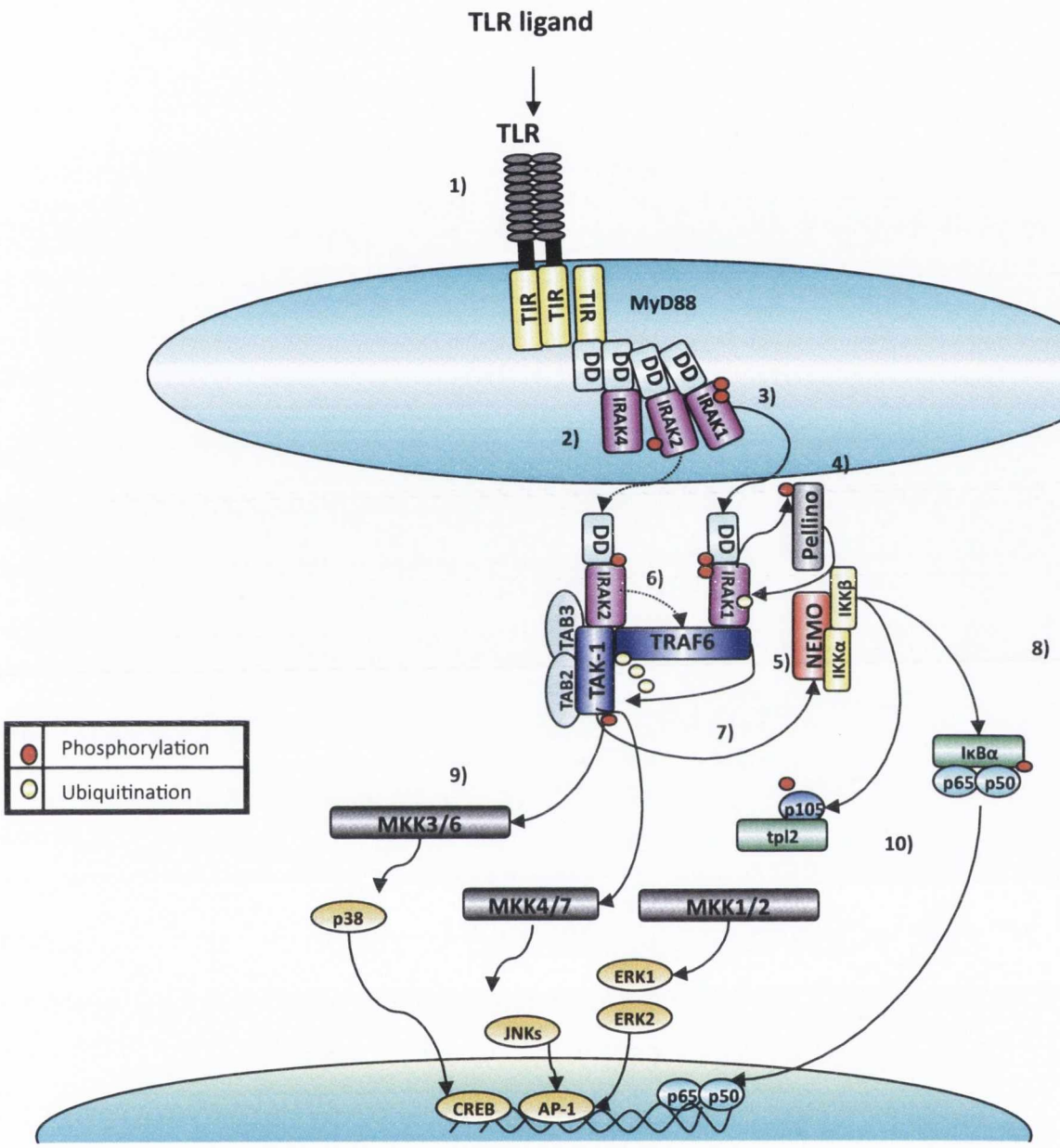

TLR ligand
TLR
1)
TIR
TIR
TIR
MyD88
DD
DD
DD
DD
IRAK4
IRAK2
IRAK1
2)
3)
4)
Pellino
DD
IRAK2
DD
IRAK1
6)
TAB3
TAB2
TAK-1
TRAF6
NEMO
IKKβ
IKKα
5)
8)
7)
Phosphorylation
Ubiquitination
9)
MKK3/6
p38
MKK4/7
MKK1/2
ERK1
ERK2
JNKs
CREB
AP-1
p105
tpl2
10)
IκBα
p65
p50
p65
p50

Figure 1.4. NFκB and MAPK signalling pathways activated by TLR4. 1) Stimulation of TLR4 by LPS,results in the recruitment of TIR adaptors to the plasma membrane. IRAK4 interacts with MyD88 through death domain interactions. **2)** IRAK-4 is thought to phosphorylate both IRAK-1 and IRAK-2 which induces their autophosphorylation activity. **3)** Hyperphosphorylated IRAK-1 (and possibly IRAK-2) are released from the receptor complex and subsequently associate with TRAF6. **4)** IRAK-1 phosphorylates Pellino1 which can ubiquitinate IRAK-1. IRAK-1 and pellino form a complex with TRAF6. **5)** NEMO binds to ubquitinated IRAK-1. **6)** IRAK-2 induces the polyubiquitination associated with TRAF6. TRAF6 stimulated polyubiquitination results in the recruitment of the TAK-1/TAB2 complex and activation of TAK-1. **7)** TAK-1 activates the IKK complex. **8)** The IKK complex phosphorylates IκBα which allows p65/p50 to translocate in the nucleus. **9)** For MAP kinase activation, TAK-1 activates MKK3/6 and MKK4/7 for p38 and JNK activation respectively. **10)** The IKK complex phosphorylates the inhibitory protein p105. Upon phosphorylation and degradation of p105, tpl2 is activated and subsequently activates MKK1/MKK2. MKK1/ MKK2 activate ERK1 and ERK2. (Dashed arows indicate less defined pathways).

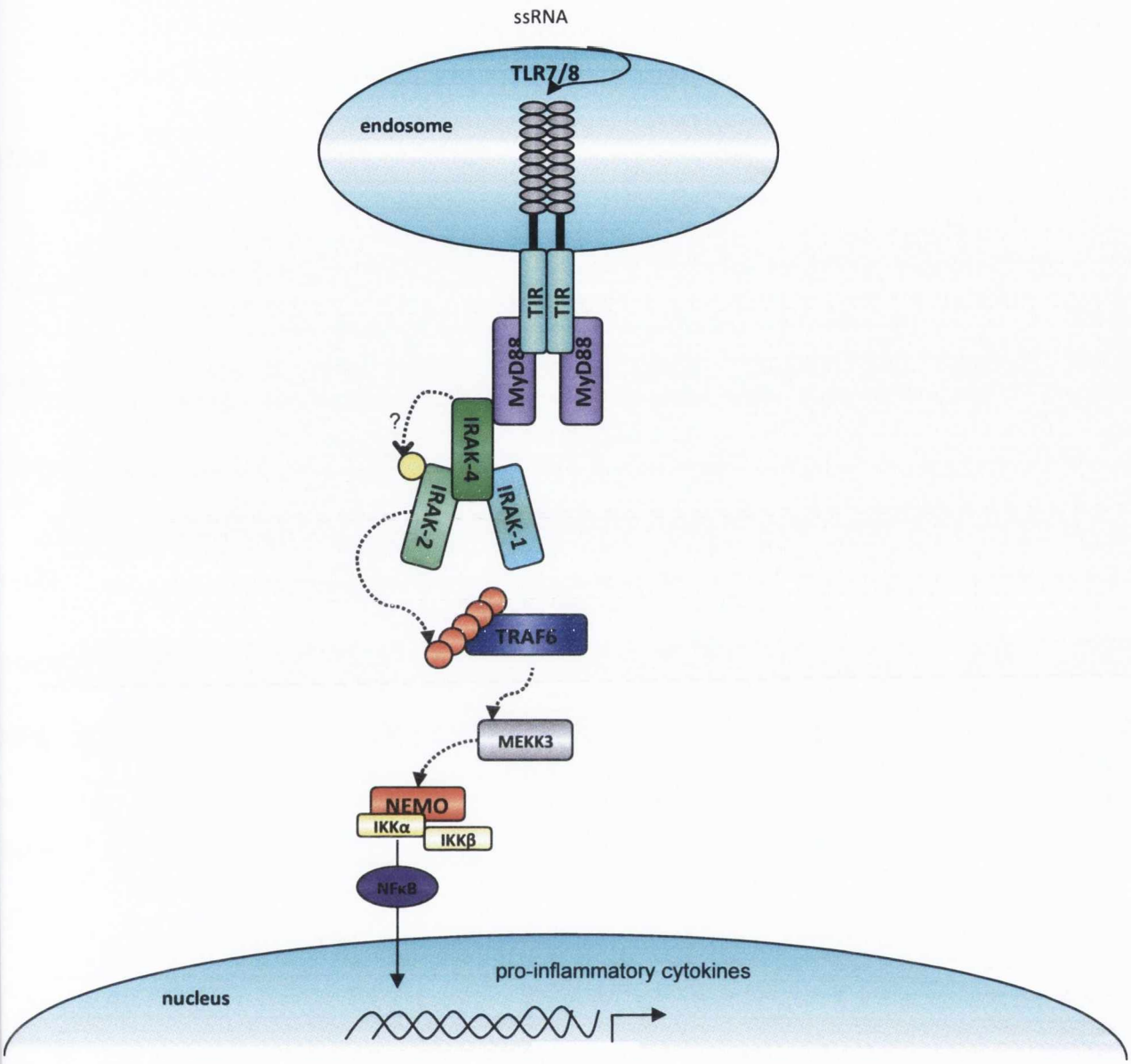
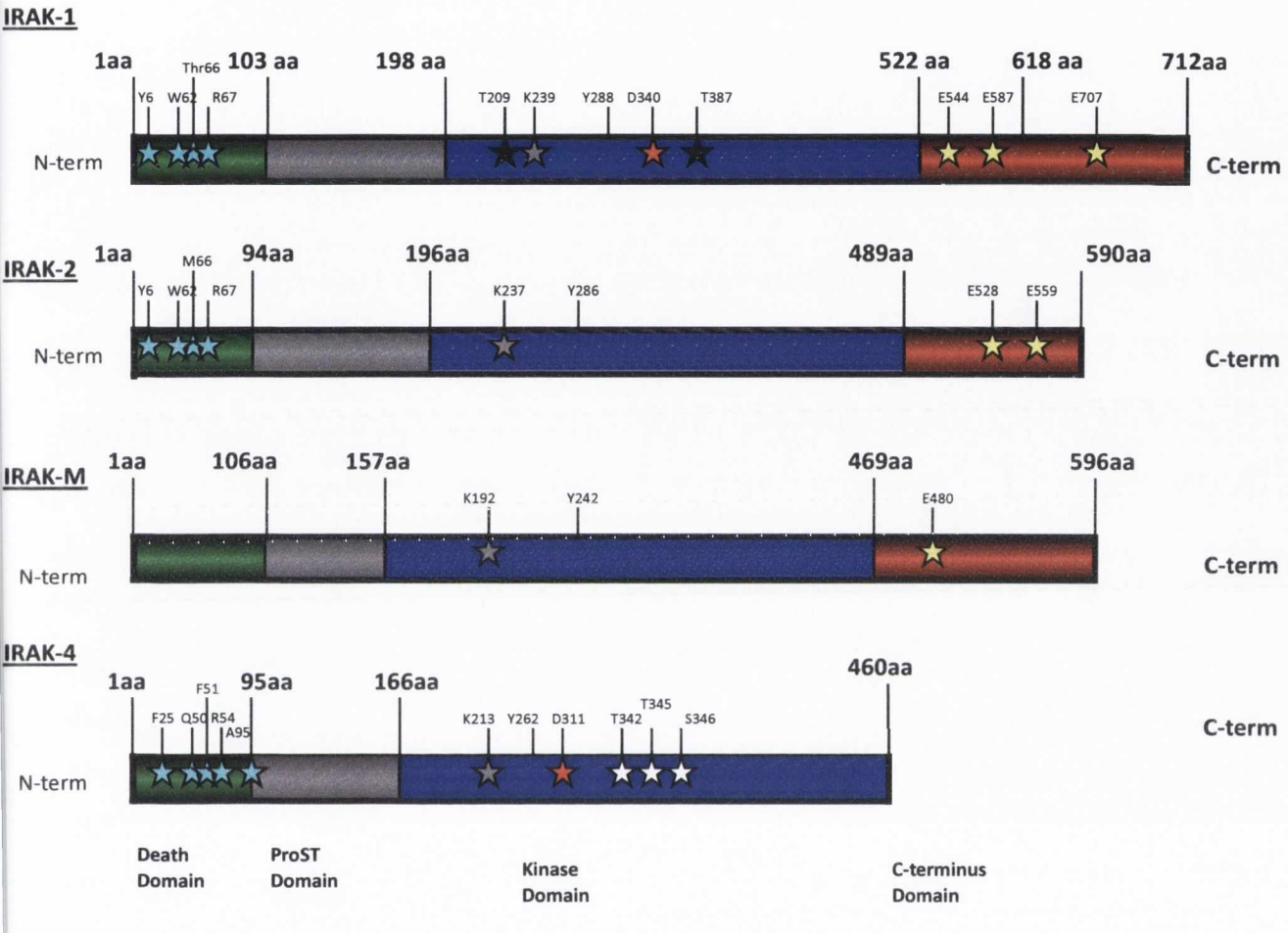


Figure 1.5 TLR8 signalling to NFκB Recognition of ssRNA in the endosomes of cells results in the dimerisation of the TLRs. The adaptor molecule MyD88 is recruited to the receptor. IRAK-4 is recruited to MyD88 and with IRAK-2 or IRAK-1 form a helical signalling complex termed the Myddosome. For activation of NFκB, IRAK-2 triggers the E3 ligase activity of TRAF6, which leads to the formation of polyubiquitin chains that are important for NFκB activation. It has been shown that TLR8 signalling to NFκB requires IRAK-1, but IRAK-1 is not phosphorylated or ubiquitinated after TLR8 stimulation, thus suggesting a different role for IRAK-1 in the TLR8 pathway. TLR8 signalling to NFκB has also been shown to not require TAK-1 but is MEKK3- dependent. MEKK3 activates the IKK complex which leads to NFκB activation.



★	Important for IRAK-2/-1: IRAK-4 interaction for Myddosome structure
☆	Important for kinase activity
★	Invariant lysine residue
★	Phosphorylated by IRAK-4
★	Critical asparate residue
★	TRAF6 binding motif

Figure 1.6 Functional domains of the human interleukin receptor associated kinases (IRAKs). Each member has a death domain, a proST domain, a conserved kinase domain, and C terminal domain. The only exception is IRAK-4 which lacks a C-terminal domain. For human **IRAK-1** the death domain contains residues Y6, W62, M66, R67 (that are also conserved in IRAK-2) and are important for IRAK-4 interaction and myddosome formation, the proST domain has been shown to be vital for autophosphorylation and the kinase domain contains Thr209 and Thr387, located in the activation loop, which are potential phosphoryation sites for IRAK-4. The invariant lysine residue in the ATP binding pocket of IRAK-1 is located at K239 and there is a critical aspartate residue at D340, both of which are critical for IRAK kinase function. The tyrosine gatekeeper is located at Y288. The C-terminus contains three TRAF6 binding motifs (E544, E587 and E707). The kinase domain of human **IRAK-2** contains a invariant lysine residue in the ATP binding pocket at K237 which is said to be important for its kinase activity. The tyrosine gatekeeper is located at Y286. Its C-terminal contains two TRAF6 binding motifs (E528 and E559). E528 is critical for IRAK-2 function. The invariant lysine residue of **IRAK-M** is located at K192. the tyrosine gatekeeper is located at Y242. IRAK-M also has a TRAF6 binding motif in its C-terminus (E480). Lastly **IRAK-4** contains a death domain, a proST region and a kinase domain. the invariant lysine residue of IRAK-4 is located at K213. There is a critical aspartate residue at D311 which is essential for IRAK-4 kinase function. Other important residues in IRAK-4 kinase domain include T342, T345, T346. The tyrosine gatekeeper is located at residue Y262.

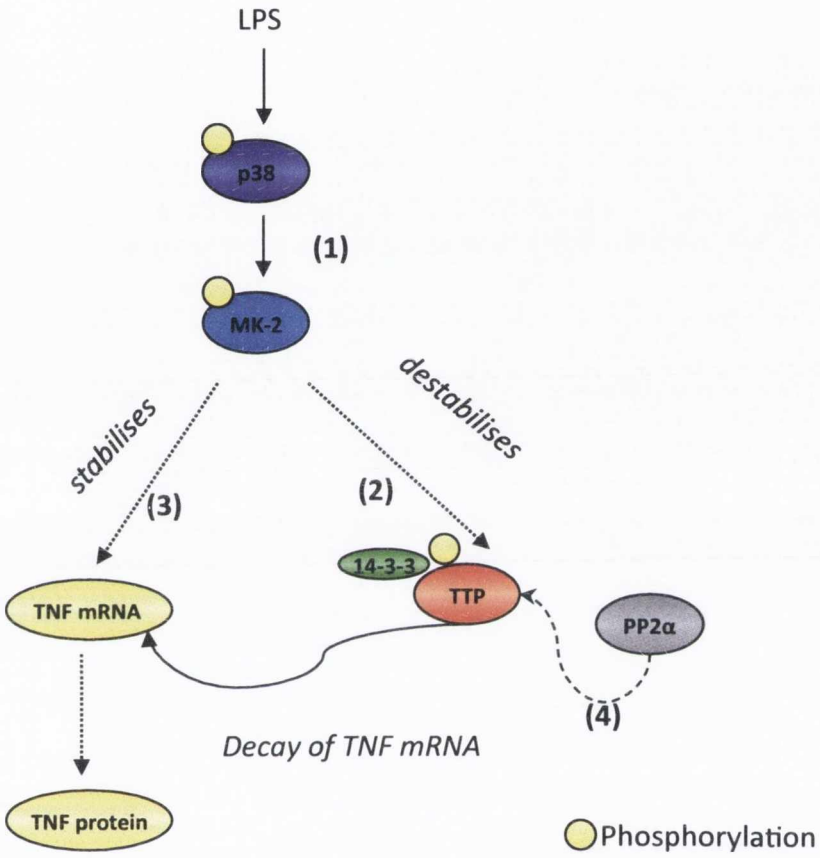


Figure 1.7 Post-transcriptional regulation of TNFα. (1) When p38 MAPK is activated upon stimulation with LPS, expression of TNFα and TTP are both upregulated. **(2)** TTP is phosphorylated by MK2 and this leads to recruitment of 14-3-3 proteins. **(3)** Phosphorylation of TTP impairs its destabilising function and therefore TNFα mRNA is expressed and translated. 14-3-3 proteins localises TTP to the cytoplasm and prevents TTP from undergoing dephosphorylation by protein phosphatase 2α (PP2α). **(4)** During the resolution of inflammation, PP2α dephosphorylates TTP,leads to dissociation of 14-3-3 protein, TTP can bind to TNF mRNA causing the destabilisation of TNFα mRNA.

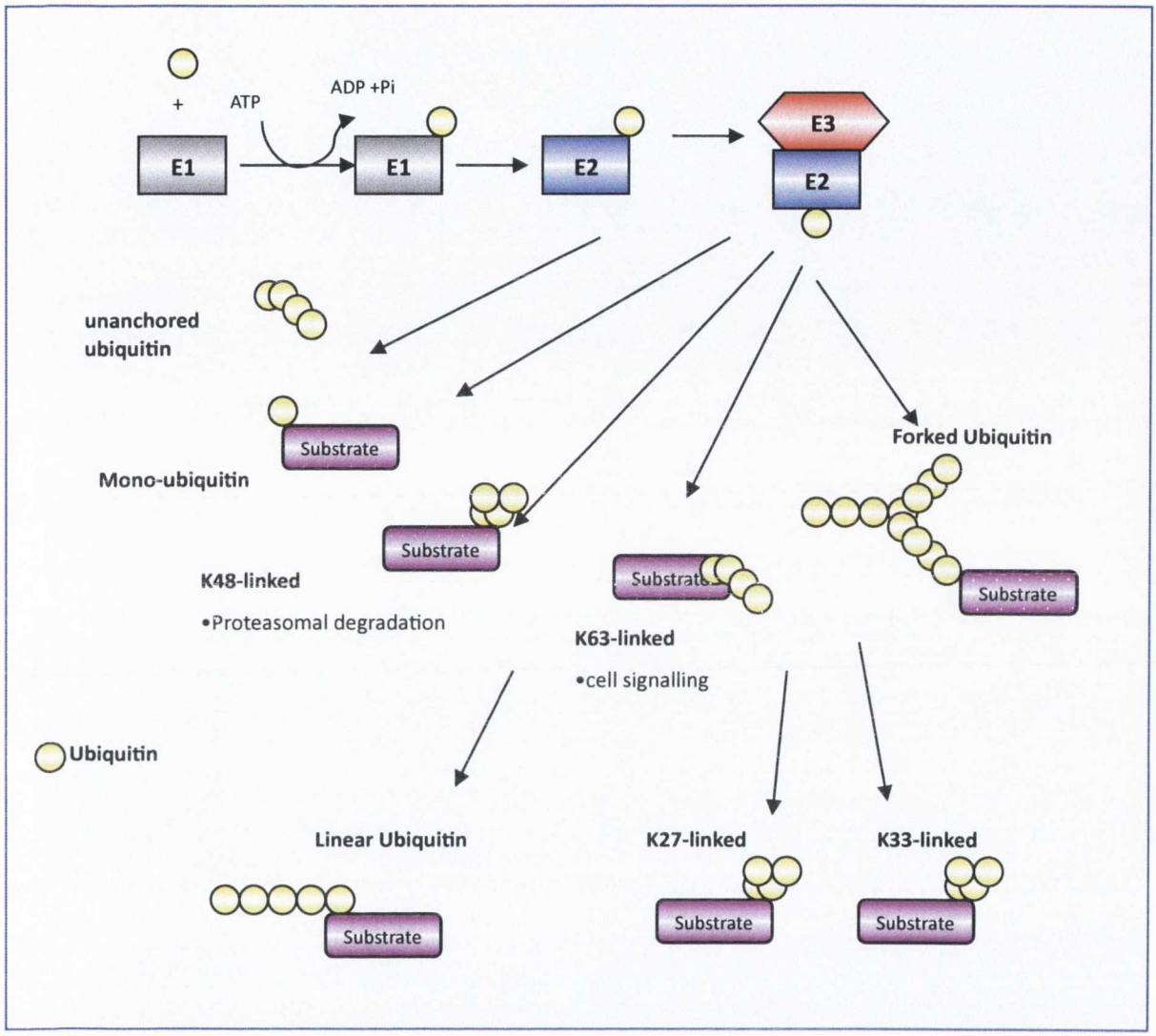


Figure 1.8 The Formation of Ubiquitin Chains There is a three step reaction that is involved, which results in the formation of ubiquitin conjugates. Firstly an E1 ubiquitin-activating enzyme activates the C-terminal glycine residue of ubiquitin. The active ubiquitin is then transferred to the cysteine residue of the active site of the E2 ubiquitin-conjugating enzyme. The E3 ligase then recruits the ubiquitin-E2 complex and the target protein and transfer the ubiquitin to the substrate. Successive conjugation of ubiquitin molecules to an internal lysine of a previous conjugated ubiquitin occurs resulting in the formation of polyubiquitin chains.

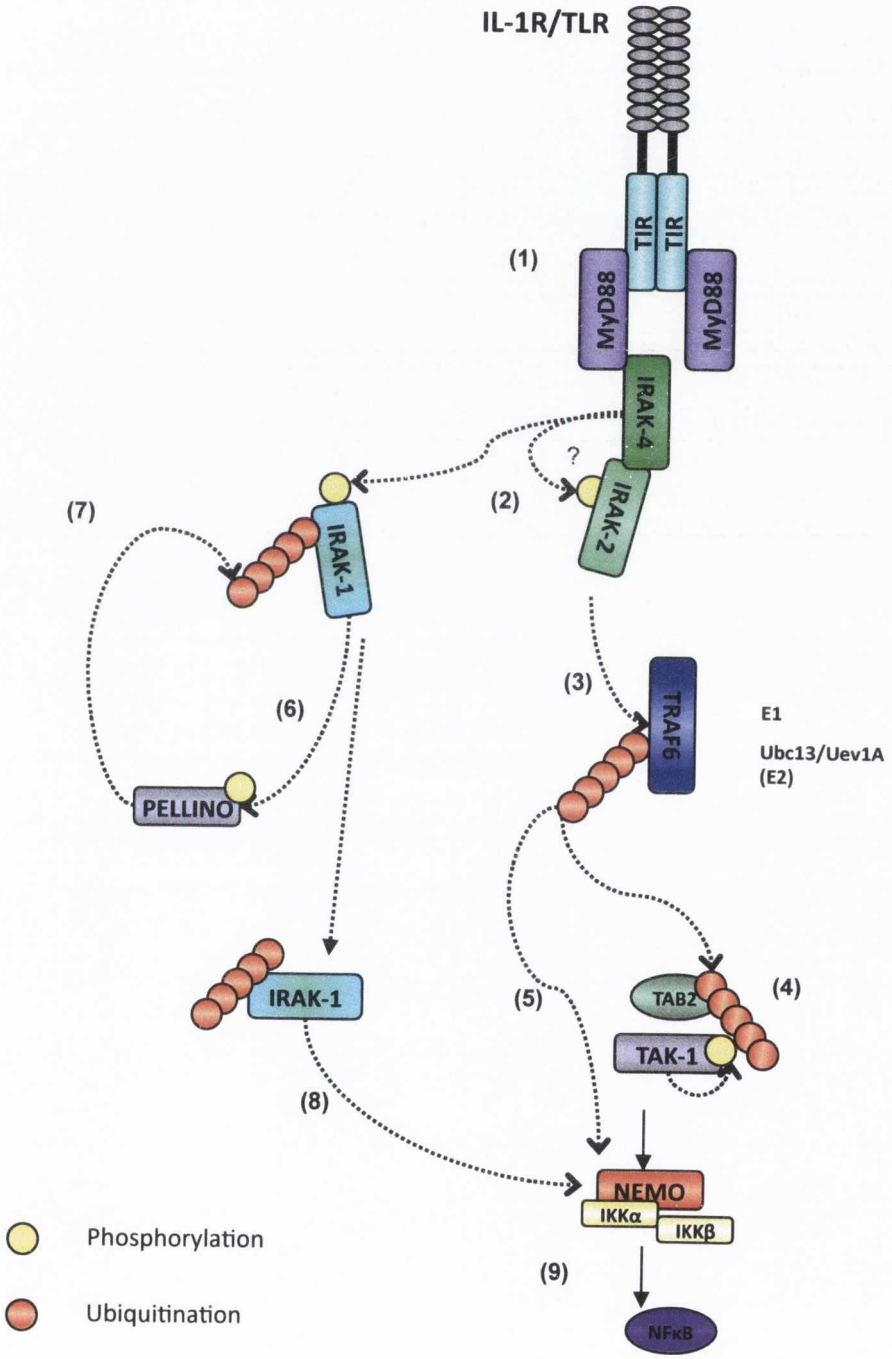

IL-1R/TLR
TIR
TIR
(1)
MyD88
MyD88
IRAK-4
?
(2)
IRAK-2
(7)
IRAK-1
(3)
TRAF6
E1
Ubc13/Uev1A
(E2)
(6)
PELLINO
IRAK-1
(5)
TAB2
(4)
TAK-1
(8)
NEMO
IKKα
IKKβ
(9)
NFκB
Phosphorylation
Ubiquitination

Figure 1.9 Role of IRAK-2 in polyubiquitin chain formation in TLR signalling to NFκB. (1) IL-1/TLR stimulation leads to receptor dimerisation allowing recruitment of MyD88 via TIR/TIR domain associations. Binding of the MyD88 TIR domain to the receptor, releases MyD88 and allows the formation of the Myddosome complex. MyD88, IRAK-4 and IRAK-2 form a complex containing 6-8 MyD88, 4 IRAK-4 and 4 IRAK-2/IRAK-1 DD. The formation of the Myddosome complex allows IRAK-4 to come in close proximity to IRAK-2 & IRAK-1.**(2)** IRAK-4 then phosphorylates IRAK-2 & IRAK-1. **(3)** IRAK-2 then triggers the E3 ligase activity of TRAF6 through an unknown mechanism that may increase TRAF6 oligomerisation. **(4)** TRAF6 then forms free K63-linked polyubiquitin chains leading to TRAF6 autoubiquitination. **(5)** The unanchored polyubiquitin chains can activate downstream signalling molecules such TAK-1 and NEMO. TAK-1 complexes are brought into close proximity by binding polyubiquitin chains and this process requires TAB2. **(6)** In parallel to this, IRAK-1 phosphorylates pellino proteins and **(7)** the pellino proteins trigger the formation of polyubiquitin chains on IRAK-1. **(8)** The polyubiquitin chains on IRAK-1 are said to facilitate the recruitment of NEMO with IRAK-1, which is required for NEMO activation. **(9)** Activation of the IKK complex by TAK-1 ultimately leads to the activation of NFκB.

Table 1.1: Knockout studies showing the effect of absence of IRAKs on TLR pathway.

	in vivo	Effect on NFκB activation	Effect on MAPK	Effect on IRF activation	Effect on mRNA stability
IRAK-1	Partially resistant to LPS-induced septic shock (180)	Partial impairment for IL-1/TLR 4 (180-182)	Impairment for IL-1 /TLR4 (180-182) No impairment of TLR2-induced p38 (208) Dramatic impairment of TLR2-induced ERK and JNK (208)	Effect on TLR7/9-induced IFN (34)	Required for IL-1-induced mRNA stabilisation (238)
IRAK-2	Mice completely resistant to LPS and CpG- induced septic shock (197)	Impairment of late TLR2/ TLR7-activation(197, 198) No effect for TLR4 (198)	Impairment of late TLR2-activation (197)	Negative role in TLR9-induced IFN (199)	Required for TLR4-induced mRNA stability (198).
IRAK-M	Reduced survival upon influenza infection (206)	Enhanced activation of TLR4 and TLR9 (137)	Enhanced activation TLR4 and TLR9 (137) Enhanced TLR2- induced p38 (209) No effect on TLR2-induced ERK and JNK (209)	N/D	N/D
IRAK-4	Mice completely resistant to LPS and CpG-induced septic shock (211)	Impairment of IL-1 and MyD88-dependent TLR (121,211) No effect on TLR3 (121)	Impairment of TLR4 induced JNK (211) Impairment of IL-1 induced p38 (211)	Effect on TLR7/8/9-induced IFN (121)	Required for TLR4 and TLR7-induced mRNA stability (216)

(N/D, not determined)

Table 1.2: Roles of IRAK-2 in different TLR pathways

TLR2	•Role for murine (C57BL/6J) IRAK-2 in late NFκB activation [197] •Role for murine (MOLF/Ei) IRAK-2 in early NFκB activation [202]
TLR3	•Role for human IRAK-2 in NFκB activation [106] •Interaction between IRAK-2 and TLR3 in human system [106]
TLR4	•Role for human IRAK-2 in NFκB activation [106] •No role for murine IRAK-2 in early or late NFκB activation [198]
TLR7	•No role for murine IRAK-2 in early NFκB activation [198]. •Role for murine IRAK-2 in TLR7-induced late or sustained activation [198]
TLR8	•Role of human IRAK-2 in early NFκB activation (this thesis)
TLR9	•Negative role for murine IRAK-2 in IFN production [199]

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 General Reagents

General laboratory chemicals were purchased from Sigma-Aldrich (Dublin, Ireland). Agar, yeast extract and tryptone were purchased from Unitech Ltd (Dublin, Ireland). Plasmid kit for DNA preparation was purchased from Qiagen. NovaBlue ™ Singles were purchased from Novagen (Merck Biosciences, Nottingham, UK).

2.1.2 Cell Culture

DMEM (Dulbecco's modified Eagle's medium) was purchased from Gibco Biosciences (Paisley, UK). RPMI was purchased from Sigma-Aldrich. Gentamicin and trypsin-EDTA were purchased from Sigma-Aldrich. Endotoxin-Free Foetal calf serum was purchased from Biosera (Ringmer, East Sussex, UK). Phosphate-buffered saline (PBS) was purchased from Sigma-Aldrich. Cell culture plastics were all purchased from Corning Inc. (Amsterdam, The Netherlands. Lymphoprep was purchased from Axis Shield (Dundee, UK).

2.1.3 Transfections

Lipofectamine was purchased from Invitrogen (Groningen, Holland). Gene Juice™ for reporter gene assays was purchased from Novagen. Coelenterazine was purchased from Biotium Incorporated (Hayward, USA). Passive Lysis Buffer was purchased from Promega (WI, USA). Luciferase mix was homemade (Tricine, $MgSo_4$, EDTA, DTT, ATP Acetyl CoEnyzme A, NaOH, Magnesium Carbonate Hydroxide. Luciferin was purchased from Biosynth (Staad, Switzerland)).

2.1.4 IL-1/TLR agonists

CL075 was purchased from Invivogen (CA, USA). CL075 (3M002) is a thiazoloquinolone derivative.



Figure 2.1 Structure of CL075

Human rIL-1β was a gift from the National Cancer Institute (Fredrick, Washington USA). Ultrapure LPS from Gram-negative bacteria (*Eschericia coli)* (>99.9 pure in respect to contaminating protein, DNA, and TLR2 agonists) was purchased from Alexis Biochemicals (Plymouth Meeting, PA).

2.1.5 SDS-PAGE and Western Blot Analysis:

Trizma base (Tris), Sodium dodecyl sulphate (SDS), ammonium persulphate (APS), bromophenol blue, glycine, dithiothreitol (DTT), Tween 20® and N,N,N',N'-tetramethyldiamine (TEMED), polyvinyidene difluoride (PVDF) and Nitrocellulose were all purchased from Sigma Aldrich. Enhanced chemiluminescence (ECL) was homemade. (ECL is made of Solution A which is composed of 1ml luminal stock (250 mM luminal stock (0.44 g/10 ml DMSO), 0.44 ml p-Courmaric acid stock (90 mM p-Courmaric acid stock 0.15 g/10 ml DMSO), 10 ml 1M Tris-HCL, pH 8.5 and a final volume brought to 100 mls. Solution B is composed of 10mL 1M Tris-HCl pH 8.5 brought up to a final volume of 100 mls. 1mL of Solution A and 1mL Solution B are added together with 0.61 µl H_2O_2 /per blot.) Luminol

Courmaric acid, H_2O_2 and Tris to make home made ECL were all purchased from Sigma-Aldrich. Scientific Imaging Film was purchased from Sigma-Aldrich. Broad range pre-stained protein marker was purchased from New England Biolabs Ltd. (MA, USA). Reblot solution was purchased from Millipore (Bedford, MA). Fixation Buffer is composed of 140ml Acetic Acid, 800mls Methanol, 60mls of glycerol and 1000ml of dH_2O and were all purchased from Sigma-Aldrich.

2.1.6 Antibodies:

Mouse monoclonal antibody to ubiquitin (P4D1) and rabbit polyclonal antibody to TRAF6 (H-274) were purchased from Santa Cruz Biotechnology (CA, USA). Anti IκBα was provided by R. Hay (University of St Andrews, Scotland). Monoclonal anti-FLAG, anti-myc and anti-β-actin were all purchased from Sigma-Aldrich. Monoclonal anti-HA antibody was purchased from Cambridge Biosciences (Cambridge, UK). For detection of *p*-p38 a rabbit phospho-specific antibody was used (Cell Signalling, Danvers, MA). To control for protein loading, a rabbit anti-p38 antibody was used (Cell Signalling Technology). All HRP-conjugated secondary antibodies were purchased from Sigma-Aldrich. For detection of endogenous IRAK-2, a rabbit pAb was generated using the following IRAK-2 peptide as an antigen: ($NHCOCH_3$) CADVYRGHRHGKPFVFK($CONH_2$) (Inbiolabs, Estonia). K63-linked and K48-linked ubiquitin specific antibodies were purchased from Millipore (Bedford, MA)

2.1.7 Cells

The human embryonic kidney (HEK) cell line stably transfected with the IL-1R1, termed HEK_293 R1 and HEK293T cells were provided by Tularik Inc. (San Francisco, CA, USA). HEK293 cells stably expressing TLR4 and TLR8 were a gift from Dr. K. Fitzgerald (University of

Massachusetts Medical School, Worcester, MA). Wild-type, IRAK2 $^{-/-}$, IRAK-1 $^{-/-}$ and IRAK-1/2 $^{-/-}$ mouse embryonic fibroblasts (MEFs) were a gift from Shuizo Akira (Osaka, Japan). Buffy Coats for PBMC preparation (Peripheral Blood Mononuclear Cells) were provided by the Irish Blood Transfusion Service. Ethical Approval was obtained from the Faculty of Health Sciences Ethics Committee for experiments involving PBMCs.

2.1.8 Reporter and Expression Plasmids

The NFκB-luciferase reporter plasmid was provided by R. Hofmeister (University of Regensburg, Germany). It consists of five κB elements and is inserted in the pGL3 Basic Vector. TK-Renilla was purchased from Promega (Madison, WI). Expression plasmids myc-IRAK2 and MyD88 were acquired from M. Muzio (Mario Negri Institute, Milan Italy) while HA-Ubiquitin was obtained from A. Mansell (Monash University, Melbourne, Australia). The empty vector control pcDNA 3.1 was purchased from Invitrogen (CA, USA). GAL4-IRF5 and GAL4-IRF7 were obtained from Kate Fitzgerald (University of Massachusetts, Worcester, MA). The pFR luciferase fusion protein was purchased from Stratagene (CA, USA). pFR luciferase reporter plasmid has a synthetic promoter with five tandem repeats of the yeast Gal4 binding sites which control the expression of the luciferase gene. The ISRE-reporter gene construct and the CHOP-GAL4 fusion vector were purchased from Stratagene. The murine TNF 3'UTR-luciferase reporter construct and the β-actin-luciferase reporter construct were kindly provided by Aihao Ding (Cornell University, New York, NY). The TNF 3'UTR (746 base pairs) and β-actin 3'UTR (640 base pairs) were generated by RT PCR and ligated downstream of the luciferase gene in the pGL3 vector. The IL-8 3'UTR-luciferase reporter construct was a gift from Peter King (Birmingham Veterans Affairs Medical Centre, Birmingham, AL, USA). The TRIF plasmid was obtained from S. Sato (Research Institute for

Infectious Disease, Osaka University, Japan). The MAL, TRAM and IRAK-4 plasmids were provided by L. O' Neill (Trinity College Dublin, Ireland). IRAK-1 plasmid was provided by Keith Ray (Stevenage, UK). Plasmids expressing K237A IRAK-2 and E528A IRAK-2 were generated in house by site directed mutagenesis. The TRAF6 expression plasmids FLAG-TRAF6 and FLAG-TRAF domain (dominant negative TRAF6 amino acids 289-522) were provided by Tularik Inc. (San Francisco, CA). C70A TRAF6 and K124R TRAF6 were provided by Justin Mc Carthy (University College Cork, Ireland). HA-WT IRAK-2 (N-terminal tag), WT IRAK-2-Ha (C-terminal tag), L503I IRAK-2, L392V IRAK-2, R43Q IRAK-2, A498G IRAK-2 and R214G IRAK-2 SNPs were provided by Alexander Weber (Bonn, Germany).

2.1.9 siRNA gene silencing

siRNA duplexes targeting the human *Irak-2* gene were obtained from Qiagen (West Sussex, UK) and targeted the following sites: IRAK-2A: 5'CAGCAACGTCAAGAGCTCTAA; IRAK-2B: 5'CCAGATCATCCTGAACTGGAA. Non silencing siRNA was used as a control (Qiagen).

2.1.10 ELISA

hTNFα, hIL-8, hIL-6 and muIL-6 Duosets were purchased from R&D systems (Minneapolis, USA). BSA was purchased from Sigma-Aldrich. Wash buffer was made up of 0.05% (v/v) Tween®20 in PBS both of which were purchased from Sigma-Aldrich. Capture Antibody, detection antibody, standard, streptavidin-HRP and substrate solution were all purchased as part of the R&D systems ELISA kit. Stop solution was composed of 2M H_2SO_4.

2.1.11 RT PCR

High Pure Isolation kits were purchased from Roche Applied Science (Burgess Hill, U.K.). Tris-HCl, KCl, $MgCl_2$, and DTT for RT buffer were all purchased from Sigma-Aldrich. dNTPs were purchased from New England Biolabs. RNAse Out was purchased from Biosciences. RT enzyme (M-MLV) was purchased from Promega. Induction of mRNA in PBMCs was assayed by quantitative real-time PCR using GoTaq qPCR Master Mix (Promega) and normalised to β-actin mRNA according to the manufacturer's instructions. For mRNA stability experiments, Actinomycin D was purchased from Sigma-Aldrich. The primers used were as follows; For TNFα: 5'-GAA CCC CGA GTG ACA AGC CTG-3' for 5'-TCA GCT CCA CGC CAT TTG CCA-3' IL8: 5' CTC TGT GTG AAG GTG CAG TTT TG-3' and 5'AAG CTT TAC AAT AAT TTC TGT GGT-3' for β-actin: 5' CGC GAG AAG ATG ACC CAG ATC-3' and 5'-GCC AGA GGC GT CAG GGA TA-3'. The p38 inhibitor SB202190 was purchased from Sigma-Aldrich.

2.1.12 Immunoprecipitation

For 1% NP40 lysis buffer, HEPES, Ipegal, NaCl, Glycerol and EDTA were purchased from Sigma-Aldrich. The protease inhibitors Aprotinin was also purchased from Sigma-Aldrich. PMSF, sodium orthovanadate and protein cocktail inhibitor. Iodoacetamide was purchased from Sigma-Aldrich. Isopeptidase T was purchased from Boston Biochem (Cambridge, MA). Protein A beads were purchased from Sigma-Aldrich.

2.2 Methods

2.2.1 Cell Culture

HEK293_R1, HEK293_T cells, HEK293_TLR4, HEK293_TLR8 and MEF cell lines were maintained in DMEM supplemented with 10% (v/v) FCS, 100 µg/ml gentamycin and 2 mM L-glutamine. All cell lines were maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO_2.

Cell lines were subcultured every two to three days. The old medium was removed and the cells were washed with 5 ml sterile PBS. Next 3mls of Trypsin-EDTA (0.05 mg/ml) was added to the cells and they were returned to the incubator (37°C, 5% CO_2) for 2mins. Following incubation 10 mls of complete medium was added to the trypsinized cells and the cells were resuspended slowly and gently by pipetting. The cell suspension was then centrifuged at 1000 g for 5 mins. The supernatant was discarded and the cell pellet was resuspended in 1 ml of complete medium. The cell density was determined by counting the cells in a 10 µl sample of cell suspension with a haemocytometer and a light microscope. Flasks were seeded at $1x10^5$ cells/ml to a volume of 15ml in a T75 flask.

For the MEF cell line the old medium was removed from the cells and the cells were washed as mentioned above. 3 mls of Trypsin-EDTA (0.05 mg/ml) was added to the cells and they were returned to the incubator (37°C, 5% CO_2) for 2 mins. The MEF cell line does not respond well to centrifugation so after incubation the cells were scraped using a cell scraper and resuspended without centrifugation. 20 mls of complete medium was added to the trypsinized cells and the cells were resuspended slowly and gently by pipetting.

For reporter gene assay cells were seeded at a density of 1.5x 10^5 cells/ml in a volume of 200 µl per well of a 96 well plate. For immunoprecipitation experiments, 100 mm dishes were seeded at a density of 1.5x10^5 cells/ml in 15 ml of complete medium.

2.2.1.1 PBMC preparation

For PBMCs, a buffy coat of blood was diluted 1:7 using sterile PBS. 15 mls of lymphoprep was added to 50 ml tubes. 35 mls of the diluted blood was added very slowly onto the lymphoprep. The 50 ml tubes were then centrifuged at 1200 g for 30 mins ensuring that the brake was off on the centrifuge. Using a sterile pipette, the buffy coat layer at the interface of the lymphoprep and the serum was collected. The buffy coat cells were then diluted in PBS in 50 ml tubes and subsequently centrifuged at 1200 g at room temperature for 8 mins. The cell pellet was then washed in PBS or medium and centrifuged as before. The pellet was then resuspended in an appropriate volume of medium. The cells were then counted and seeded out.

2.2.2 Plasmid Transformation

Competent (Nova Blue singles) *E coli* cells were thawed on ice. The cells were divided into 5 µl aliquots. 1 µl of a 1/10 dilution of DNA was added directly to the cells on ice. The mixture was mixed gently to ensure the cells and DNA mixture was evenly suspended. The cells were incubated on ice for 5 mins. The tubes were heated for exactly 30 secs in a 42˚C water bath. The tubes were placed on ice for 2 mins. 50 µl of SOC medium was added to each tube and then plated on Amp resistant agar plates (LB agar and ampicillin 1:1000). The plates were incubated overnight at 37˚C.

2.2.3 Plasmid DNA Preparation

Using the Qiagen EndoFree Plasmid Maxi Kit the following protocol was used to grow DNA plasmids. All buffers were taken from this kit. After plasmid transformation (2.2.2) a single colony was picked and was added to 100ml of LB broth containing the antibiotic Ampicillin. It was incubated at 37°C for 12-16 hours in a shaking incubator at 300 rpm. The bacteria was then harvested by centrifugation at 5000g for 20 minutes. The supernatant was decanted.

2.2.3.1 Clearing of Bacterial Lysate

The bacterial pellet was resuspended in 10 ml Buffer P1. 10 mls of Buffer P2 was added and incubated at room temperature for 5 minutes. 10 ml of Buffer P3 was added and inverted 4-6 times. Upon addition of these buffers the pellet was lysed. The lysate was added into a QIAfilter Cartridge which was supplied with the kit. It was incubated at room temperature for 10 mins. The plunger was inserted into the cartridge and the cell lysate was filtered into a 50 ml tube. A Buffer ER was added to the filtered lysate and mixed. The tube was then incubated on ice for 10 mins.

2.2.3.2 Binding, washing and eluting of plasmid DNA

A Qiagen-tip 500 column was firstly equilibrated by adding 10 ml of Buffer QBT. The filtered lysate was then added to the column and allowed to run through by gravity flow. The column was subsequently washed twice with 30 ml of Buffer QC. The DNA was eluted with 15 ml Buffer QN.

2.2.3.3 Precipitate, wash and redissolving plasmid DNA

The DNA was precipitated by adding 10.5 ml of room temperature isopropanol to the eluted DNA and mixed. It was then centrifuged at >10,000 g for 30 min at 4°C. The supernatant was then decanted. The DNA pellet was washed with 5 mls of endotoxin free room temperature 70% ethanol and centrifuged at >10,000g for 10 mins. The supernatant was carefully decanted ensuring not to disturb the cell pellet. The pellet was air-dried for 5-10 mins and redissolved in a suitable amount of endotoxin free Buffer TE. The concentration of plasmid DNA was measure using a nano-drop machine.

2.2.4 Transfection of Cells for Reporter Gene Assays

Cells were seeded at $1.5x10^5$ cells/ml in 96 well plates as described above. Plasmid DNA was transfected into cells using Gene Juice™ for 96 well plates. A total of 230 ng DNA was transfected per well. The transfection mix was made up in triplicate. 3.5 times the amount of DNA was first added to a V bottomed 96 well plate. A master mix of 0.8 µl Gene Juice ™ in 9.2 µl serum free medium (DMEM) was added per transfection, the gene juice was mixed by pipetting and the mixture was incubated for 5 mins at room temperature. 35 µl of gene juice mix was added to DNA (3.5 times 10 µl per transfection) and incubated for 15 mins at room temperature. 10 µl of the transfection mix was added to the flat bottomed 96well plate that contained the cells in 200 µl of DMEM at a density of $1.5X10^5$ cells/ml.

Approximately 18 hours later the cells that require stimulation were stimulated. The cells were then incubated at 37°C and harvested 6 hours later.

2.2.5 Transfection of siRNA in 96 well plates

The required volume of siRNA was added into appropriate well of V-bottomed 96well plate. The siRNA was diluted in an end volume of 25 µl per transfection. 0.5 µl of lipofectamine 2000 (Invitrogen) was used per transfection for the HEK293 cells and 0.2 µl of lipofectamine was used for the PBMC experiments. The Lipofectamine was diluted in 25 µl SFM (DMEM for HEK293 and RPMI for PBMCs) per transfection. The Lipofectamine and SFM mix was incubated for 5 mins at room temperature. 87.5 µl of SFM and Lipofectamine mix was then added to each well of siRNA/SFM. The plate was then incubated for 20 mins at room temperature. 50 µl was then pipetted into each well of cells.

2.2.6 Quantitative Real Time PCR

PBMCs were seeded at $2X10^6$/ml in either 12 well plates or T175 flask 1 hr before treatment with siRNA. The RNA isolation was carried out using High-Pure Isolation RNA kits (Roche Applied Science). Cells were harvested and spun down at 10,000 g. The cell pellet was washed in 100 µl 1XPBS and 200 µl of lysis buffer was added. Lysates were transferred to High Pure Filter Tubes and centrifuged at 10,000 g for 15 secs. After this spin, the flow through was discarded from the collection tube. 10 µl/sample of DNAse I was added to 90 µl DNAse Incubation Buffer and the mix was added to the filter tube and incubated for 10 mins at room temperature. 500 µl of Wash Buffer 1 was added to the filter tubes and centrifuged for 15 secs at 10,000 g. 500 µl of Wash Buffer 2 was then added to the filter tube and the tubes were centrifuged as described above. A final wash with 200 µl of Wash Buffer 2 was performed and the tubes were centrifuged for 2 mins to remove any residual buffer from the filter. The tubes were transferred to clean sterile eppendorf tubes and the RNA was eluted by adding 50 µl of Elution Buffer and subsequently centrifuging for 1 min at 10,000 g.

RNA concentration was determined by spectrophotometry using a Nano Drop 2000 (Thermo Scientific).

Reverse Transcriptase PCR was carried out using a master containing 2 µl 5X RT Buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$ and 50 mM DTT), 0.5 µl of dNTP mix (containing 10 mM of each dNTP), 0.5 µl of random primer, 0.25 µl of RNase Out, 0.25 µl RT M-MLV Enzyme and 1.5 µl of RNAse free water. 5 µl of the master mix was then added to clean PCR tubes. 5 µl of isolated RNA sample was added to the PCR tubes with master mix. A reverse transcription reaction was performed using the following cycle: 10 mins at room temperature, 30 mins at 42°C, 3 mins at 95°C. Samples were then stored at -20°C.

qRT-PCR was carried out using Go-Taq® qPCR Master Mix. For each mRNA sample analysed, a separate reagent mix was prepared which contained per sample 2.5 µl Go-Taq® qPCR Master Mix, 0.5 µl of each of the forward and reverse primers of the gene of interest.

2.2.7 mRNA induction

PBMCs were seeded at $1X10^6$ cells/ml in 12 well plates. Cells were transfected with 25 pmol siRNA with 0.2 µl Lipofectamine 2000/per transfection. Cells were transfected with an identical siRNA treatment 24 hrs later. After another 24 hrs cells were stimulated with either 100 ng/ml LPS or 2.5 µg/ml CL075 for the indicated times. When required, the p38 inhibitor (SB 202190) was added 1 hr before stimulation.

2.2.8 mRNA stability

To examine the stability of mRNA, PBMCs were seeded at $1X10^6$ cells/ml. Cells were transfected with siRNA using 0.2 µl Lipofectamine 2000/per transfection and subsequently transfected with siRNA again 24 hrs later. After another 24hrs cells were stimulated with

either 100 ng/ml LPS or 2.5 μg/ml CL075 for 2 hours followed by treatment of 5 μg/ml Actinomycin D (Act D) and either LPS and CL075. Cells were harvested and RNA analysis was carried out as described above.

2.2.9 Immunoprecipitation

HEK293 cells or MEF cells were seeded at a density of $1X10^5$ cells/ml in 100 mm dishes and were incubated at 37°C for 24 hrs prior to transfection or stimulation. For transfection the cells were transfected with a total of 8 μg of DNA using lipofectamine 2000 (Invitrogen). The cells were then returned to the incubator and left for 24 hrs before harvesting or stimulation. For endogenous IPs, the cells were seeded as described above and left 24 hrs before stimulation. After stimulation with respective TLR agonists or IL-1, cells were harvested on ice by washing the cells firstly with 2 mls of ice cold PBS. The PBS was then removed and the cells were scraped into a further 1 ml of ice cold PBS. The cells were then pipetted into a 1.5 ml eppendorf and centrifuged for 10 mins at 10,000 g. The supernatant was discarded and the cell pellet was resuspended with 850 μl 1% NP40 lysis buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 200 mM NaCl,1% NP40, 10% (v/v) glycerol containing protease inhibitors (10 μl/ml Aprotinin, 10 μl 1 mM PMSF and 10 μl 1 mM sodium orthovanadate and protein cocktail inhibitor) for 1 hr on ice. For ubiquitination experiments Iodoacetamide was also added to the cells, which inhibits ubiquitin specific proteases. After 1 hr, 50 μl of the cell lysate was removed and the remaining 800 μl was used in immunoprecipitation. The 50 μl of lysate that was removed initially was boiled in SDS-containing sample buffer and was set aside for analysis by SDS-PAGE.

Lysates were immunoprecipitated with antibody which was precoupled to protein A-sepharose beads. The protein A beads were washed three times with the lysis buffer. 40 µl of the beads were added to an eppendorf per sample. The required amount of antibody was added (1-4 µl per reaction). The antibody was coupled to the beads with continuous rolling at 4°C overnight. Each 800 µl lysed sample was added to the 40 µl of precoupled antibody and beads. The immunoprecipitation was then incubated with continuous rolling at 4°C for two hours. The samples were then centrifuged for 10 mins at 10000 g and washed four times in lysis buffer. 30 µl SDS-sample buffer (62.5 mM Tris, pH 6.8, 2 % (w/v) SDS, 50 mM DTT 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue with the DTT being added just before use) was added and the samples were boiled for 5 mins in order to prepare the sample for western blotting.

2.2.10 Western Blotting

The lysates and immunoprecipitation were analysed by sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) electrophoresis. 10% SDS-PAGE was used to resolve the samples. The gels were run at 100 V for approximately 1.5 hrs. Following electrophoresis, the gels were transferred to either PVDF or nitrocellulose membranes using a wet transfer system. Nitrocellulose was used for examining endogenous ubiquitination. The transfer was carried out for 1 hr at 100 V. Following transfer, the PVDF membranes were allowed to dry in order to fix the proteins into the membranes. The blots were then reactivated in methanol and then rinsed in water. A blocking solution of 5% (w/v) milk in PBS-Tween was used to block the PVDF blots at room temperature for 1 hr. For detection of *p*-p38 or IκBα the membranes were blocked with a blocking solution of 3% (w/v) BSA in TBS-Tween. The blots were then washed three times either PBS-Tween or TBS-Tween (for detection of

phosphorylated proteins) and then incubated with the primary antibody solution diluted in the blocking solution overnight at 4°C with continuous rolling. The blots were subsequently washed three times with PBS-Tween/TBS-Tween prior to incubation with the secondary antibody for one hour at room temperature. Next the blots were washed four times in total, three washes with PBS-Tween/TBS Tween and the final wash in PBS. Enhanced chemiluminescence (ECL) solution was added to the membranes. (ECL is made of Solution A which is composed of 1 ml luminal stock (250 mM luminal stock (0.44 g/10 ml DMSO)),0.44 ml p-Courmaric acid stock (90 mM p-Courmaric acid stock 0.15 g/10ml DMSO), 10 ml 1M Tris-HCL, pH 8.5 and a final volume brought to 100 mls. Solution B is composed of 10 ml 1M Tris-HCl pH 8.5 brought up to a final volume of 100 mls. 1 ml of Solution A and 1 ml Solution B are added together with 0.61µl H_2O_2 /per blot. The ECL was then added to the blots and left for 2 mins. The membranes were then placed in a cassette and film was placed over the membranes in the cassette and developed to detect the HRP-tagged antibodies.

2.2.11 ELISA

Cytokine production was measured using the "sandwich" Enzyme-Linked Immuno-Sorbent Assay (ELISA) method. Polysterine microplates were prepared in the following way: the capture antibody was diluted to the working concentration in PBS and a 100 µl per well was added to 96-well microplate (high binding). The plate was sealed and incubated overnight at room temperature. The next day each well was aspirated and washed with wash buffer (0.1% PBS-Tween) three times in total, ensuring complete removal of liquid. The plate was subsequently blocked by adding 300 µl of reagent diluent (1% (w/v) BSA/PBS-Tween) to each well and the plate was incubated for a minimum of 1 hr at room temperature. The plate was aspirated and washed as before The ELISA assay procedure was then carried out:

100 µl of sample or standard was diluted in reagent diluent and added per well. The plate was then sealed and incubated for 2 hrs at room temperature. The plate was aspirated and washed as before. 100 µl of detection antibody, which was diluted in reagent diluent, was added to each well. The plate was sealed and covered with an adhesive strip and incubated 2 hrs at room temperature. The plate was aspirated and washed as before. 100 µl of the working dilution of streptavidin-horseradish peroxidase (HRP) was added per well. The plate was covered and incubated for 20 mins at room temperature away from direct light. The plate was again aspirated and washed as before. 100 µl of substrate solution was added to each well and the plate was placed away from direct light. 50 µl of stop solution was then added per well. The colour change was read on the Multiscan Accent spectrometer (BioSciences, Dublin, Ireland). The optical density was set at 540nm- 450nm, this subtraction corrected for optical imperfections in the plate thus making the reading more accurate.

2.2.12 Luciferase reporter gene assay

After transfection of 96 well plate, the medium was removed from the plate and cells were lysed with 50 µl passive lysis buffer (Promega) with shaking for 15 mins. 20 µl of the lysates were placed into two different plates to analyse Firefly and *Renilla* luciferase activity. For Firefly-luciferase, 40 µl of luciferase assay mix 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H2O$, 2.67 mM $MgSO_4$, 0.1 M EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferin and 530 mM ATP) was added to 20µl of lysates. For *Renilla* luciferase activity 40 µl of coelentrazine (2 µg/ml in 1xPBS) was used as a substrate. The samples were analysed using a Reporter microplate luminometer (Turner designs). Firefly luminescence readings were corrected for *Renilla* activity and expressed as fold induction over unstimulated empty vector control.

2.3.1 TLR activation of NFκB transcription factor activation in a reporter gene assay.

In order to examine the activation of NFκB by TLR8, HEK293 cells stably transfected with the TLR8 receptor were used. These cells were stimulated with the TLR8 ligand CL075 and a reporter gene assay examining NFκB was used. NFκB reporter gene plasmid contains a luciferase gene downstream from the promoter of the interest. Activation of the promoter of interest leads to expression of the luciferase gene, which is then assayed as a measure of the induction of the promoter of interest. As a constitutively active internal control, 20 ng per transfection of *Renilla*-luciferase gene reporter construct was added to the cells. HEK293_TLR8 cells were seeded at $1X10^5$/ml and transfected 16-18 hrs later with 60 ng of NFκB and 20 ng of TK-Renilla. Up to 150 ng of empty vector control pcDNA3.1 was added to bring the final concentration of DNA to 230 ng. The cells were transfected with 0.8 µl of Gene Juice ™ and diluted with 9.2 µl SFM. 16-18 hrs later the cells were stimulated with a dose response of the TLR8 ligand CL075 namely 0.1, 0.5, 1, 2.5 and 5 µg/ml. NFκB activity was measured by luciferase reporter gene activity as described above (2.2.12).The above protocol was used for examining TLR8 activation of the ISRE also.

2.3.2 Examining the activation of p38 MAP kinase via the CHOP assay

HEK293_T cells were seeded at $1x10^5$ /ml. For p38 MAP kinase reporter assay, 0.25 ng of CHOP-GAL4 fusion vector, 20 ng of TK-renilla and 60 ng of pFR-luciferase were transfected into HEK293_T cells 16-18 hrs later. Up to 150 ng of empty vector control pcDNA3.1 was added to bring the final concentration of DNA to 230 ng. The cells were transfected with 0.8 µl of Gene Juice™ and diluted with 9.2 µl SFM. 16-18 hrs, cells were harvested and lysed with passive lysis buffer and luciferase activity was measured (2.2.12).

2.3.3 Examining the activation of IRF5 and IRF7.

To examine the activation of IRF5 or IRF7, HEK293_TLR8 were seeded at $1X10^5$/ml and transfected 16-18 hrs later with either 1, 2.5 or 5 ng of GAL4-IRF5 or GAL4-IRF7, 60 ng of pFR-luciferase and 20 ng of TK Renilla. 150 ng of empty vector control pcDNA3.1 was added to bring the final concentration of DNA to 230 ng. The cells were transfected with 0.8 µl of Gene Juice ™ and diluted with 9.2 µl of SFM. 16-18 hrs later the cells were stimulated with 2.5 µg of the TLR8 ligand CL075 and harvested 24 hrs later. Cells were lysed with passive lysis buffer and IRF5 and IRF7 activation was measure by luciferase reporter gene assay (2.2.12).

2.3.4 siRNA transfection in 96 well plate

HEK293_TLR8 or HEK293_R1s were seeded at $1X10^5$/ml and treated with siRNA as described above (2.2.5) 24 hrs later. An identical second transfection of siRNA with lipofectamine 2000 was carried out 24 hrs later. Cells were also transfected with 60 ng of NFκB, 20 ng of TK-Renilla and up to 150 ng pcDNA 3.1 (empty vector control). To examine the efficacy of the siRNA cells were also transfected with 50ng of WT IRAK-2. After a further 24 hrs, the cells were harvested. In order to examine the role for IRAK-2 in TLR8 signalling to NFκB, HEK293_TLR8 were seeded at $1X10^5$/ml and treated with siRNA as described above (2.2.5) 24 hrs later. An identical second transfection of siRNA with lipofectamine 2000 was carried out 24 hrs later. Cells were also transfected with 60 ng of NFκB, 20 ng of TK-Renilla and 150 ng pcDNA 3.1 (empty vector control). After a further 24 hrs the cells were stimulated with 2.5 µg/ml CL075 and harvested 6 hrs later. NFκB activity was measured by luciferase reporter gene assay.

2.3.5. Detection of myc-tagged IRAK-2 and endogenous IRAK-2 protein

HEK293_TLR8 or PBMC cells were seeded in 96 well plates at $1X10^5$/ml and subject to siRNA treatment as described above. Cells were lysed in passive lysis buffer and 10 µl of lysate was subject to immunoblotting for detection of protein levels. For Myc-tagged IRAK-2 protein, the blots were blotted with a monoclonal α-myc antibody (1:1000) and secondary antibody (1:4000) and detected using ECL. For detection of endogenous IRAK-2, lysates were subject to immunoblotting and probed with an α-IRAK-2 antibody (1:3000) and secondary rabbit antibody (1:8000). In both cases blots were re-probed with monoclonal β-actin (1:1000) and secondary mouse antibody (1:5000) to ensure equal protein levels.

2.3.6 siRNA transfection for 6 well plate in PBMCs

PBMCs were seeded in 6 well plates at $2X10^6$ /ml. Cells were transfected with 50 pmol of siRNA 1 hr later. A second identical transfection of siRNA with lipofectamine 2000 (Invitrogen) diluted in serum free RPMI was carried out 24 hrs later. After a further 24 hrs the cells were stimulated with 2.5 µg/ml CL075 or 100 ng/ml LPS and harvested at indicated time points.

2.3.7 siRNA transfection for 12wp in PBMCs

PBMCs were seeded at $1X10^6$ /ml in 12 well plates. Cells were transfected with 25 pmol with 0.2 µl lipofectamine 2000 diluted with serum free RPMI/per transfection. Cells were transfected with an identical siRNA transfection 24 hrs later. After another 24 hrs, cells were stimulated with either 2.5 µg/ml CL075 or 100 ng/ml LPS. Cells were harvested at the times indicated.

2.3.8 Assay of 3'UTR reporter genes

HEK293_R1 cells were seeded at $1X10^5$/ml and transfected 16-18 hrs later with either 60 ng of TNF, IL-8 or β-actin 3'UTR reporter construct. The TK-Renilla construct was included to normalise the data for transfection efficiency. Where indicated 10 or 50 ng of MyD88, MAL, TRIF, TRAM, WT TRAF6, C70A K124R or DN TRAF6 were co transfected with reporters. Similarly, either 5-25 ng of WT IRAK-2, or 25 ng E528A IRAK-2 or K237A IRAK-2 were cotransfected with reporters. The total amount of DNA transfected was kept constant at 230ng by the addition of empty vector control pcDNA3.1. The cells were transfected with 0.8 µl of Gene Juice ™ and diluted with 9.2 µl SFM. Cells were harvested and luciferase activity was measured 24 hrs following transfection.

2.3.9 Detecting endogenous ubiquitination of TRAF6

MEFs were seeded in 100 mm dishes. 24 hrs later the cells were subject to serum starvation for three hrs and subsequently stimulated with 100 ng/ml of IL-1. The cells were harvested at the time points indicated (0, 2, 5 and 10 mins), lysed on ice and subject to immunoprecipitation. 4 µl of TRAF6 antibody was used for immunoprecipitating each sample (see 2.2.9 for Immunoprecipitation protocol). Lysates and Immunoprecipitated samples were subject to immunoblotting (see 2.2.10 for immunoblotting protocol). For detecting endogenous ubiquitin, gel was transferred using nitrocellulose and transferred overnight at 20 V. The membrane was then subject to fixation for 30 mins using a fixation buffer. The membrane was then washed 3X PBS. A blocking solution of 20% FCS/PBS-Tween was used for blocking the nitrocellulose membranes. The blots were then washed three times in PBS-Tween and then incubated with the primary monoclonal ubiquitin antibody

(1:1000) solution diluted in the blocking solution overnight at 4°C with continuous rolling. The blots were subsequently washed three times with PBS-Tween prior to incubation with the secondary mouse antibody (1:4000) for one hour at room temperature. Next the blots were washed four times in total, three washes with PBS-Tween and the final wash in PBS. Enhanced chemiluminescence (ECL) solution was added to the membranes and left for 2 mins. The blots were then exposed to film and the film developed.

2.3.10 Detection of IRAK-2-dependent formation of ubiquitin chains conjugated to TRAF6

HEK293_T cells were seeded in 100 mm dishes as described above (2.2.1). The cells were transfected using Gene Juice™ with a total of 8 µg of DNA per dish (1 µg HA-ubiquitin plasmid, 0.5 µg of IRAK-2, and 6.5 µg pcDNA empty vector). 24 hrs later the cells were then harvested and subject to immunoprecipitation (2.2.9) with TRAF6 antibody (4 µl per sample). The immunoprecipitated samples were then subject to immunoblotting as described above (2.2.10 or for detection of endogenous ubiquitin (2.3.9)). Blots were probed with either HA-antibody (1:1000) or mouse monoclonal antibody to ubiquitin and secondary mouse antibody (1:8000). The samples were also probed for TRAF6 to ensure equal pull down of TRAF6 in the IP. The lysates were probed for HA-ubiquitin, myc-IRAK-2 or HA-IRAK-2 also to ensure equal protein levels. ECL was added to the membranes and then left for two minutes. The blots were then exposed to film and the film developed.

2.3.11 SDS precipitation for ubiquitin assay

For SDS precipitation experiments, cells were immunoprecipitated as described above (2.2.9). After immunoprecipitation of the cells, the beads were incubated in 20 µl of 1% SDS solution and incubated in 70°C for 5 mins. Beads were then spun down and 20 µl was

removed and diluted to a final concentration of 0.1% SDS. This was then repeated once more to ensure all removal of protein from the beads. The 0.1% solution was then re-immunoprecipitated with fresh beads conjugated to TRAF6.

2.3.12 Detection of free polyubiquitin chains

Cells were lysed for 1.5 hrs in 850 ml 1% NP40 lysis buffer. Cells were spun down for 10 mins at 10,000 g. 50 µl of lysate was removed and 3xSB was added to this lysate. 800 µl was then added to 4 µl of TRAF6 antibody and rotated for 1 hr at 4˚C. 40 µl of protein A beads were then added to lysate and antibody for another 1-2 hr. Beads were then washed x3 in 1% NP40 lysis buffer (with inhibitors). The beads were then resuspended in 1 ml lysis buffer and split in half and spun down and all supernatant was removed. To one half, 10 µl of IsoT in buffer was added and to the control samples 10 µl of lysis buffer was added. Both samples were incubated at 30˚C for 1 hr. The beads were then washed three times and resuspended in 30 µl of 3xSB.

Chapter 3:

The role of IRAK-2 in TLR8 signalling

3.1 Introduction

The IRAK family are defined as intracellular kinases that play a significant role in the innate immune system as they are involved in regulating TLR and IL-1 signalling pathways [157]. The family comprises of four members IRAK-1, IRAK-2, IRAK-M and IRAK-4 [157]. IRAK-2, discovered in 1997, was initially implicated in TLR and IL-1 signalling. Initial reports showed that IRAK-2 when overexpressed activated NFκB [161]. In addition, it has been shown to interact with TLR signalling members such as MyD88 and MAL [85, 161]. Despite these initial observations, the relative role for IRAK-2 in innate immune signalling remained largely unknown for some time. Given that IRAK-2 is structurally and functionally similar to IRAK-1, it has always been assumed that the function of IRAK-2 was redundant with that of IRAK -1.

The importance of IRAK-2 in TLR-induced NFκB activation was discovered through studies of Vaccinia Virus (VACV). VACV, a large dsDNA virus, is a member of the poxviridae family and is known to encode proteins that antagonise important components of host-viral defence. A52 is one such protein that is encoded by VACV. A52, shown to be important for virus virulence, inhibited all TLR-induced NFκB pathways [195]. It was shown to interact with both TRAF6 and IRAK-2 (but not IRAK-1). Furthermore A52 was shown to inhibit TLR-induced NFκB activation solely through this interaction with IRAK-2 [106].

Thus given that A52 specifically targeted IRAK-2 to inhibit TLR-induced NFκB activation this suggested that IRAK-2 may have an important role in NFκB activation

that was not redundant with IRAK-1. Furthermore, we hypothesised that there was a dichotomy in IRAK-1 and IRAK-2 function and that these proteins are not functionally redundant as originally presumed. For example it now seems that IRAK-1 is more important in IRF activation than NFκB activation in certain contexts.

One particular study of interest showed that IRAK-1 was completely dispensable for TLR7/TLR9-mediated NFκB and MAP kinase in pDCs [167]. In these cells IRAK-1 was shown to directly phosphorylate IRF7 *in vitro* and the kinase activity of IRAK-1 was shown to be critical for transcriptional activation. Thus this study was the first indication that IRAK-1 may have a more essential role in TLR-induced IRF activation than NFκB [167]. It is worth noting that A52 had no effect on TLR-induced IRF activation, which suggested that IRAK-2 is not required for the TLR-IRF axis.

Studies from this lab have now shown an important requirement for IRAK-2 in NFκB activation. Knockdown of human IRAK-2 expression by siRNA impaired NFκB by TLR3 and TLR4 [106]. Here I wished to extend these studies and examine the role of IRAK-2 in the TLR8 pathway. While signal transduction of other TLR pathways, such as TLR3 and TLR4 has been studied extensively, less is known about TLR8 and the mechanisms of its signalling pathways. TLR8, along with TLR7, are located endosomally and are known to recognise ssRNA. While the function of TLR7 has been partially established due to the availability of a TLR7 $^{-/-}$ mice, there is no functional murine TLR8. With the use of TLR7 and TLR8 selective agonists it is known that while both TLR7 and TLR8 can induce IFNα, TLR8 preferentially induces

proinflammatory cytokines such as TNFα, IL-6, IL-8 and IL-12, whereas TLR7 strongly induces IFNα and IP-10 [113].

TLR8 signalling was also of interest since IRAK-1 has been shown to be dispensable for activation of TLR7-induced NFκB activation in mice [167]. Thus IRAK-2 may have a more important role than IRAK-1 in NFκB activation in the TLR7/8/9 axis.

3.2 Results

3.2.1 TLR8 signalling in human transformed cells.

Previous work in HEK293 cells stably expressing either TLR3 or TLR4 revealed a critical role for IRAK-2 in TLR3- or TLR4-induced NFκB-dependent reporter gene activity [106]. Thus, in order to examine the role of TLR8 in this system, firstly it was necessary to establish which signalling pathways were activated in response to the TLR8 ligand CL075. CL075 (3M002) is a thiazoloquinolone derivative that preferentially stimulates TLR8. It is known to activate NFκB [113]. A reporter gene assay was used to test the activation of NFκB. For this, HEK293 cells that were stably transfected with the TLR8 receptor were transfected with an NFκB-luciferase reporter plasmid. The cells were then stimulated 24 hrs later with an increasing dose of CL075. Cells were harvested six hrs after stimulation and subsequently lysed in passive lysis buffer. The results clearly show that TLR8 activates NFκB in a dose-dependent manner (Figure 3.1). The optimal dose of CL075 was 5 μg/ml although over a number of experiments it was revealed that 2.5 μg/ml CL075 could activate NFκB to a similar degree as 5 μg/ml and thus was used in subsequent experiments.

3.2.2 CL075 activates the ISRE in human transformed cells

Having shown that CL075 activates NFκB, it was next examined whether CL075 induces activation of the Interferon Stimulated Response Element (ISRE). The ISRE is found in many interferon responsive genes and can be stimulated by active IRF5 and IRF7, which are downstream targets of TLR8 signalling (REF). HEK293_TLR8 cells were

transfected with an ISRE-luciferase plasmid as described in methods (see section 2.3.1). The cells were stimulated with increasing doses of CL075 24 hrs later. The cells were harvested 6 hrs later and lysed in passive lysis buffer. Figure 3.2 shows that CL075 induced the ISRE-luciferase activity in a dose-dependent manner. TLR8 activation of the ISRE was shown to be quite weak compared to the positive control poly I:C.

3.2.3 CL075 activates IRF5 and IRF7.

As mentioned previously TLR8 has a key role in antiviral immunity. It would be expected therefore that TLR8 activates IRFs. Schoenemeyer *et al.* showed that neither TLR7 nor TLR8 activated IRF3, while TLR7/8-induced IRF5 and IRF7 were required for IFN induction [69]. This suggested that IRF5 and IRF7 were important mediators of the anti viral response for TLR7/8 signalling [69]. In order to test whether IRF5 and IRF7 activation was detectable in this system, a GAL4 transactivation assay was used. This assay utilises hybrid proteins consisting of the yeast GAL4 DNA-binding domain fused to IRF5 or IRF7 lacking its own DNA-binding domain. In this assay the GAL4-dependent pFR-luc reporter requires phosphorylation of the corresponding IRF-GAL4 fusion protein. Here HEK293_TLR8 cells were transfected with either 1, 2.5 or 5 ng of GAL4-IRF5 (Figure 3.3 (A)) or GAL4-IRF7 (Figure 3.3 (B)) and co transfected with PFR-luciferase reporter. Cells were stimulated 24 hrs later and harvested after another 24 hrs. As shown in Figure 3.3 TLR8 activated both IRF5 and IRF7 quite robustly in response to CL075.

Thus CL075, the TLR8 ligand, was shown to activate the NFκB axis, IRF5 and IRF7. Keating *et al.* showed previously that IRAK-2 has an important role in TLR3 and TLR4-induced NFκB activation [106]. Knockdown of IRAK-2 expression with siRNA led to a decrease in NFκB activation upon stimulation of TLR3 with poly I:C and TLR4 with LPS [106]. Therefore in order to examine the role for IRAK-2 in TLR8 induced NFκB, two IRAK-2 sequence specific siRNA oligonucleotides that targeted the human Irak-2 gene were chemically synthesised by Qiagen. The following sites were targeted:

IRAK-2A 5' CAGCAACGTCAAGAGCTCTAA

IRAK-2B 5' CCAGATCATCCTGAACTGGAA

3.2.4 IRAK-2 gene silencing blocks signalling to NFκB by over-expressed IRAK-2

In order to test the efficacy of two IRAK-2 sequence-specific siRNA oligonucleotides, the ability of siRNAs to reduce expression of over-expressed IRAK-2 was first assayed. To do this, HEK293_TLR8 cells were transfected with siRNA as described in methods (section 2.2.5) together with an expression vector encoding IRAK-2-myc. Cells were harvested 24 hrs later. To quantify IRAK-2 expression, cells were lysed, subject to SDS-PAGE and immunoblotted with an anti-myc antibody. The results clearly show that IRAK-2-myc protein expression from a transfected expression vector was completely ablated in the presence of either IRAK-2 A or IRAK-2 B siRNA (Figure 3.4 (A) lanes 5-8 upper panel) compared to cells treated with a non-silencing control siRNA (Figure 3.4 (A) lanes 2, 3 and 4 upper panel). In lanes 2-4 where the cells were treated with no siRNA or a negative non silencing control siRNA, the myc-IRAK-2 protein was highly expressed. In lanes 5-8, where the cells were transfected with

siRNA targeting IRAK-2, protein expression can be seen to be completely ablated. Immunoblotting with an anti-β-actin antibody confirmed that equal protein amounts were loaded in each lane (Figure 3.4 (A), lower panel).

The efficacy of the two IRAK-2 sequence specific siRNA oligonucleotides was also tested by examining the effect of these siRNAs on activation of an NFκB-luciferase reporter by over-expressed IRAK-2-myc (Figure 3.4 (A)). HEK293_TLR8 cells were transfected with siRNA and a reporter gene assay was used to examine the effect on NFκB activation (see 2.2.5 in methods). The cells were lysed 24 hrs later. While the negative control siRNA had no effect on over-expressed IRAK-2-induced NFκB activity, NFκB activity was completely blocked in the cells treated with siRNA targeting the IRAK-2 gene (Figure 3.4 (B)).

3.2.5 IRAK-2 is required for signal transduction from TLR8 to NFκB

Since previous commercial IRAK-2 antibodies used in the lab proved not to be effective in detecting endogenous IRAK-2 by immunoblotting, a new antibody was generated using the following IRAK-2 peptide as an antigen: ($NHCOCH_3$) CADVYRGHRHGKPFVFK($CONH_2$) (Inbiolabs). This antibody proved to be effective in detecting endogenous IRAK-2 (Figure 3.5, (A)). Thus using this antibody it was possible to demonstrate knockdown of endogenous IRAK-2 protein levels. Although both oligonucleotides were shown to decrease IRAK-2 expression (Figure 3.4) IRAK-2B appeared to inhibit more consistently. Therefore in this experiment and subsequent experiments only IRAK-2B was used. HEK293_TLR8 cells were treated

with either 5 or 10 pmol siRNA as described in methods. Cells were stimulated with CL075 and immunoblotted with anti-IRAK-2 antibody (Figure 3.5 (A), upper panel). The results showed that IRAK-2 protein levels decreased in the presence of siRNA targeting IRAK-2 compared to cells that were treated with negative or no siRNA (Figure 3.5 (A), upper panel). The higher dose of siRNA (10 pmol) achieved greater knockdown of IRAK-2 protein levels (Figure 3.5 (A), upper panel, lane 4). Re-probing the immunoblot with anti-β-actin antibody confirmed equal protein loading in all lanes (Figure 3.5 (A), lower panel).

Since the IRAK-2 siRNA was shown to be capable of inhibiting activation of NFκB by over-expressed IRAK-2 and reducing endogenous IRAK-2 expression, it was further used to test the role of endogenous IRAK-2 in TLR8 activation of NFκB. After siRNA treatment, cells were stimulated with CL075 and harvested 6 hrs later. In samples which received no siRNA treatment, CL075 stimulated NFκB as normal. However treatment of cells with siRNA targeting IRAK-2 diminished stimulation of the NFκB reporter gene by the TLR8 agonist very potently and in a dose-dependent manner (Figure 3.5 (B)). Crucially, the samples that were treated with the negative control non-silencing siRNA activated NFκB in a similar way to the non treated stimulated cells. Notably, treatment with the IRAK-2 siRNA oligo had no effect on control levels of NFκB activation. Thus here we demonstrate a significant role for IRAK-2 in TLR8-induced NFκB activation.

In order to demonstrate further that IRAK-2 is required for TLR8 signalling to NFκB, we examined the effect of IRAK-2 siRNA on IκBα, an inhibitory subunit of NFκB, thus its degradation can be used as a marker for NFκB activation. HEK293_TLR8 cells were seeded at $1X10^5$ /ml in 6 well plates and transfected with siRNA described in methods. Cells were stimulated with CL075 for 30 mins and harvested. Cells were lysed, subject to SDS-PAGE and immunoblotted with an antibody against IκBα. Degradation of the NFκB inhibitory subunit IκBα in response to CL075 was inhibited compared to control treated cells confirming that TLR8-induced NFκB activation was also impaired in IRAK-2 siRNA treated cells (Figure 3.6, upper panel, compare lane 3 and lane 4). This is the first demonstration that there is prevention of IκB degradation by IRAK-2 siRNA in human cells. Immunoblotting with an anti β-actin antibody confirmed that equal protein was loaded in each lane (Figure 3.6, lower panel). Thus this shows that the role of IRAK-2 is upstream of IκB degradation in the TLR8 pathway to NFκB.

3.2.6 IRAK-2 is required for TLR4- and TLR8-induced cytokine induction

The data thus far shows that IRAK-2 was required for CL075-induced NFκB activation in reporter gene assays (Figure 3.5). Furthermore we showed that IRAK-2 was required upstream in TLR8 signalling to NFκB by detecting reduced IκB degradation in IRAK-2 siRNA-treated cells (Figure 3.6). Therefore, in order to extend our findings that IRAK-2 is required for NFκB activation, we examined the contribution of IRAK-2

to an NFκB-dependent gene. IL-8 is a proinflammatory chemokine that is NFκB-dependent.

Thus, before testing the role for IRAK-2 in TLR8-induced IL-8 production, firstly we wanted to examine the role for IRAK-2 in TLR4-induced IL-8 production in HEK293_TLR4 cells. Previously it has been shown that IRAK-2 was required for TLR4-induced NFκB in reporter gene assay [106]. In addition, IRAK-2 has also been shown to be required for LPS-induced IL-8 induction in PBMCs [106]. Therefore we wanted to confirm the role for IRAK-2 in TLR4-induced IL-8 in HEK293_TLR4 cells. Cells were treated with siRNA, stimulated with LPS (24 hrs) and assayed by ELISA. As predicted, targeted knockdown of IRAK-2 led to impaired LPS-induced IL-8 production in HEK293_TLR4 cells compared to control treated samples (Figure 3.7).

The role for IRAK-2 in TLR-induced IL-8 production was then examined. HEK293_TLR8 cells treated with either control or IRAK-2 siRNA and stimulated with CL075 (24 hrs). Supernatants from these cells were assayed by ELISA. There was a significant decrease of IL-8 production in cells treated with IRAK-2 siRNA compared to non siRNA-treated cells (Figure 3.8). It was clear from this that IRAK-2 is required in TLR8-induced IL-8 gene induction in human cells.

Thus IRAK-2 is required for both TLR4 and TLR8-induced NFκB cytokine induction in human cells.

This, and previous work [106] clearly demonstrates the role of IRAK-2 in TLR pathways to NFκB in human cell lines. However the relative importance of IRAK-2 in

the murine system was uncertain until recently. In 2008 IRAK-2 knockout mice were generated [197].

3.2.7 Absence of murine IRAK-2 and IRAK-1 inhibits cytokine responses in MEFs

Thus murine embryonic fibroblasts (MEFs) from IRAK-2 knockout mice became available and we used these to examine what effect the absence of IRAK-2 had on TLR responses. It was previously unknown whether the MEFs would respond to CL075, although a study examining TLR expression in MEFs showed that MEFs produce mRNA for TLR7 and TLR8 [269]. If the cells were responsive to CL075, this would serve as a good model for investigating the role of IRAK-2 in TLR7 in the murine system (as TLR8 is inactive in the mouse). In order to test responsiveness of the MEFs to CL075 and to assess the role of IRAK-2 in TLR gene induction in these cells the induction of IL-6 was examined. IL-6 is an NFκB-dependent cytokine. The cells were stimulated with LPS, CL075 and also IL-1. Wild-type (WT) or IRAK-2 KO MEFs were stimulated with 100 ng/ml IL-1, 100 ng/ml LPS or 2.5 μg/mL CL075, the supernatants from this experiment were harvested 24 hrs later and IL-6 production was assayed by ELISA. Figure 3.9 shows that the cells were responsive to all three agonists tested; IL-1, CL075 and LPS. In each case, the WT MEFs showed strong induction of IL-6 (Figure 3.9). In murine embryonic fibroblasts (MEFs) from IRAK-2 $^{-/-}$ mice both CL075 and LPS-induced IL-6 was strongly impaired compared to wild type MEFs (Figure 3.9 (a) and (b)) Thus as is the case for TLR4 and TLR8 in human cells, IRAK-2 was shown to be required for cytokine responses in the TLR4 and TLR7 in the mouse. Murine cells do not express a functional TLR8 and it was demonstrated that

CL075 can activate TLR7 in the mouse. IL-1-induced IL-6 was also impaired in IRAK-2 $^{-/-}$ MEFs compared to wild type MEFs, indicating a role for IRAK-2 in IL-1 signalling also.

3.3 Discussion

In this chapter I examined a role for IRAK-2 in TLR8 signalling. TLR8 is known to have a critical role in the innate immune system, in particular in antiviral immunity [113]. The mechanisms of TLR8 signalling remain the least defined of the TLRs. TLR8 is shown here to activate both NFκB and the IRF axis which would give rise to a broad innate immune response. A critical role for IRAK-2 in TLR3- and TLR4-induced NFκB activation has previously been demonstrated [106]. We extend these findings here to reveal an essential role for IRAK-2 in TLR8 signalling to NFκB. TLR8 signalling was examined as IRAK-1 has been shown to be dispensable for TLR7-induced NFκB activation in mice [167]. Therefore IRAK-2 may have a more important role than IRAK-1 in the TLR7/8/9 axis. The efficacy of the IRAK-2 siRNA oligos was tested firstly on protein expression of over-expressed myc-tagged IRAK-2 (See Figure 3.4). Over-expressed IRAK-2 protein was completely ablated in the presence of the siRNA, in comparison to no siRNA or the non silencing negative control. The siRNAs were further tested in a reporter gene assay. Previously it has been shown that over-expressed IRAK-2 drives NFκB activation [161] and here in the presence of these siRNA oligos, IRAK-2 gene silencing resulted in the blocking of NFκB signalling. This was important as it showed that the siRNA oligos were working optimally.

The lack of any effective commercially available IRAK-2 specific antibodies had made the detection of endogenous IRAK-2 difficult in the past. Thus a new rabbit polyclonal anti-IRAK-2 antibody was synthesised by Inbiolabs and this proved successful. Treatment of HEK293_TLR8 cells with siRNA targeting IRAK-2 led to a

lower abundance of IRAK-2 protein, which was detectable with the endogenous IRAK-2 antibody (Figure 3.5, (A))

The presence of IRAK-2 siRNA decreased both protein expression of IRAK-2 and TLR-induced NFκB activation. Thus, this revealed a role for IRAK-2 in TLR8-induced NFκB activation. In addition the effect of IRAK-2 siRNA on IκB degradation was examined. It was demonstrated that in the presence of IRAK-2 siRNA, IκB degradation was reduced compared to the negative control lanes (Figure 3.6). Significantly, this shows that for the first time the effect of IRAK-2 siRNA on IκBα degradation. This data reveals that IRAK-2 plays a role upstream of IκB degradation on the NFκB axis in TLR signalling.

A role for IRAK-2 in TLR8 signalling was further highlighted by examining the effect of IRAK-2 siRNA on a NFκB-dependent cytokine IL-8. Previously it had been shown that IRAK-2 was required for LPS-induced IL-8 cytokine production in PBMCs [106]. It was revealed here that IRAK-2 was required for both TLR4- and TLR8-induced IL-8 production in HEK293 cells. In addition to this, a role for murine IRAK-2 in TLR-cytokine responses was demonstrated using IRAK-2 $^{-/-}$ MEFs. Thus IRAK-2 is required for cytokine induction in both mouse and human cells.

In 2008 a study from Kawagoe *et al.* revealed the function of IRAK-2 in the murine system [197]. Their study showed that when macrophages from IRAK-2 $^{-/-}$ mice were tested with various TLR ligands (MALP2, LPS, R848, CpG DNA), production of IL-6 and TNF and of the chemokine KC were severely impaired relative to the wild-type cells [197]. Critically, a role for IRAK-2 *in vivo* has also been revealed [197]. IRAK-2 $^{-/-}$ mice

were highly resistant to LPS- and CpG-induced septic shock which indicates a physiologically relevant role for IRAK-2 in TLR signalling in murine cells.

Interestingly, while the IRAK-2$^{-/-}$ mice were resistant to TLR-induced septic shock, in cells derived from these mice early TLR2 signalling events was intact, with IRAK-2 only being required for late and sustained NFκB activation. Only when these IRAK-2 $^{-/-}$ mice were crossed with IRAK-1 $^{-/-}$ mice was a dramatic impairment of NFκB activation by TLR2 observed. A second study with independently generated IRAK-2 $^{-/-}$ mice revealed that IRAK-2 was required for late TLR7-induced NFκB activation [198]. Thus while I have a shown a role for IRAK-2 in TLR8 signalling in NFκB in human cells, this role may differ to the function of IRAK-2 in TLR7. It has been shown with TLR7 and TLR8 specific agonists that TLR7 preferentially triggers type I IFNs while TLR8 preferentially induces proinflammatory cytokines such as TNF and IL-6, therefore the signalling pathways between TLR7 and TLR8 may differ [113]. In addition, a negative role for IRAK-2 in TLR9-induced IFN has recently been described [199].

In further contrast between my data and this study [198], murine IRAK-2 was not required for either early or late TLR4-induced NFκB activation. Therefore the role of IRAK-2 in TLR signalling to NFκB may be Toll and/or species specific.

While it was shown that IRAK-2 was not required for TLR4-induced NFκB in the murine system, it was revealed that IRAK-2 did have a crucial role in TLR4-mediated cytokine production in mice [198]. Thus it appeared that while IRAK-2 in the murine system was shown to have only a subtle role in NFκB activation it was essential for post-transcriptional regulation of cytokines [198] . Therefore the impairment of the

chemokine IL-8 in human cell lines in the presence of IRAK-2 siRNA may be suggestive of a role for IRAK-2 in post-transcriptional regulation in addition to its role in NFκB activation in the human system. A recent study has shown that another IRAK family member, IRAK-1 mediates not only NFκB activation but also IL-1-induced mRNA stabilisation [238]. Signals leading to mRNA stabilisation and NFκB activation diverged at IRAK-1 thus showing a role in IRAK-1 in both pathways. Thus the role of IRAK-2 in TLR signalling events is still unclear and may differ between human cell lines and primary murine cells.

In conclusion, in this chapter I have demonstrated a critical role for IRAK-2 in TLR8-mediated NFκB activation. IRAK-2 was required for activation of NFκB as IκB degradation was impaired in cells treated with IRAK-2 siRNA compared to control treated samples. In addition, IRAK-2 was required for proinflammatory cytokine responses in both mouse and human system.

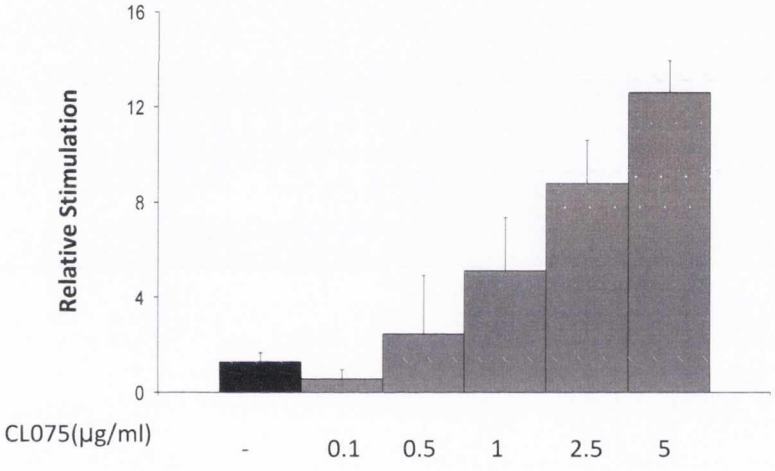


Figure 3.1 CL075 activates NFκB in human cells. HEK293_TLR8 cells were seeded at a density of Ix10^5/ml and transfected 16-18 hours later with NFκB_luc plasmid. The cells were stimulated for 6 hours with the indicated concentrations (µg/ml) of the TLR8 ligand CL075. The data was normalised for transfection efficiency based on renilla values as described in methods. The data is expressed as the mean fold induction ± SD for a single experiment performed in triplicate and is representative of three experiments.

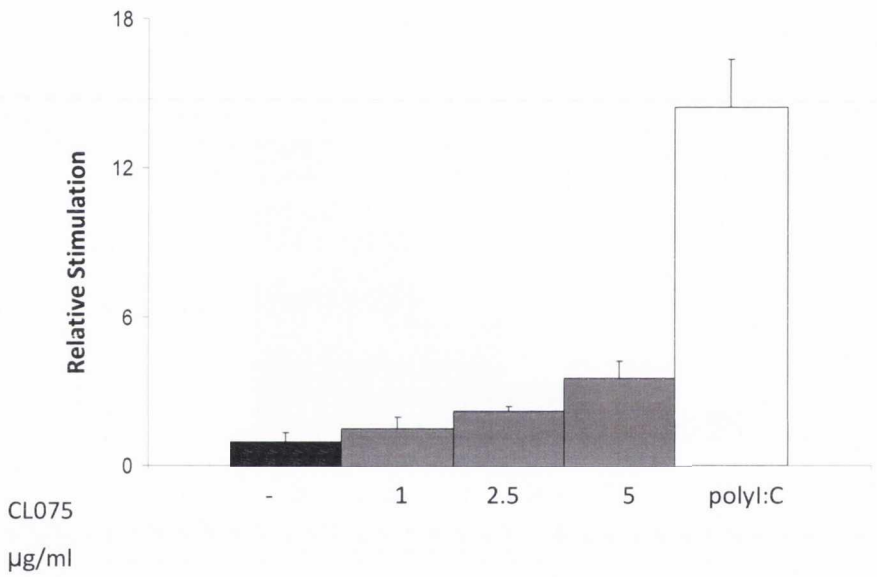


Figure 3.2 CL075 activates the ISRE in human cells. HEK293_TLR8 cells were seeded at a density of $1x10^5$/ml and transfected 16-18 hours later with ISRE. The cells were stimulated for 6 hours with the indicated concentrations (µg/ml) of the TLR8 ligand CL075 respectively. Poly (I:C) was used as a positive control. The data was normalised for transfection efficiency based on renilla values as described in methods. Data is expressed as the mean fold induction ± SD for a single experiment performed in triplicate and is representative of three experiments.

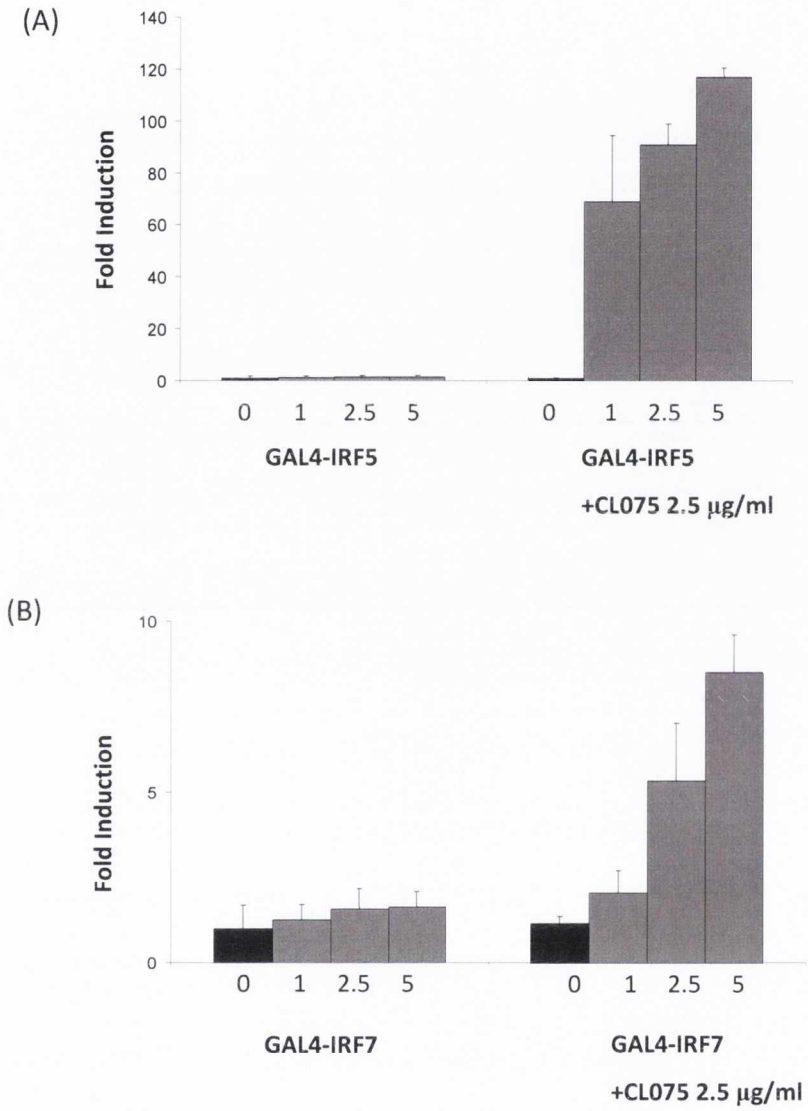


Figure 3.3 CL075 activates IRF5 and IRF7. HEK293_TLR8 cells were seeded at $1x10^5$/ml and transfected 16-18 hours later with either GAL4-IRF5 (A) or GAL4-IRF7 (B). GAL4-IRF5 or GAL4-IRF7 was transfected with 1, 2.5, 5 ng respectively. Cells were stimulated with 2.5 µg/ml CL075 and harvested 24 hrs later. Data are expressed as the mean fold induction ± SD for a single experiment preformed in triplicate and is representative of three experiments.

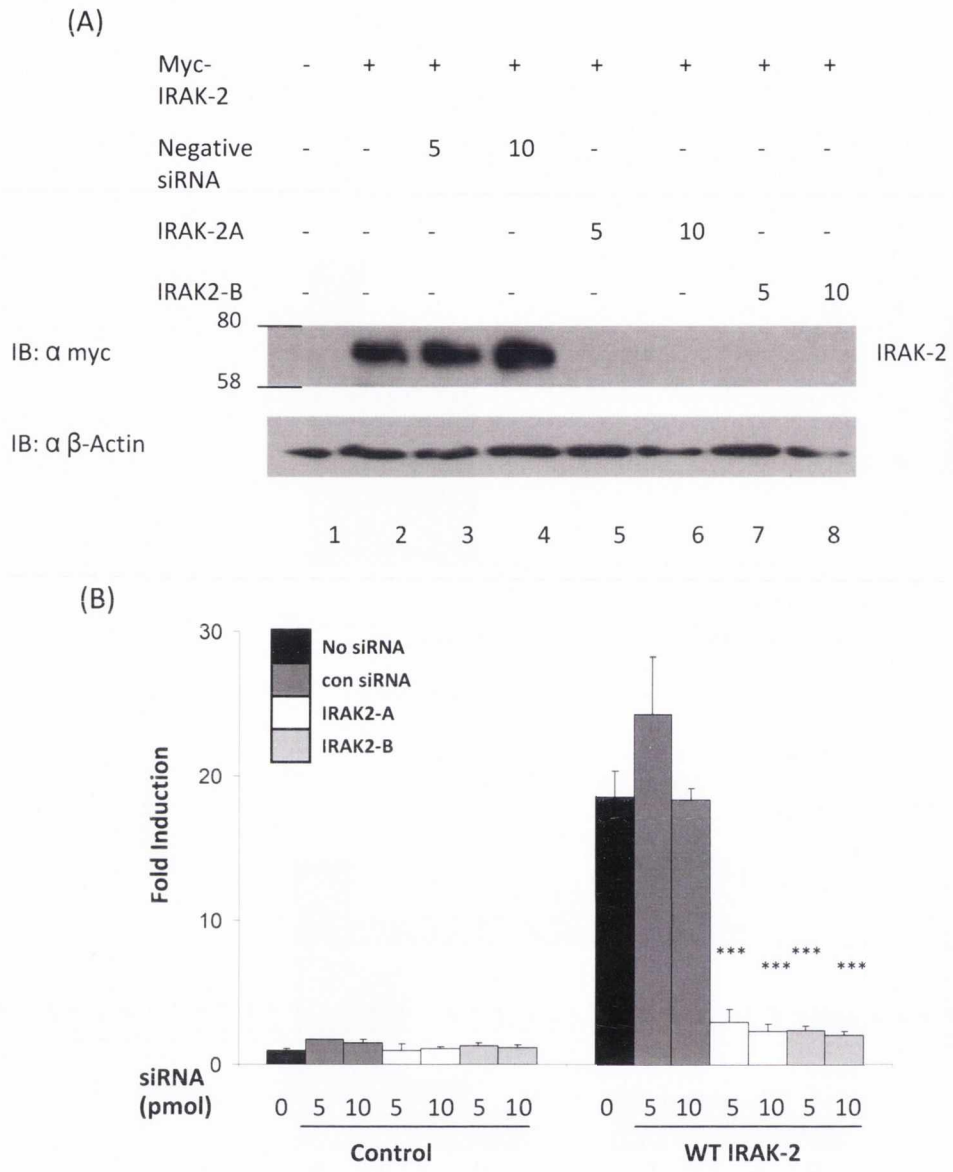


Figure 3.4 IRAK-2 gene silencing blocks signalling to NFκB. HEK293_TLR8 cells were seeded at $1x10^5$/ml and transfected 16-18 hours later with either 5 or 10 pmol of non silencing siRNA or siRNA targeting the IRAK-2 gene (IRAK-2A or IRAK-2B). Cells were treated a second time of 5 or 10 pmol siRNA 24 hours later. (A) Cell lysates assayed for IRAK-2 myc expression by immunoblotting with anti-myc antibody (upper panel). Cells were immunoblotted with anti-β-actin to confirm equal protein levels (lower panel). (B) Cells were also transfected with NF-κB luc plasmid, pcDNA3.1, TK-renilla and where indicated pcDNA3.1(control) or 50ng WT IRAK-2. Data is expressed as the fold induction ± SD for a single experiment performed in triplicate representative of three experiments. ***p<0.0005 compared to control siRNA

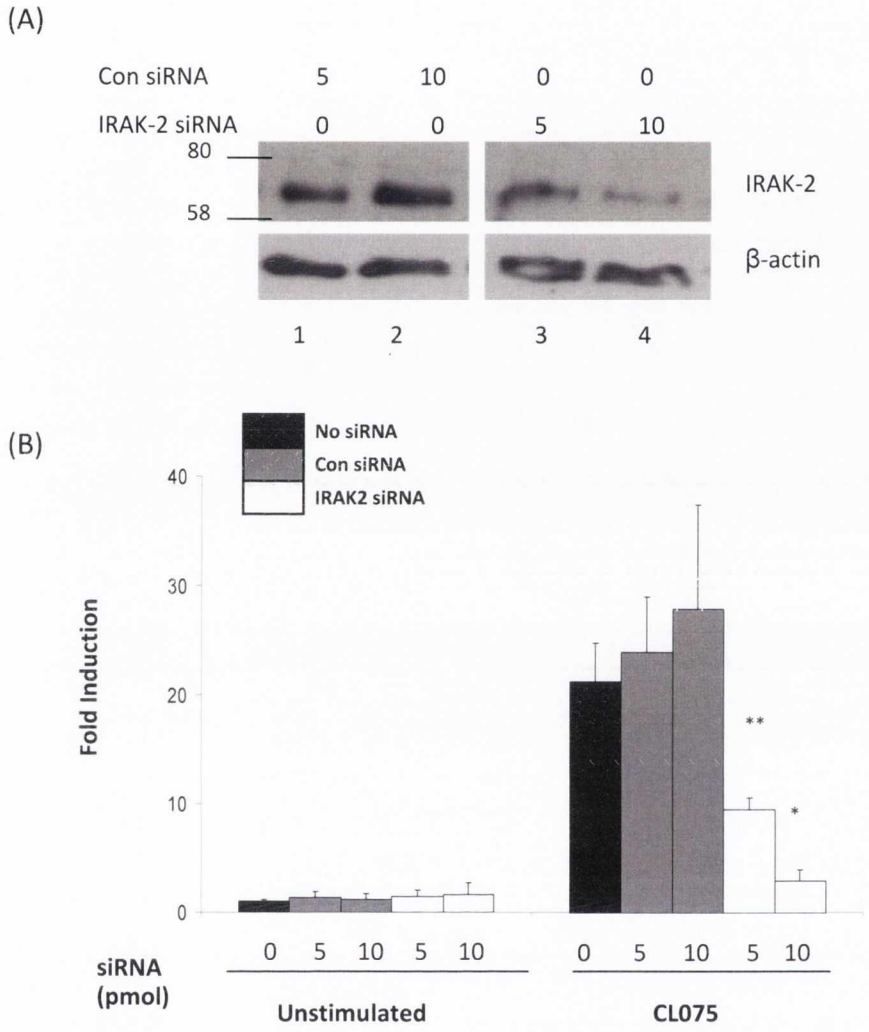


Figure 3.5 IRAK-2 is required for TLR8 signalling to NFκB. HEK293_TLR8 cells were seeded at $1x10^5$/ml and transfected 16-18 hours later with either 5 or 10 pmol of non silencing control siRNA or siRNA targeting the IRAK-2 gene. Cells were treated with second time with 5 or 10 pmol siRNA 24 hours later. 48 hours later, cells were stimulated with 2.5 μg/ml CL075 (6 hours). Cell lysates were examined for endogenous IRAK-2 levels by immunoblotting for IRAK-2 (upper panel) (A). Cell lysates were immunoblotted with β-actin to confirm equal protein levels (lower panel). Cells were also transfected with NF-κB luc plasmid, pcDNA and normalised with TK-renilla (B). Data is expressed as the fold induction ± SD for a single experiment performed in triplicate representative of three experiments. *$p<0.05$, **$p<0.005$ compared to control siRNA.

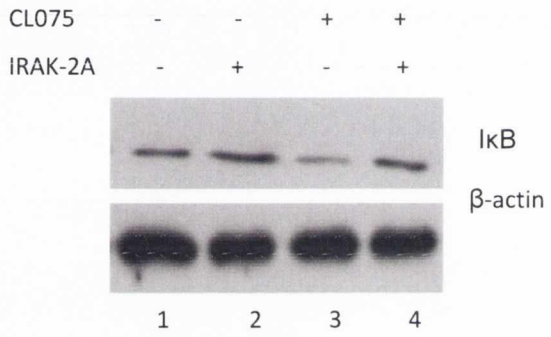


Figure 3.6 IRAK-2 gene silencing reduces the degradation of IκB. HEK293_TLR8s were transfected with 50 pmol of either control non-silencing siRNA (-) or siRNA targeting the IRAK-2 gene (+). Cells were stimulated with 2.5 μg/ml CL075 for 30 minutes and harvested. Lysates were assayed for IκBα degradation by immunoblotting with IκBα Ab (1:1000 α-IκBα, 1: 8000 α-mouse) (upper panel) or probed with β-actin Ab to confirm equal protein levels (lower panel). The experiment is representative of two independent experiments.

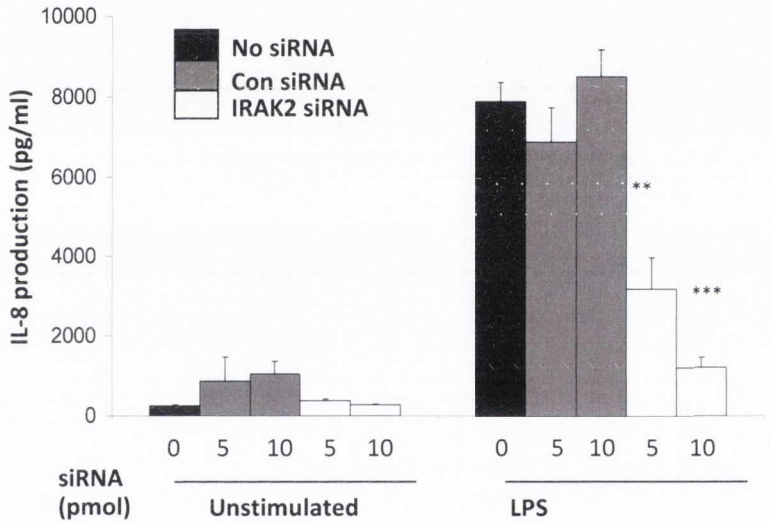


Figure 3.7 IRAK-2 is essential for LPS-induced IL-8 cytokine induction in human cells. HEK293_TLR4 cells were seeded at $1x10^5$/ml and were transfected 16-18 hours later with either 5 or 10 pmol of non-silencing siRNA or siRNA targeting the IRAK-2 gene. Cells were treated a second time with 5 or 10 pmol siRNA 24 hours later. Cells were stimulated with 100ng/ml LPS and IL-8 production in cell supernatants was assayed by ELISA (24 hrs). Data is expressed as mean ± SD from an experiment performed in triplicate and is representative of three experiments. **p<0.005, ***p<0.0005 compared to control siRNA

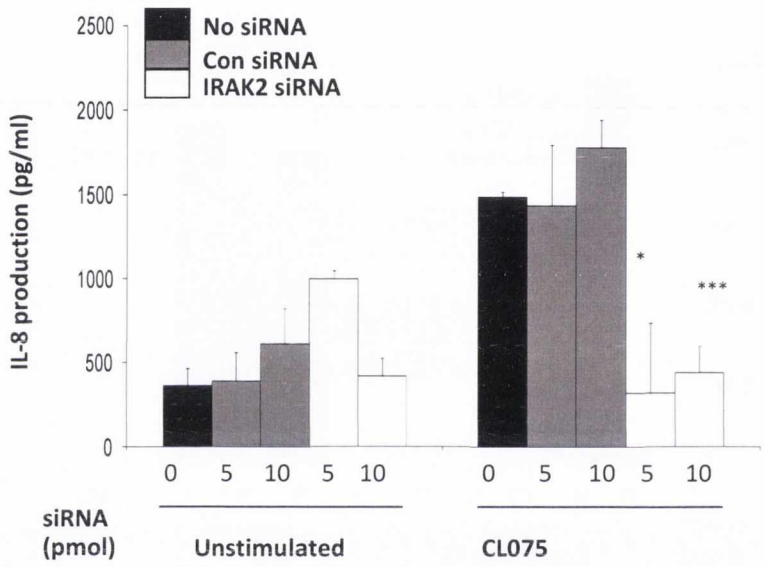


Figure 3.8 IRAK-2 is essential for CL075-induced IL-8 cytokine induction in human cells. HEK 293_TLR8 cells were seeded at $1x10^5$/ml and were transfected 16-18 hours later with either 5 or 10 pmol of non-silencing siRNA or siRNA targeting the IRAK-2 gene. Cells were treated a second time with 5 or 10 pmol siRNA 24 hours later. Cells were stimulated with 2.5 μg/ml of CL075 and IL-8 production in cell supernatants was assayed by ELISA (24 hrs). Data is expressed as mean ± SD from an experiment performed in triplicate and is representative of three experiments. **p<0.005, ***p<0.0005 compared to control siRNA

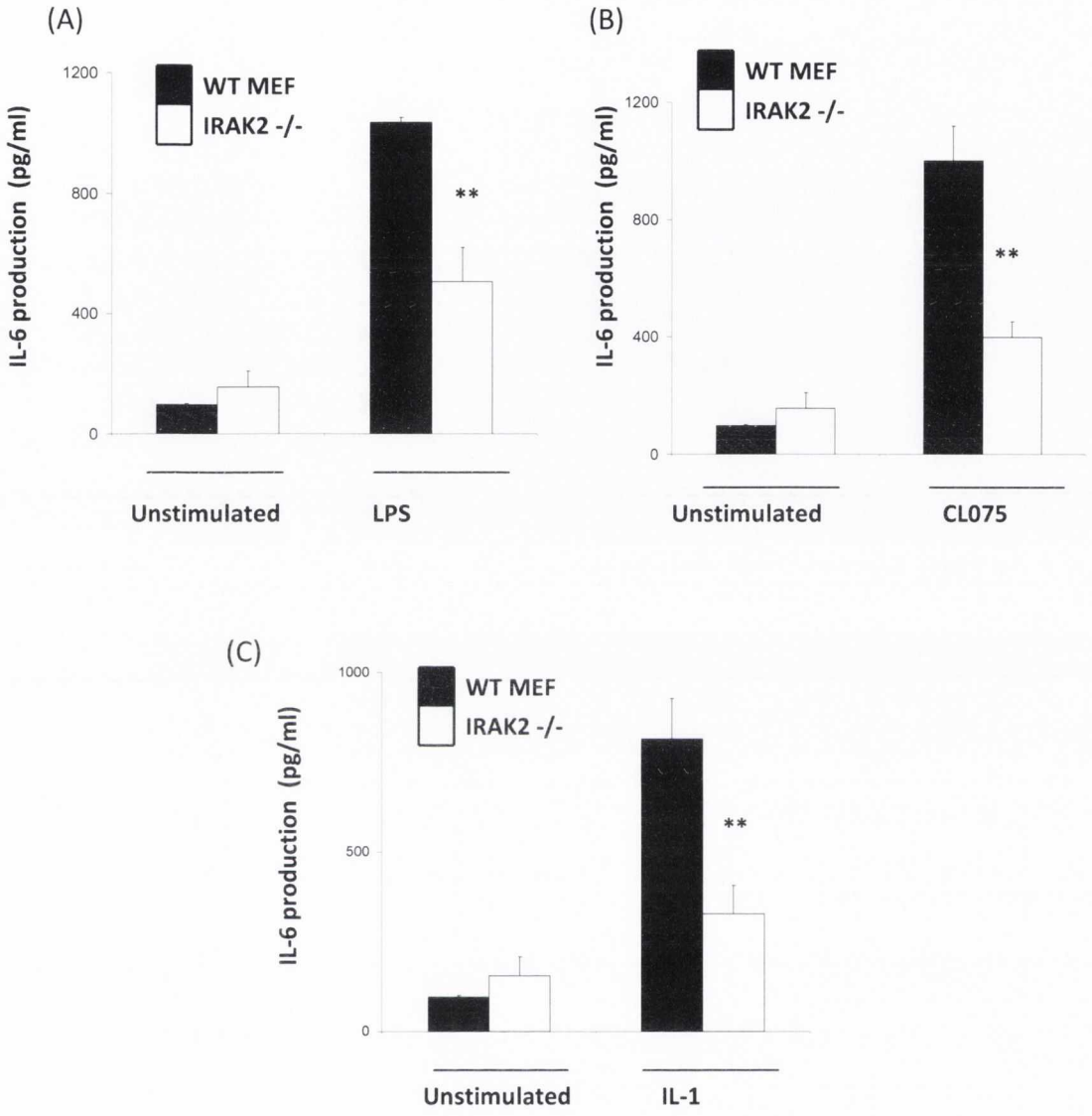


Figure 3.9 Absence of IRAK-2 impairs IL-1/TLR responses in MEFs MEFs were seeded at $1x10^5$/ml and stimulated with LPS 100 ng/ml(A), CL075 2.5 µg/ml (B) or IL-1 20 ng/ml and harvested 24 hours later. The supernatants were assayed by ELISA. The data is expressed as the fold induction ± SD for a single experiment performed in triplicate representative of three experiments. p**<0.005 compared to WT controls

Chapter 4: The role of IRAK-2 in TLR signalling in primary human cells

4.1 Introduction

Previously it was described that IRAK-2 was required for TLR3- and TLR4-mediated NFκB-dependent reporter gene activation in a human cell line [106]. Those findings were extended in the previous chapter by demonstrating a critical role for IRAK-2 in TLR8-induced NFκB activation.

In 2008 IRAK-2 $^{-/-}$ mice were generated and shown to be highly resistant to LPS- and CpG-induced septic shock [197]. 80% of LPS-challenged IRAK-2 $^{-/-}$ mice survived in comparison to the wild-type mice that succumbed to septic shock and all died. Interestingly even though the IRAK-2 $^{-/-}$ mice were resistant to TLR-induced septic shock, in cells derived from these mice, early TLR2 signalling events were intact, with IRAK-2 only being required for late NFκB activation and not MAPK activation. A second group independently generated knockout mice and showed that for TLR7 or TLR9 signalling, IRAK-2 was not required for early NFκB activation but required for sustained activation [198, 199]. Results from IRAK-2 $^{-/-}$ macrophages from this group also revealed that for TLR4, there was no defect in either early or late activation of NFκB [198].

Thus while in human cell lines it appeared that IRAK-2 was important for NFκB activation, there seemed to be a more subtle requirement for IRAK-2 in TLR-induced NFκB in the murine system. Although there was a redundancy of IRAK-1 and IRAK-2 in early NFκB activation, IRAK-2 was shown to be essential for TLR-induced cytokine and chemokine gene expression in murine cells. In IRAK-2 $^{-/-}$ macrophages, TLR2,

TLR4, TLR7 and TLR9-induced proinflammatory cytokine and chemokine production was significantly impaired relative to wild type control [197-199].

One way TLR signalling regulates gene expression is by stabilising otherwise unstable mRNAs that encode proinflammatory cytokines. Many cytokines are rendered unstable due to the presence of adenosine uridine rich elements (ARE) in their 3'UTR. mRNAs can be regulated post-transcriptionally via these ARE-regions by ARE-binding proteins that can positively or negatively regulate mRNA stability. Previous studies have revealed a requirement for IRAK family members in post-transcriptional regulation of cytokine induction in the murine system [216, 238]. Of note, the role for human IRAKs in post-transcriptional regulation is unknown. IRAK-1 has been shown to be required for IL-1-induced KC mRNA stability [238]. Furthermore IRAK-4 has been shown to be required for cytokine and chemokine mRNA stability pathway in the murine system [216]. With the generation of the IRAK-2 $^{-/-}$ mouse it was shown that IRAK-2 also has a role in post-transcriptional regulation of cytokine and chemokine gene expression [198].

TLR4-induced stability of IL-6 and KC mRNA was shown to be regulated by IRAK-2. It was found that the role of IRAK-2 in mRNA stability was ligand specific as there was no role for IRAK-2 in TLR7-induced post-transcriptional control [198]. In addition to its role in post-transcriptional regulation, IRAK-2 was also shown to regulate LPS-induced TNFα gene translation. A contrasting report with IRAK-2 knockout mice showed no effect of IRAK-2 on mRNA stability for TLR-2 induced cytokines and

chemokines tested thus suggesting a specific role for IRAK-2 in TLR4-mediated post-transcriptional regulation [198].

Murine and human IRAK-2 show 67% sequence identity [200]. They also share the same domain structure and are highly conserved in their DD and kinase domains and therefore are unlikely to be significantly structurally different [200]. IRAK-2 in mice is known to have four splice variants (IRAK-2a ,-2b, -2c and 2d) and different functions have been attributed to different isoforms, since IRAK-2c and IRAK-2d have been proposed as having a negative role on TLR signalling, while IRAK-2a is a positive regulator of signalling [201]. In contrast there is no evidence of splice variants for human IRAK-2, so IRAK-2 in mice and humans may function differentially.

Therefore due to the possible differential role of IRAK-2 in primary murine cells and human cell lines, a comprehensive analysis of the role of human IRAK-2 in a more physiological relevant setting of primary human cells (PBMCs) was explored. In this chapter, the contribution of IRAK-2 to TLR signalling in both transcriptional and post-transcriptional regulation in human PBMCs is examined.

4.2 Results

4.2.1 IRAK-2 is required for TLR4- and TLR8-mediated cytokine induction in primary human cells

It has previously been shown that knockdown of IRAK-2 expression by siRNA inhibited human TLR3-, TLR4- and TLR8- dependent NFκB reporter gene activity in HEK 293 cells [106]. In contrast to this, reported data from murine IRAK-2 $^{-/-}$ cells has shown that IRAK-2 is dispensable for early NFκB activation. Thus, I wanted to assess the requirement for human IRAK-2 in early TLR signalling in the more physiological setting of primary human PBMCs.

Firstly the efficacy of the IRAK-2 siRNA oligonucleotides that target IRAK-2 in PBMCs was confirmed by examining their effect on endogenous IRAK-2 protein levels. PBMCs were transfected with IRAK-2 siRNA as described in methods. Endogenous IRAK-2 protein levels were almost undetectable in PBMCs treated with IRAK-2 siRNA relative to the control-siRNA treated samples either in the presence or absence of stimulation with CL075 (3M002) (Figure 4.1, A).

PBMCs are known to respond to both LPS and CL075, via TLR4 and TLR8 respectively, leading to the production of inflammatory cytokines. It is thought that it is the monocyte population in PBMCs that primarily respond to LPS and CL075 as it is known that TLR4 and TLR8 are preferentially expressed in monocytes [270]. When PBMCs were treated with CL075, the production of IL-8 was reduced to basal levels in the cells treated with the control siRNA (Figure 4.1, B). Both CL075-induced TNFα and IL-6 production was significantly impaired in PBMCs treated with IRAK-2 siRNA compared to control treated samples (Figure 4.2 (A) and (B) respectively). This

demonstrates for the first time, to my knowledge, a role for IRAK-2 in multiple cytokine responses to CL075 in primary human cells.

Given the dramatic reduction of TLR8-induced cytokine levels observed when treated with IRAK-2 siRNA compared to control treated samples, we wanted to further explore the contribution of IRAK-2 in TLR signalling pathways to NFκB in PBMCs. The previous chapter showed a role for IRAK-2 in TLR4-induced IL-8 signalling in the HEK293 human cell line (Figure 3.7). As shown for those transformed cells, IRAK-2 was also required for LPS-induced IL-8 production in primary human cells (Figure 4.3 (A)). Furthermore targeted knockdown of IRAK-2 led to significantly impaired LPS-induced TNFα and IL-6 release compared to cells treated with a control siRNA (Figure 4.3, (B) and (C).

Thus IRAK-2 has a vital role in cytokine production for both endosomal (TLR8) and non-endosomal TLR (TLR4) pathways in primary human cells.

Thus, my data from primary human cells showed a requirement for IRAK-2 in TLR-induced cytokine induction. Previous work showed that TLR-dependent cytokine induction from macrophages from inbred mice IRAK-2 $^{-/-}$ mice was also impaired although NFκB and MAP kinase activation was largely unaffected [198]. In contrast, in macrophages from a wild-derived mouse strain, RNA interference targeting IRAK-2 did inhibit TLR-induced NFκB and MAP kinase activation [202]. Thus the mechanistic basis for the requirement for IRAK-2 for cytokine induction in the more physiological setting of primary human cells was not obvious and therefore I next explored the role of IRAK-2 in TLR-induced signalling pathways in PBMCs.

4.2.2 IRAK-2 is essential for TLR-mediated NFκB and p38 MAP kinase activation in primary human cells.

In order to examine the role for IRAK-2 in TLR-induced NFκB in primary human cells the effect of IRAK-2 siRNA on IκBα was examined. Cells were seeded at $1x10^6$/ml in 6 well plates and transfected with siRNA as described in methods (see section 2.3.6). Cells were stimulated with CL075 and harvested at the time points indicated (Figure 4.4, (A)). Cells were lysed, subject to SDS-PAGE and immunoblotted with an antibody against IκBα. Treatment of PBMCs with CL075 led to NFκB activation within 5 minutes of stimulation as measured by degradation of IκBα (Figure 4.4 (A) upper panel). When cells were pre-treated with IRAK-2 siRNA, TLR8-dependent degradation of IκBα was prevented at every time point tested (5-30 mins) compared to cells treated with control siRNA (Figure 4.4 (A)). Cells were immunoblotted with an antibody against β-actin to confirm equal protein levels (Figure 4.4, (A), lower panel). This is in contrast to the role for IRAK2 in the murine system as it has been shown that in IRAK-2 $^{-/-}$ macrophages IRAK-2 is not required for early TLR7-induced NFκB activation but is required for late/sustained NFκB activation [198]. Thus this demonstrates for the first time a role for IRAK-2 in early TLR8-induced NFκB activation in primary human cells, which is not the case for TLR7 in murine cells [198].

It was next determined whether IRAK-2 was involved in p38 activation in TLR8-stimulated primary human cells. p38 activation was measured by detection of phosphorylated p38 by immunoblotting with a phospho-p38 specific antibody. Upon stimulation with CL075, p38 phosphorylation was detected within 5 minutes and was sustained until at least 20 minutes (Figure 4.4 (B)). Crucially, when PBMCs were

treated with siRNA specifically targeting IRAK-2 prior to stimulation, a dramatic reduction of p38 phosphorylation was observed relative to control-siRNA treated samples (Figure 4.4 (B), upper panel). Equal protein levels were detected by measuring total p38 (Figure 4.4 (B), lower panel).

A role for IRAK-2 in early TLR4-mediated NFκB activation was also observed in primary human cells. Cells were immunoblotted with an antibody against IκBα and reduced IκBα degradation was observed in cells treated with IRAK-2 siRNA compared to control siRNA-treated cells, at 20 and 30 minutes (Figure 4.5 (A), upper panel). Equal protein levels were observed using an antibody to β-actin (Figure 4.5, (A), lower panel) Again this contrasts with what was observed in macrophages from IRAK-2 $^{-/-}$ mice where IRAK-2 had no role in either early or late TLR4-induced NFκB activation.

IRAK-2 was also shown to be critical for TLR4-MAP kinase activation. As was the case with CL075, LPS induced p38 phosphorylation within 5 minutes of stimulation and was sustained for at least 20 minutes (Figure 4.5 (B)). A striking reduction of p38 phosphorylation occurred upon treatment with IRAK-2 siRNA compared to control siRNA-treated samples (Figure 4.5 (B)). This is also different to what was found in the murine system where there was only a slight reduction in p38 phosphorylation upon LPS stimulation of IRAK-2 $^{-/-}$ macrophages [198]. Thus together these results reveal a critical and novel role for IRAK-2 in early signalling events for TLR pathways in primary human cells.

As described above, IRAK-2 has a critical role in TLR-induced IL-8 and TNFα induction. Furthermore, we observed the novel critical role for IRAK-2 in early NFκB and p38 MAP kinase activation in PBMCs. NFκB and p38 are known to be involved in the regulation of the induction of IL-8 and TNFα mRNA, therefore it was hypothesised that IRAK-2 may have a role in the regulation of IL-8 and TNFα mRNA levels.

4.2.3 IRAK-2 is required for TLR-mediated TNFα and IL-8 mRNA induction in PBMCs

In order to test the role of IRAK-2 in mRNA induction, PBMCs were seeded at $1x10^6$/ml in 12 well plates and were treated with either IRAK-2 siRNA or control siRNA as described in methods (see section 2.3.7). Cells were stimulated with CL075, mRNA was subsequently isolated from samples and mRNA levels were quantified by RT-PCR. A time course of CL075-induced TNFα transcript indicated that TNFα mRNA induction peaked at 1-2 hours after stimulation (Figure 4.6, black squares). When cells were treated with IRAK-2 siRNA there was a significant reduction in the levels of CL075-induced TNFα at 2 hours compared to the cells treated with control siRNA (Figure 4.6). Therefore IRAK-2 is required for TLR8-induced TNFα mRNA induction.

To detect the possible role for IRAK-2 in LPS-induced TNFα mRNA regulation, PBMCs were treated with either IRAK-2 siRNA or control siRNA and stimulated with LPS at various timepoints. As was the case for CL075, a time course of LPS-induced TNFα mRNA showed that TLR4-induced TNFα mRNA peaked at 2 hours stimulation (Figure 4.7). Upon treatment of PBMCs with siRNA targeting IRAK-2, there was potent inhibition of TNFα mRNA levels compared to control siRNA samples (Figure 4.7). Thus IRAK-2 has a role in both TLR4 and TLR8 induction of TNFα mRNA in primary human cells. This was again in contrast to previous work in murine IRAK-2 $^{-/-}$

macrophages where LPS-induced TNFα mRNA was not impaired at any time point examined [198].

The possible requirement for IRAK-2 in IL-8 mRNA regulation was next examined. When cells were stimulated with CL075 for 2 hours there was a significant decrease in IL-8 mRNA in cells treated with IRAK-2 siRNA compared to those treated with a control siRNA (Figure 4.8 (A)). In addition to this, there was a striking 80% reduction of LPS-induced IL-8 mRNA levels observed in the presence of IRAK-2 siRNA (Figure 4.8 (B)). Thus, we observed that not only was TNFα mRNA IRAK-2-dependent in PBMCs, but IL-8 mRNA induction by TLR4 and TLR8 was also shown to require IRAK-2. Thus, here I demonstrate for the first time to my knowledge a role for IRAK-2 in the regulation of both TLR4 and TLR8-induced TNFα and IL-8 mRNA in primary human cells. This is in direct contrast to the murine system where there is no role for IRAK-2 in the regulation of TNFα mRNA.

4.2.4 IRAK-2 is required for the stabilisation of TNFα but not IL-8 mRNA in primary human cells

IRAK family members have been proposed to have roles in post-transcriptional gene regulation. In particular IRAK-1 and IRAK-4 have been shown to regulate stability of some transcripts [216, 238] while the role of human IRAK-2 in regulating mRNA stability is untested. Of note, mechanisms are known to exist for TLRs to increase the stability of both IL-8 and TNFα mRNA although the signalling molecules involved have not been fully defined [79, 271, 272]. Given that the reduction of IRAK-2

expression by siRNA reduced the total amount of IL-8 and TNFα mRNA detected after either TLR4 or TLR8 stimulation, the potential role of IRAK-2 in the regulation of the stability of these transcripts was investigated.

PBMCs were transfected with either control or IRAK-2 siRNA, then stimulated with either LPS and CL075 alone for 2 hours to induce gene expression, followed by treatment with Actinomycin D (to block transcription) for a time course of 10-30 mins. This time course was chosen as it has been previously shown that TNFα mRNA has a short half life of 20-30 mins in primary human cells [273]. mRNA was then isolated from the PBMCs and the effect of reduced IRAK-2 expression on the rate of mRNA decay was then determined. It appeared that IRAK-2 was not required for the TLR8 pathway that induces IL-8 mRNA stability as the rate of decay in IRAK-2 siRNA treated samples was identical to cells treated with control siRNA treated samples (Figure 4.9). Similarly, IRAK-2 was not required for TLR4-induced IL-8 mRNA stability pathway as the rate of decay observed in IRAK-2 siRNA treated samples was similar to control treated samples (Figure 4.10)

In contrast to the lack of effect on IL-8 mRNA decay, in the presence of IRAK-2 siRNA, the decay of CL075-induced TNF mRNA was accelerated compared to cells treated with the control siRNA (Figure 4.11). This indicated that IRAK-2 may have a specific role in the regulation of TNFα mRNA stability and not IL-8. Indeed, when the requirement for IRAK-2 in TLR4-induced TNFα mRNA stability was examined, the rate of decay of TLR4-induced TNFα mRNA was even more marked and statistically significant compared to the effect observed for TLR8 (Figure 4.12). Thus there is an

essential role for human IRAK-2 in the post-transcriptional regulation of the TNFα gene.

The production of human TNFα in response to LPS is regulated at both transcriptional and post-transcriptional levels. p38 is known to be a key regulator of TNFα however, how exactly p38 regulates TNFα expression is still somewhat controversial. Initial studies using the pyridinyl imidazole-based inhibitor SB202350 showed that p38 specifically regulated the translational control of TNFα expression [274, 275]. In contrast to this other studies have shown that p38 MAPK controls TNFα mRNA stability [272, 276]. Since it was shown that IRAK-2 was required for LPS-induced p38 phosphorylation (Figure 4.5 (B)) the potential role of p38 in post-transcriptional regulation of TNFα in PBMCs was examined.

4.2.5 Role for p38 in regulation of TNF induction

Thus, to explore the role for p38 in the regulation of TNFα in PBMCs, PBMCs were treated with either the p38 inhibitor SB202190 or a DMSO control, 1 hr before stimulation with LPS. For mRNA detection the cells were harvested 2 hrs later. We observed that in the presence of the SB inhibitor, there was an impairment of LPS-induced TNFα mRNA compared to the DMSO control (Figure 4.13 (A)). For detection of TNFα protein, PBMCs were treated with the SB inhibitor or the DMSO control, 1 hour before stimulation and cells were harvested 24 hours later. There was a dramatic reduction of TNFα protein in the presence of the SB inhibitor compared to the DMSO control (Figure 4.13 (B)). This shows that p38 regulates LPS-induced TNFα both at mRNA and protein levels in PBMCs.

I next examined, whether, like IRAK-2, p38 was required for TNF mRNA stability. Thus the effect of the p38 specific inhibitor SB 202190 on LPS-induced TNFα mRNA stability in PBMCs was tested. SB202190 caused accelerated decay of LPS-induced TNFα mRNA (Figure 4.14) in comparison to DMSO control-treated samples. Therefore LPS induction of TNFα mRNA stability is p38-dependent. IRAK-2 siRNA treated samples caused comparable decay to the SB 202190 treated samples of TNFα mRNA (compare Figure 4.12 and Figure 4.14). Thus the LPS-induced TNFα mRNA stability pathway in primary human cells is both p38- and IRAK-2-dependent. The role for IRAK-2 in mediating TNFα mRNA stability in response to LPS is in contrast to the lack of requirement for murine IRAK-2 in this process as demonstrated in IRAK-2 $^{-/-}$ macrophages [198]. Further, the mechanism whereby IRAK-2 regulates TNFα mRNA stability maybe via p38 activation.

4.2.6 To examine regulation of TNF mRNA stability via the TNF 3'UTR

To further explore mechanistically how IRAK-2 might regulate the stability of the TNF mRNA and not IL-8 mRNA we focused on the 3'UTR of both mRNA transcripts. Some cytokines, including TNFα and IL-8, are rendered unstable due to the presence of adenosine and uridine-rich elements (ARE) in their 3'UTR. Firstly in order to assay the role of IRAK-2 in mRNA stability pathways converging on cytokine 3'UTR, luciferase reporters whereby the luciferase transcript was under the control of the TNFα 3'UTR or IL-8 3'UTR, were transfected into HEK293_R1 cells. Cells were stimulated with LPS and harvested 6 hours later. Stimulation with LPS slightly activated the TNF 3'UTR

and the IL-8 3'UTR (Figure 4.15 (A) and (B)) but this activation was not above 2-fold. CL075 also failed to induce the TNF and IL-8 3'UTR (data not shown).

Thus, since we were unable to significantly activate the 3'UTR reporters via a ligand-driven signal [277]. I next examined whether the pathway could be activated through ectopic expression of the TIR adaptors. MyD88 is a crucial signalling component of the TLR pathway. It contains a C-terminal Toll-IL-1-R (TIR) homology domain and an N-terminal death domain. It has been shown to be an essential adaptor protein in the signalling from the TIR-containing receptor to the death domain-containing IRAKs. For TLR signalling, a MyD88-dependent pathway, which would operate for both TLR4 and TLR8, has been shown to mediate a stabilising signal that converges on some cytokine 3'UTRs [271]. Interestingly, ectopic expression of MyD88 activated mRNA stabilising pathways leading to increased production of luciferase protein expressed from the reporters under the control of the TNFα and IL-8, but not the βactin 3'UTR which was used as a control. MyD88 expression led to a >15 fold induction of both the TNF 3'UTR and IL-8 3'UTR (Figure 4.16).

4.2.7 The TNF 3'UTR pathway is regulated via a MyD88-dependent pathway

TLRs signal through a MyD88-dependent and MyD88-independent pathway [87]. The MyD88-independent pathway operates via the TIR adaptors TRIF and TRAM [87]. Given that TLR4 is known to signal through both of these pathways we wanted to examine whether TIR adaptors other than MyD88 also regulate mRNA stability via the TNF 3'UTR. Previously it has been shown that the ability of the TLR members to regulate the ARE-containing mRNAs is through differential use of the various TIR

adaptors [271]. Here HEK293_R1 cells were transfected with the TNF 3'UTR luciferase reporter and co-transfected with either 10 or 25ng of MyD88, MAL, TRIF or TRAM expression vector. Figure 4.17 shows that MyD88 induced the activation of the TNF 3'UTR 8 fold, while the other TIR adaptors; MAL, TRIF and TRAM also activated the TNF 3'UTR but to a lesser extent than MyD88 (< 4fold). This is consistent with previous findings that show IL-1R and TLR4 pathways, that are MyD88-dependent, have a greater role in mRNA stabilisation than the TLR3 pathway (which is MyD88-independent) [271]. Thus while TLR4 uses both the MyD88-dependent and MyD88-independent pathway, it is the MyD88-dependnet pathway that is more likely to be critical in the regulation of mRNA stabilisation.

4.2.8 IRAK-2 controls a MyD88-dependent pathway to mRNA stability via the TNF 3'UTR

Thus MyD88 is the strongest inducer of the TNF 3'UTR pathway in comparison to the other TIR adaptors. Therefore we next examined whether there was a role for IRAK-2 on this TLR-MyD88-TNF 3'UTR pathway using IRAK-2 siRNA. In cells that were treated with control siRNA, MyD88-induced 3'UTR reporter activity was barely affected (Figure 4.18 (A)). Similarly, IRAK-2 siRNA did not significiantly affect the β-actin 3'UTR (Figure 4.18 (C)). However, in cells treated with IRAK-2 siRNA prior to expression of MyD88, there was a very significant decrease in the activation of TNFα 3'UTR compared to cell treated with control siRNA (Figure 4.18 (A)). This result demonstrates that IRAK-2 participates in the MyD88-dependent pathway that regulates the TNFα 3'UTR, and further confirmed the role of IRAK-2 in TNFα mRNA stability. Significantly, and in contrast to this, MyD88-dependent induction of the IL-8

3'UTR was not reduced in the presence of IRAK-2 siRNA (Figure 4.18 (B)), confirming the lack of a role for IRAK-2 in IL-8 mRNA stabilisation (Figure 4.9 and Figure 4.10).

Consistent with the requirement for IRAK-2 in the ability of MyD88 to stimulate the TNFα 3'UTR, when HEK293_R1 cells were transfected with a plasmid encoding WT IRAK-2, together with the reporter construct containing the TNF 3'UTR, expression of WT-IRAK-2 dose dependently enhanced the reporter activity of the transcript containing the TNF 3'UTR (Figure 4.19 (A)) while not activating the βactin 3'UTR (Figure 4.19 (B)). This further confirms the role for IRAK-2 in the regulation of the TNFα mRNA stability pathway.

4.2.9 To examine the role for IRAK-1 and IRAK-4 in post-transcriptional regulation

The IRAK family members, IRAK-1 IRAK-2 and IRAK-4 all have in common that they have been shown to have a role in post-transcriptional regulation: IRAK-1 has shown to be required for IL-1-induced stabilisation of KC (the murine equivalent of IL-8) [238], we have shown that IRAK-2 has a role in regulating LPS-induced TNFα mRNA stabilisation and IRAK-4 is known to regulate mRNA stability of various cytokines [216]. Therefore we wanted to compare the possible roles for IRAK-1 and IRAK-4 to IRAK-2 in the regulation of TNF mRNA via the TNF 3'UTR. HEK293_R1 cells were transfected with the luciferase reporter containing the TNF 3'UTR and 25ng of either WT IRAK-2, WT IRAK-1 or WT IRAK-4. 25ng of MyD88 was also transfected as a positive control.

Figure 4.20 shows that ectopic expression of MyD88 induced the activation of the TNF 3'UTR 7 fold and expression of IRAK-2 activated the TNF 3'UTR reporter 4 fold. IRAK-1 could also activate the TNF 3'UTR (approximately 2 fold) but this was to a lesser extent than either MyD88 or WT IRAK-2. This is in consistent with a previous study which reported that IRAK-1 was dispensable for the MyD88-dependent transcript stabilisation of TNF [142]. Expression of IRAK-4 failed to activate the TNF 3'UTR.

The role for IRAK-1 and IRAK-4 in IL-8 mRNA regulation via the IL-8 3'UTR was also examined. Cells were transfected with the reporter construct containing the IL-8 3'UTR and also transfected with either 25ng MyD88, IRAK-2, IRAK-1 or IRAK-4. As shown previously MyD88 could enhance the activity of the IL-8 3'UTR reporter. Expression of IRAK-1 was shown to induce the IL-8 3'UTR 5 fold and this was stronger than expression of MyD88. WT IRAK-2 and WT IRAK-4 induced activation of the IL-8 3'UTR only two fold (Figure 4.20 (B)).

4.3 Discussion

Previous work has shown a role for IRAK-2 in TLR3-, TLR4- and TLR8-mediated NFκB-dependent reporter gene activation in a human cell line [106]. Furthermore, in the previous chapter I have demonstrated a role for IRAK-2 in TLR8-induced IκB degradation and IL-8 production in human cell lines. In contrast to this, reported data from IRAK-2$^{-/-}$ cells has shown that murine IRAK-2 is not required for early NFκB activation. Hence, the physiological role of IRAK-2 in NFκB activation was not clear and thus here for the first time I provide a detailed analysis of the role of IRAK-2 in primary human PBMCs.

Since transfection of primary cells with siRNA is known to be difficult, firstly it was necessary to confirm the efficacy of the IRAK-2 siRNA oligonucleotides by examining their effect on IRAK-2 protein levels in PBMCs. Endogenous IRAK-2 protein levels were almost undetectable in PBMCs treated with IRAK-2 siRNA relative to the control-siRNA treated samples. It was somewhat surprising that expression of IRAK-2 protein levels were almost completely eliminated in the presence of IRAK-2 siRNA in a mix cell population of PBMCs. However it is possible that IRAK-2 may be expressed highly in the monocyte subpopulation which are more easily transfectable than other cell types and furthermore monocytes are known to express high levels of both TLR4 and TLR8 [270].

Recent studies using cells from IRAK-2 $^{-/-}$ mice have shown that early NFκB activation was normal for TLR2, TLR7 and TLR9, although delayed activation was impaired, while for TLR4 there was no defect in NFκB. However, in the primary cells studies

here I demonstrated an essential role for IRAK-2 in early NFκB activation for both TLR4 and TLR8. The role for IRAK-2 in TLR8-induced NFκB activation was particularly striking as IκB degradation was impaired at every time point tested (Figure 4.4, (A))

Human IRAK-2 was also shown to be more important for p38 MAPK kinase activation, since in contrast to the lack of role for IRAK-2 reported in the IRAK-2 $^{-/-}$ studies for TLR2, TLR4 and TLR7, IRAK-2 was shown to be critical for TLR4- and TLR8-induced p38 MAPK kinase activation, as p38 phosphorylation was dramatically reduced in the presence of IRAK-2 siRNA compared to control-treated samples. Thus the requirement for IRAK-2 in TLR4 and TLR8-induced NFκB was not restricted to HEK293 but was also shown to be essential for early signalling events in PBMCs. Interestingly, although IRAK-2 has been shown to be required for TLR-induced cytokine production in both human and murine cells, there seems to be a more subtle requirement for IRAK-2 in TLR signalling in the murine system.

As mentioned previously, murine IRAK-2 is known to contain four splice variants (IRAK-2a,- 2b, -2c and -2d). Different functions have been attributed to the different isoforms of murine IRAK-2, since IRAK-2c and IRAK-2d have been proposed as having a negative role on TLR signalling, while IRAK-2a is a positive regulator of signalling [201]. In contrast to this, since there is no evidence of splice variants for human IRAK-2, so the human protein is most like the murine IRAK-2a, the positive regulator. Intriguingly, a recent study examining the innate immune response of wild-derived mice versus classical inbred strains revealed a more important role for IRAK-2 in the wild-derived mice in TLR signalling events, compared to the in-bred mice, which was

attributed to differential expression of IRAK-2 splice variants between the two strains [202]. The classical inbred strain, C57BL/6J, was shown to express high levels of the inhibitory isoform of IRAK-2, IRAK-2c, which inhibits the proinflammatory isoform IRAK-2a [202]. This may explain the more dominant role of IRAK-1, rather than IRAK-2, in early signalling events in inbred mice [197]. However, as time progresses, expression of the inhibitory isoform (IRAK-2c) decreased which likely allows IRAK-2a, which is no longer being inhibited, to function in a proinflammatory manner. Thus IRAK-2a in inbred mice is responsible for late and sustained NFκB activation (Figure 4.21). Wild-derived mice differ significantly from experimental models as the genetic diversity of wild-derived mice has arisen in an evolutionary context and these mice display a higher degree of polymorphisms. In the wild-derived strain MOLF/Ei, a natural mutation occurs in the promoter of IRAK-2c leading to significantly less IRAK-2c being expressed, and thus IRAK-2a's positive function predominates. Since humans only express the IRAK-2a isoform, the wild derived mice are a better model for human IRAK-2 function than inbred mice. Indeed, siRNA targeting IRAK-2a in macrophages from wild-derived mice showed that IRAK-2a was required for early activation of NFκB and p38 MAPK by TLR2 [202]. Thus TLR-induced p38 and NFκB responses are similarly impaired by IRAK-2 siRNA in MOLF/Ei macrophages and human PBMCs.

Further differences in the murine and human system emerged upon examining cytokine induction and post-transcriptional regulation: I showed here that human IRAK-2 regulates early TNFα and IL-8 mRNA induction in response to LPS and CL075 whereas lack of murine IRAK-2 did not affect early or late TNFα mRNA induction

after LPS treatment [198]. In addition, here I demonstrate an essential role for IRAK-2 in the regulation of LPS-induced TNFα mRNA stability in PBMCs. In contrast to this, in the murine system, IRAK-2 was shown to be dispensable for TNFα mRNA stability regulation but was required for the translation of TNFα mRNA [198].

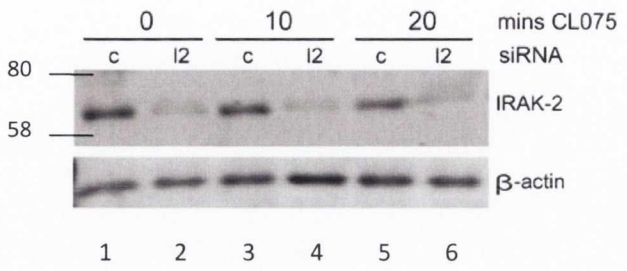
The regulation of TNFα mRNA stability in the human system was shown to be both IRAK-2- and p38-dependent. Although it is known that p38 can regulate both TNFα and IL-8 mRNA stability, IRAK-2 is specifically required for the stability of TNFα mRNA. Therefore it is possible that there are further IRAK-2-dependent, p38-independent mechanisms of stabilising TNFα mRNA.

Further evidence that IRAK-2 is required for the stabilisation of TNFα mRNA was shown via examination of the TNF 3'UTR. Many cytokines are rendered unstable due to the presence of ARE in their 3'UTR. mRNAs can be regulated post-transcriptionally via these ARE-regions by ARE-binding proteins that can positively or negatively regulate mRNA stability [79]. It was shown that IRAK-2 was required for a MyD88-dependent stabilisation pathway regulating the TNF 3'UTR but not the IL-8 3'UTR While both TNFα and IL-8 mRNA are known to be regulated via ARE within their 3'UTRs, these AREs are subject to differential post-transcriptional control depending on their sequences which defines which ARE-binding proteins target their AREs, and hence the deadenylation kinetics [79]. Although the TNFα and IL-8 3'UTRs both contain multiple AREs, their AREs vary in terms of abundance, sequence and relative orientation to each other. Thus it is possible that IRAK-2 modulates the activity of specific ARE-binding proteins that target the TNFα, but not the IL-8 3'UTR.

A MyD88-dependent pathway, which would operate for both TLR4 and TLR8 in human cells, has previously been shown to mediate both IL-8 and TNF mRNA stability [142, 271]. Here, IRAK-2 was found to be required for this MyD88-dependent pathway to TNFα mRNA stability for both TLR4 and TLR8, but not to be required for IL-8 mRNA stability. This suggests that either different MyD88 complexes exist that differentially regulate mRNA stability, or that the pathways regulating TNFα and IL-8 mRNA stability bifurcate downstream of MyD88, with IRAK-2 at least one step removed from MyD88. I have shown here that overexpression of IRAK-1 could induce activation of the IL8 3'UTR more potently than overexpression of IRAK-2. Thus, it is possible that different Myddosomes exist that regulate different post-transcriptional events. For example, since IRAK-1 has been implicated in regulating mRNA stability of the mouse chemokine KC, it may be that a MyD88-IRAK-4-IRAK-1 Myddosome regulates murine KC and/or human IL-8 mRNA stability, whereas a MyD88-IRAK-4-IRAK-2 Myddosome regulates TNFα mRNA stability [105]. Consistent with this idea, murine IRAK-4 has been shown to be required for both KC and TNFα mRNA stability [216].

Thus I have demonstrated a previously unrecognised role for IRAK-2 in early TLR signalling events and in post-transcriptional regulation of TNFα in primary human cells.

(A)



(B)

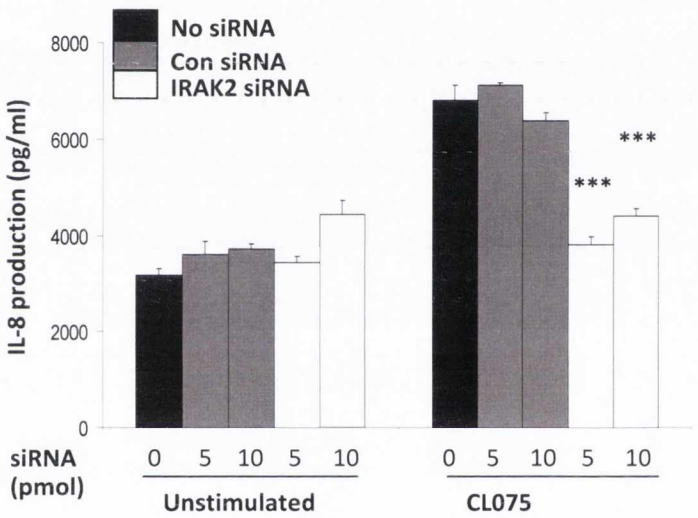


Figure 4.1 IRAK2 siRNA decreases IL-8 cytokine production by TLR8 in primary human cells. PBMCs were transfected with either 5 or 10 pmol of non-silencing siRNA or siRNA targeting the IRAK-2 gene. Cells were treated a second time with 5 or 10 pmol siRNA 24 hours later. A) Cells were stimulated with 2.5 μg/ml of CL075 for times indicated and cell lysates were examined for endogenous IRAK-2 levels by immunoblotting for IRAK-2 (1:3000 α-IRAK-2 and 1:8000 α-rabbit) (upper panel, A). Cell lysates were immunoblotted with β-actin to confirm equal protein levels (lower panel, A). B) Cells were stimulated with CL075 2.5μg/ml and IL-8 production in cell supernatants was assayed by ELISA (24 hrs) (B). Data in (A) is representative of three independent experiments. Data in (B) is expressed as mean ± SD from an experiment performed in triplicate and is representative of three experiments. ***$p<0.0005$ compared to control siRNA.

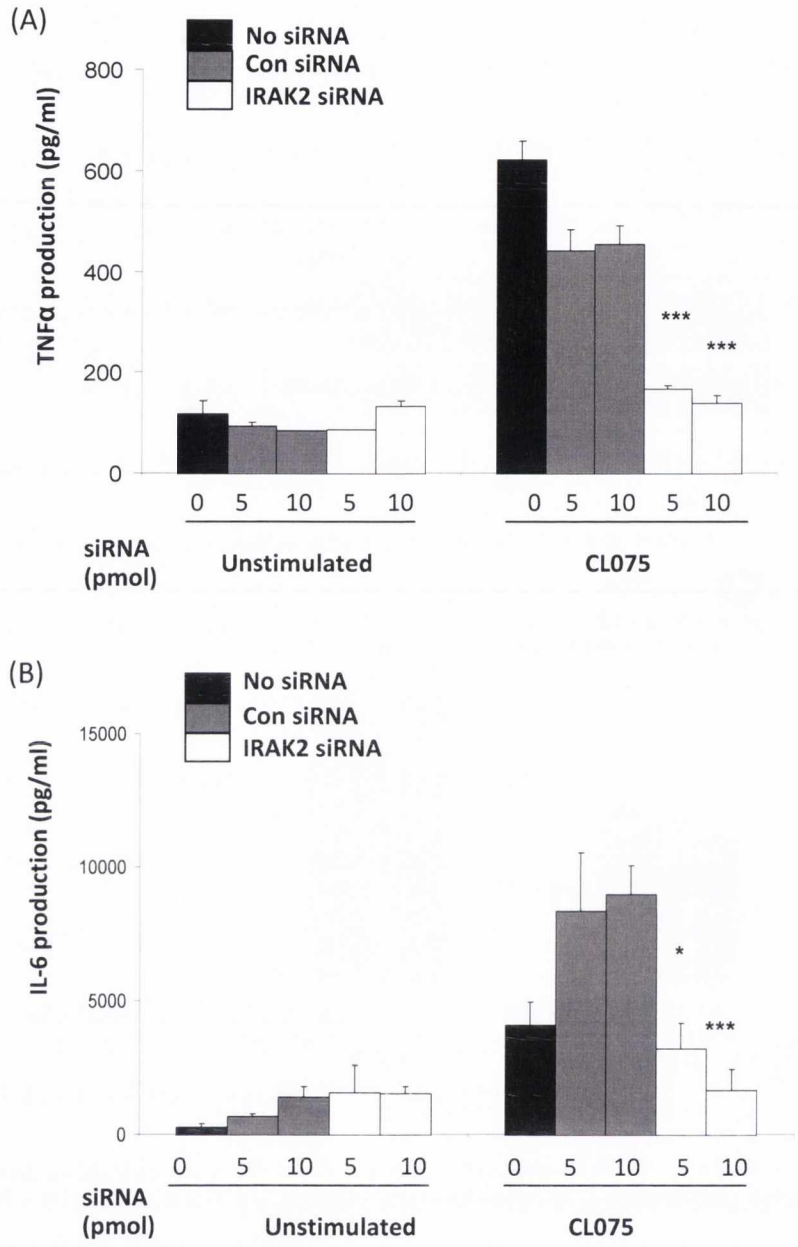


Figure 4.2 IRAK2 siRNA decreases TNFα and IL-6 cytokine production by human TLR8 in primary human cells. PBMCs were transfected with either 5 or 10 pmol of non-silencing siRNA or siRNA targeting the IRAK-2 gene. Cells were treated a second time with 5 or 10 pmol siRNA 24 hours later. Cells were stimulated with 2.5 μg/ml of CL075 and TNFα (A) and IL-6 (B) production in cell supernatants was assayed by ELISA (24 hrs). Data is expressed as mean ± SD from an experiment performed in triplicate and is representative of three experiments. *p<0.005, ***p<0.0005 compared to control siRNA

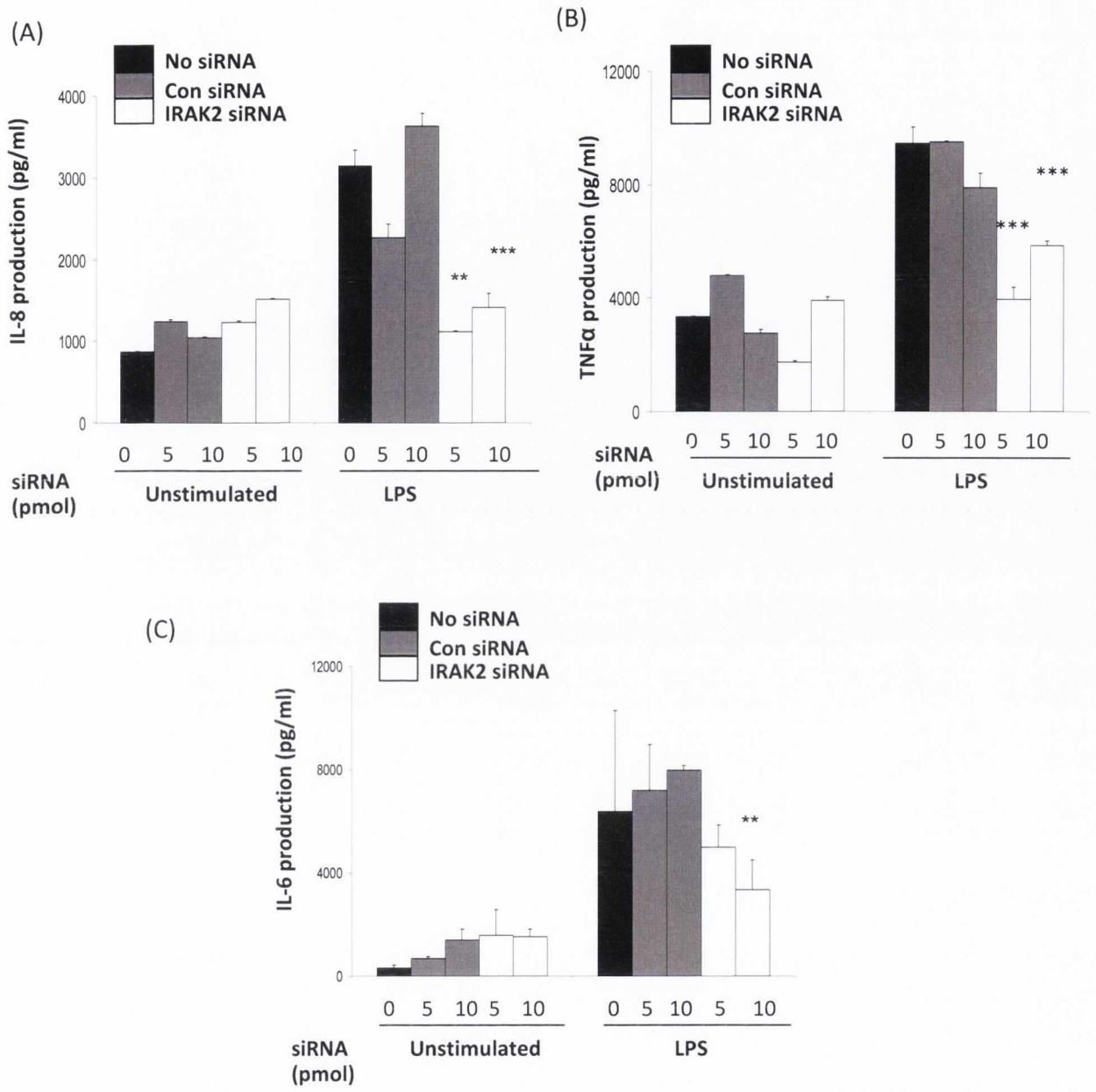


Figure 4.3 IRAK-2 is required for LPS-induced cytokine induction in primary human cells. PBMCs were transfected with either 5 or 10 pmol of non-silencing siRNA or siRNA targeting the IRAK-2 gene. Cells were treated a second time with 5 or 10 pmol siRNA 24 hours later. Cells were stimulated with 100ng/ml LPS and IL-8 (A), TNFα (B) and IL-6 (C) production in cell supernatants was assayed by ELISA (24 hrs). Data is expressed as mean ± SD from an experiment performed in triplicate and is representative of three experiments. **p<0.005, ***p<0.0005 compared to control siRNA

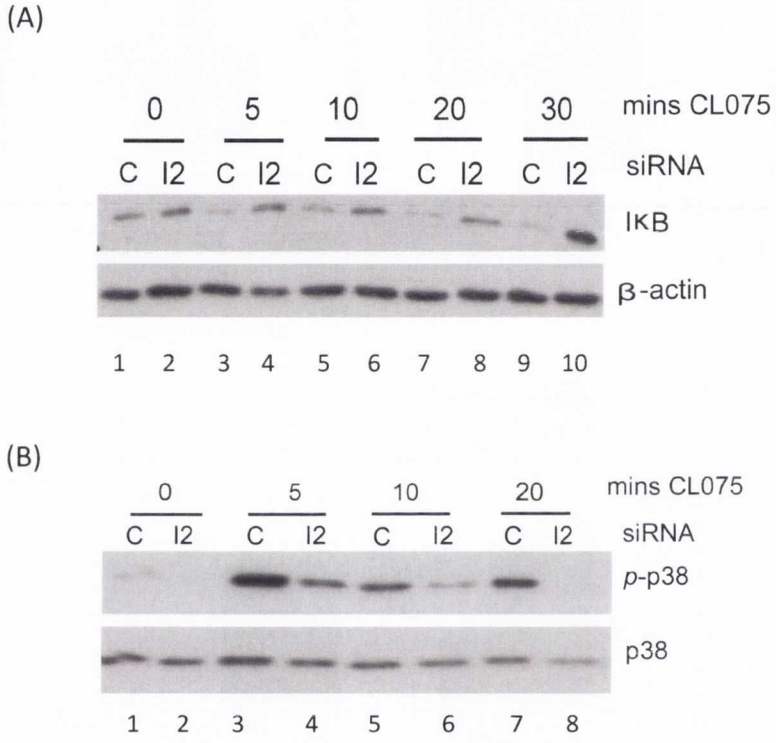
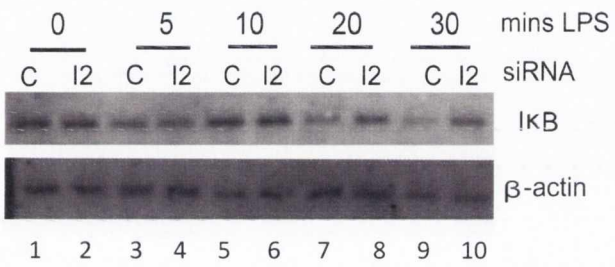


Figure 4.4 IRAK-2 is required for early TLR8-mediated NFκB and p38 MAP kinase activation. PBMCs were transfected with 50 pmol of either control non-silencing siRNA (C) or siRNA targeting the IRAK-2 gene (I2). Cells were stimulated with 2.5 μg/ml CL075 and harvested at the time points indicated. Lysates were assayed for IκBα degradation by immunoblotting with IκBα Ab (A, upper panel) or probed with β-actin Ab to confirm equal protein levels (A, lower panel). Lysates were also assayed for *p*-p38 (B, upper panel) or for p38 as a loading control (B, lower panel). Each immunoblot is representative of three experiments.

(A)



(B)

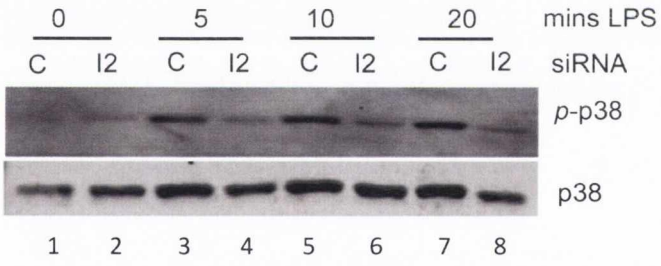


Figure 4.5 IRAK-2 is required for early TLR4-mediated NFκB and p38 MAP kinase activation. PBMCs were transfected with 50 pmol of either control non-silencing siRNA (C) or siRNA targeting the IRAK-2 gene (I2). Cells were stimulated with 100ng/ml LPS and harvested at the time points indicated. Lysates were assayed for IκBα degradation by immunoblotting with IκBα Ab (A, upper panel) or probed with β-actin Ab to confirm equal protein levels (A, lower panel). Lysates were also assayed for *p*-p38 (B, upper panel) or for p38 as a loading control (B, lower panel). Each immunoblot is representative of three experiments.

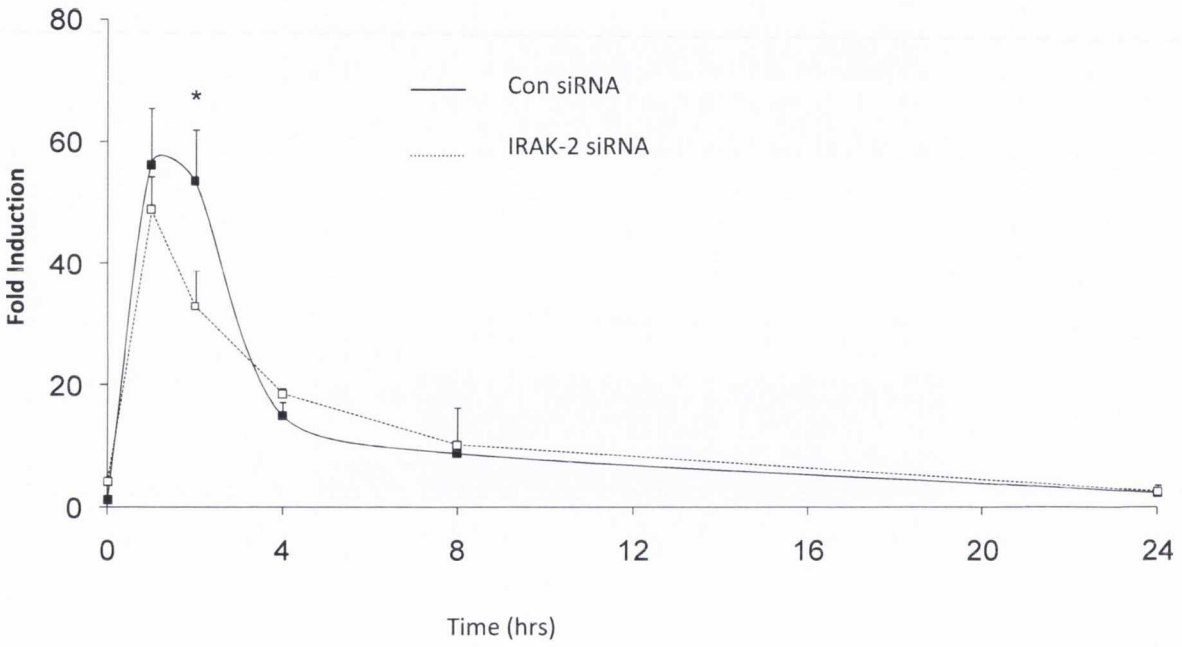


Figure 4.6 IRAK-2 is required for TLR8-mediated TNF mRNA induction in PBMCs. PBMCs were transfected with 25pmol of either control non-silencing siRNA (black squares) or siRNA targeting the IRAK-2 gene (white squares). Cells were stimulated with 2.5 μg/ml CL075 and harvested at the time points indicated. TNFα mRNA expression was assayed by quantitative RT-PCR. The data is mean ±SD of triplicate samples and is representative of three experiments. *$p<0.05$ compared to control siRNA.

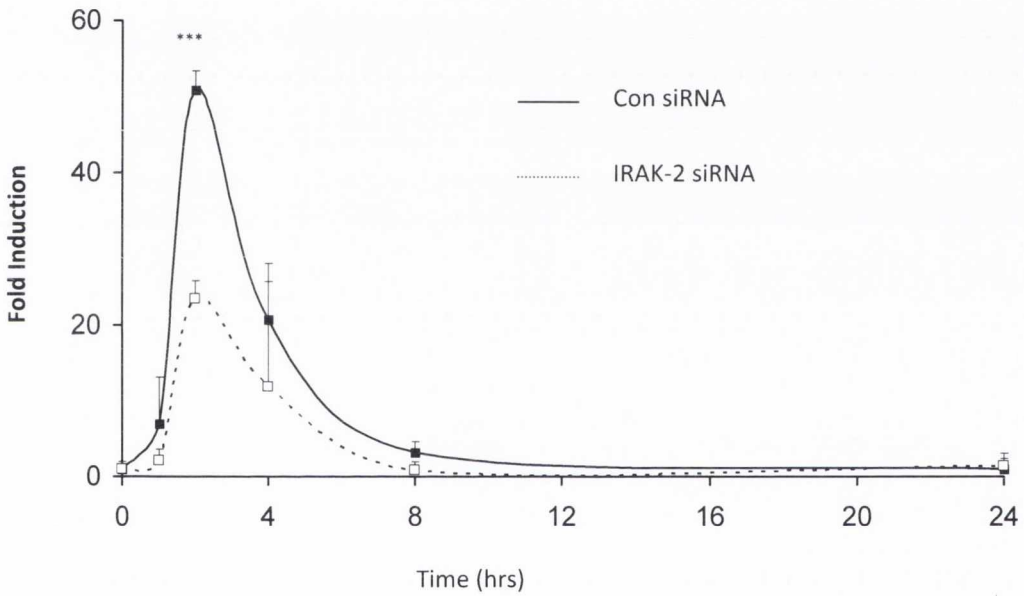


Figure 4.7. IRAK-2 is required for TLR4-mediated TNF mRNA induction in PBMCs.
PBMCs were transfected with 25pmol of either control non-silencing siRNA (black squares) or siRNA targeting the IRAK-2 gene (white squares). Cells were stimulated with 100ng/ml LPS and harvested at the time points indicated. TNFα mRNA expression was assayed by quantitative RT-PCR. The data is mean ±SD of triplicate samples and is representative of three experiments. ***$p<0.0005$ compared to control siRNA.

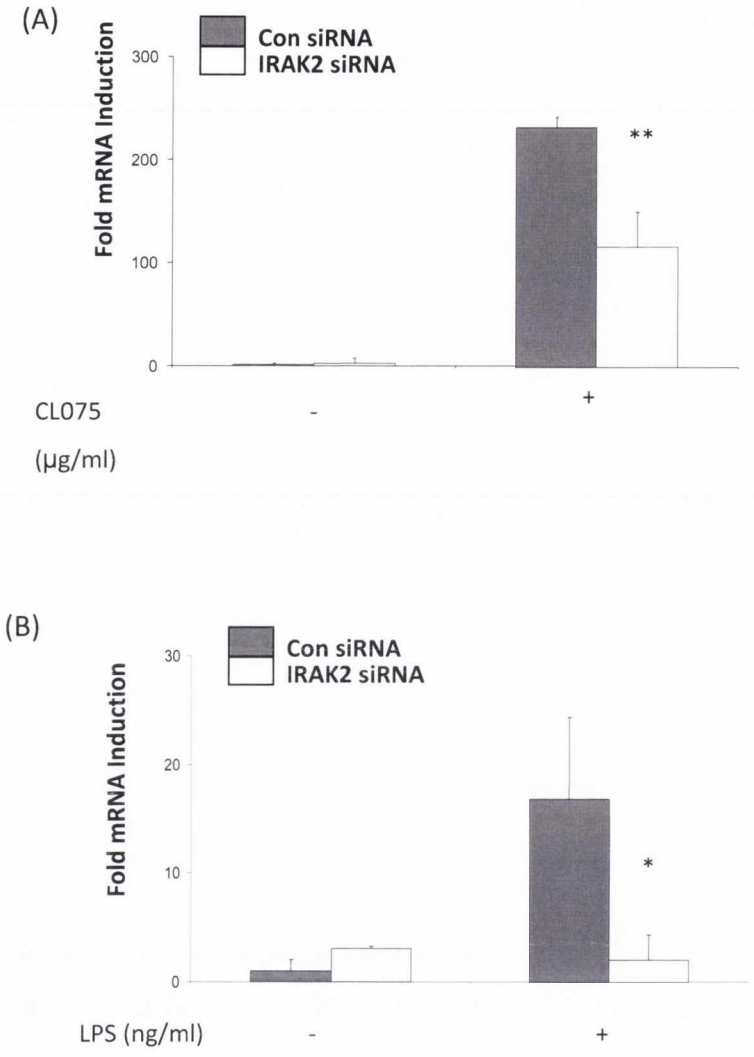


Figure 4.8 IRAK-2 is required for TLR8 and TLR4-mediated IL-8 mRNA induction in PBMCs. PBMCs were transfected with 25pmol of either control non-silencing siRNA (grey bars) or siRNA targeting the IRAK-2 gene (white bars). Cells were stimulated either with (A) 2.5 µg/ml CL075 or (B) 100ng/ml LPS and harvested 2 hours later. IL-8 mRNA expression was assayed by quantitative RT-PCR. The data is mean ±SD of triplicate samples and is representative of three experiments.

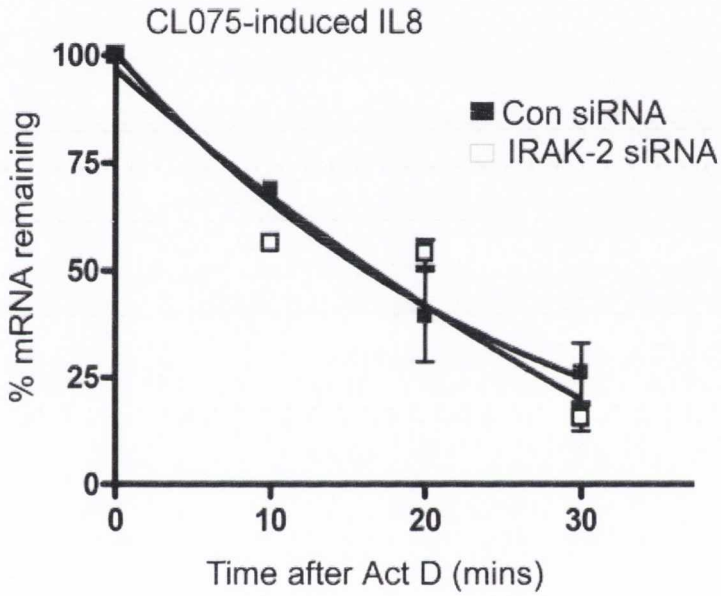


Figure 4.9. IRAK-2 is not required for TLR8-induced IL-8 mRNA stability.

PBMCs were transfected with 25pmol of either control non-silencing siRNA (black squares) or siRNA targeting the IRAK-2 gene (white squares). Cells were stimulated with 2.5 μg/ml CL075 for 2h and then 5 μg/ml actinomycin D (Act D) was added. Cells were harvested at the indicated time points and IL-8 mRNA expression was assayed by quantitative RT-PCR. Data is represented as % mRNA remaining compared to cells not treated with Act D, and is the mean ±SD of three independent experiments.

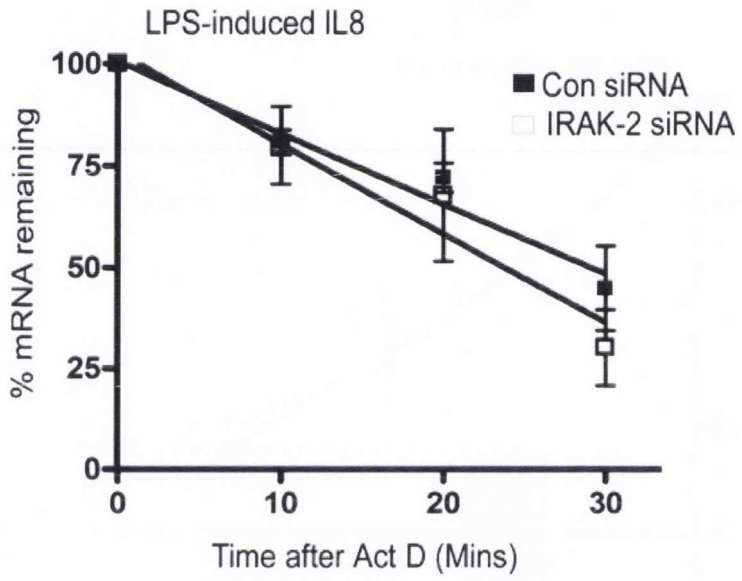


Figure 4.10. IRAK-2 is not required for TLR4-induced IL-8 mRNA stability.
PBMCs were transfected with 25pmol of either control non-silencing siRNA (black squares) or siRNA targeting the IRAK-2 gene (white squares). Cells were stimulated with 100ng/ml LPS for 2h and then 5 μg/ml actinomycin D (Act D) was added. Cells were harvested at the indicated time points and IL-8 mRNA expression was assayed by quantitative RT-PCR. Data is represented as % mRNA remaining compared to cells not treated with Act D, and is the mean ±SD of three independent experiments.

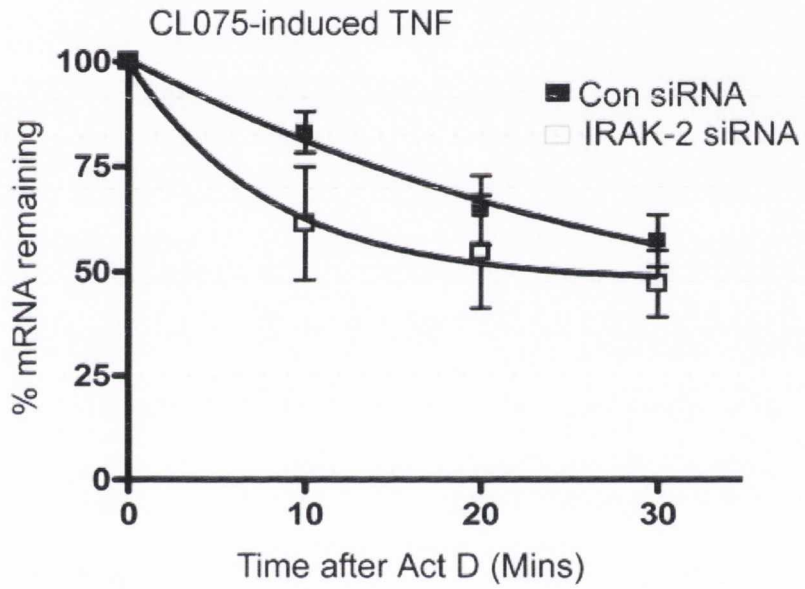


Figure 4.11. Role for IRAK-2 in TLR8-induced TNFα mRNA stability. PBMCs were transfected with 25pmol of either control non-silencing siRNA (black squares) or siRNA targeting the IRAK-2 gene (white squares). Cells were stimulated with 2.5 μg/ml CL075 for 2h and then 5 μg/ml actinomycin D (Act D) was added. Cells were harvested at the indicated time points and TNFα mRNA expression was assayed by quantitative RT-PCR. Data is represented as % mRNA remaining compared to cells not treated with Act D, and is the mean ±SD of three independent experiments.

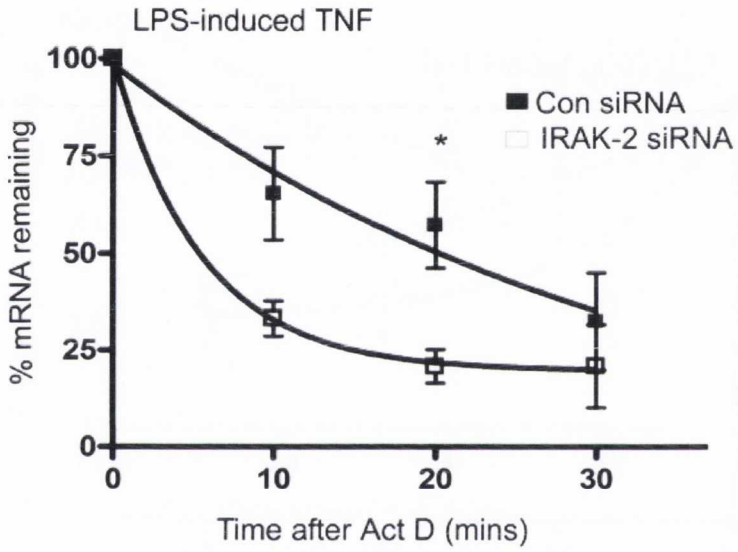


Figure 4.12. Essential role for IRAK-2 in TLR4-induced TNFα mRNA stability. (A) PBMCs were transfected with 25pmol of either control non-silencing siRNA (black squares) or siRNA targeting the IRAK-2 gene (white squares). Cells were stimulated with 100ng/ml LPS for 2h and then 5μg/ml actinomycin D (Act D) was added. Cells were harvested at the indicated time points and TNFα mRNA expression was assayed by quantitative RT-PCR. Data for all experiments is represented as % mRNA remaining compared to cells not treated with Act D, and is the mean ±SD of three independent experiments. p *<0.05 compared to control siRNA.

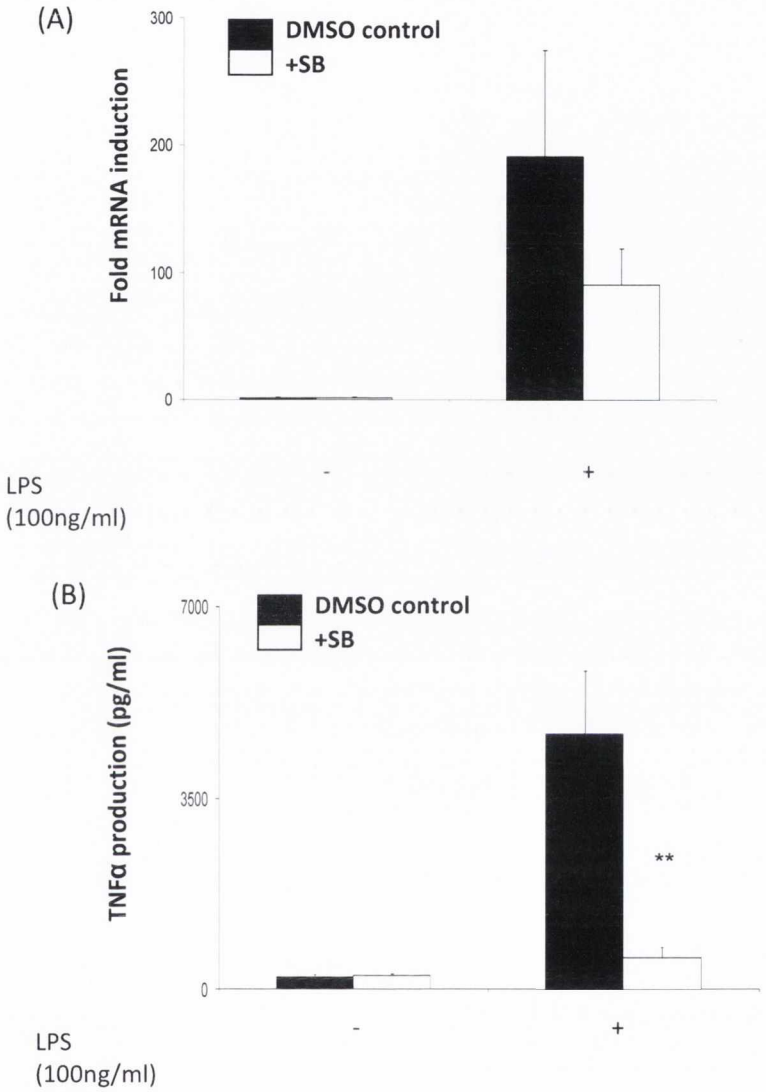


Figure 4.13. p38 has a role in regulating LPS-induced TNF mRNA and protein. PBMCs were seeded at a density of $1x10^6$/ml. In both cases, either 1μM SB 202190 inhibitor or 0.1% DMSO was added for 1 hour before stimulation. (A) Cells were stimulated for 2 hours with 100ng/ml LPS and TNFα mRNA expression was assayed by quantitative RT-PCR (B) Cells were stimulated with 100ng/ml LPS and TNFα production in cell supernatants was assayed by ELISA (24 hrs). In both cases the data is mean ±SD of triplicate samples and is representative of three experiments. **$p<0.005$ compared to control siRNA.

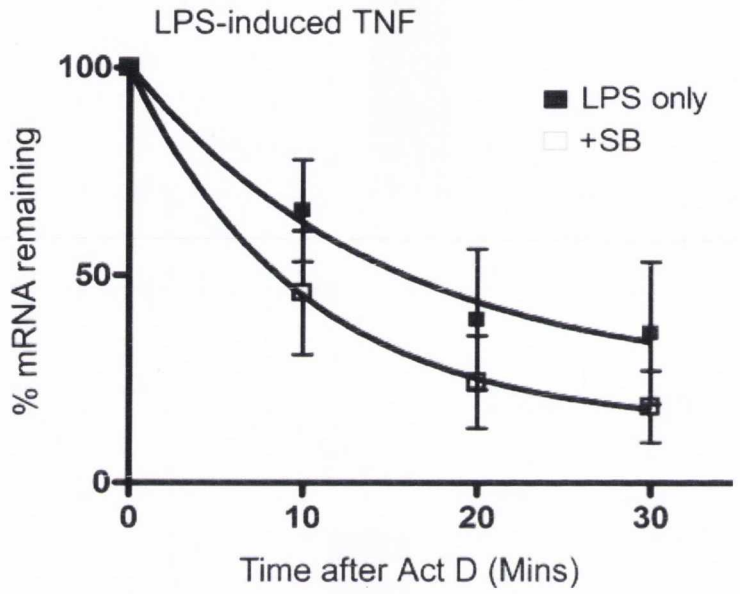


Figure 4.14. p38 is required for TLR4-induced TNFα mRNA stability. PBMCs were stimulated with 100 ng/ml LPS for 2h. Cells were then treated with 5 μg/ml Act D and 100 ng/ml LPS (black squares) or treated with 5 μg/ml Act D, 100 ng/ml LPS and 1 μM SB 202190 (white squares) and harvested at the indicated time points. Cells were harvested at the indicated time points and TNFα mRNA expression was assayed by quantitative RT-PCR. Data for all experiments is represented as % mRNA remaining compared to cells not treated with Act D, and is the mean ±SD of three independent experiments.

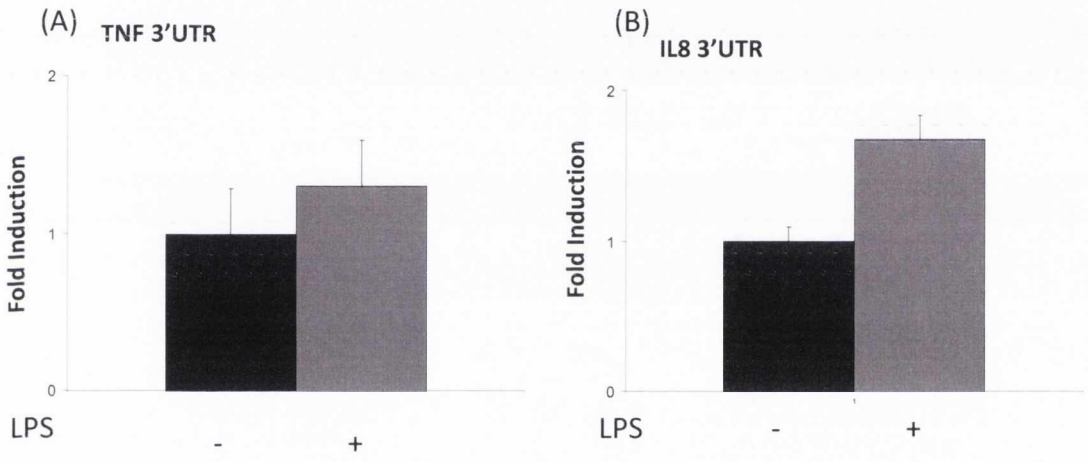


Figure 4.15 The effect of LPS stimulation of TNF 3'UTR and IL8 3'UTR activity. HEK293_R1 cells were transfected with either TNFα (A) or IL-8 3'UTR (B) reporter constructs. Cells were stimulated with 100 ng/ml LPS and activity of the 3'UTRs was measured by luciferase reporter gene assay 6h later. Data is shown as fold induction. The data is mean ± SD of triplicate samples and is representative of three separate experiments.

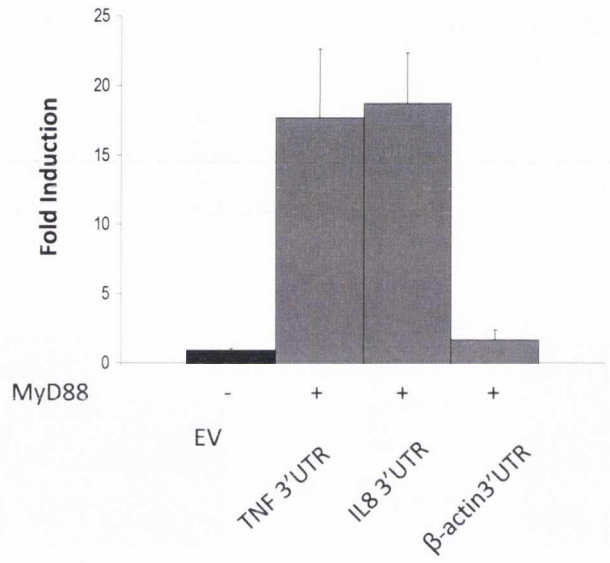


Figure 4.16 MyD88 regulates TNFα mRNA stability via the TNF α 3'UTR. HEK293_R1 cells were transfected with 10 ng MyD88 or pcDNA 3.1 empty vector (EV), together with either 60 ng of TNFα, IL-8 or β-actin 3'UTR reporter constructs. In all cases activity of the 3'UTRs was measured by luciferase reporter gene assay 24 h later. Data is shown as mean fold induction. The data is mean ± SD of triplicate samples and is representative of three separate experiments.

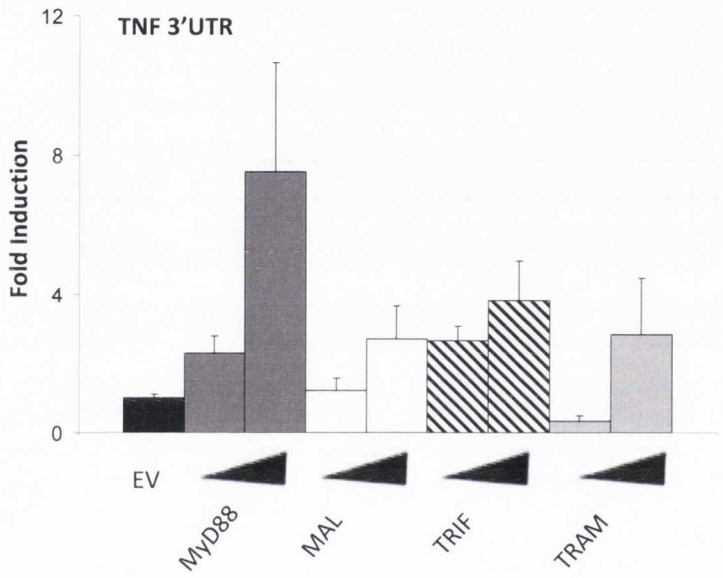


Figure 4.17 TIR adaptors ability to regulate TNF 3'UTR. HEK293_R1 cells were transfected with 60 ng of TNFα pcDNA3.1,TK renilla and either EV, or 10 or 25 ng MyD88, MAL, TRIF or TRAM. In all cases the activity of the 3'UTRs was measured by luciferase reporter gene assay 24h later. Data is shown as mean fold induction. The data is mean ± SD of triplicate samples and is representative of three separate experiments.

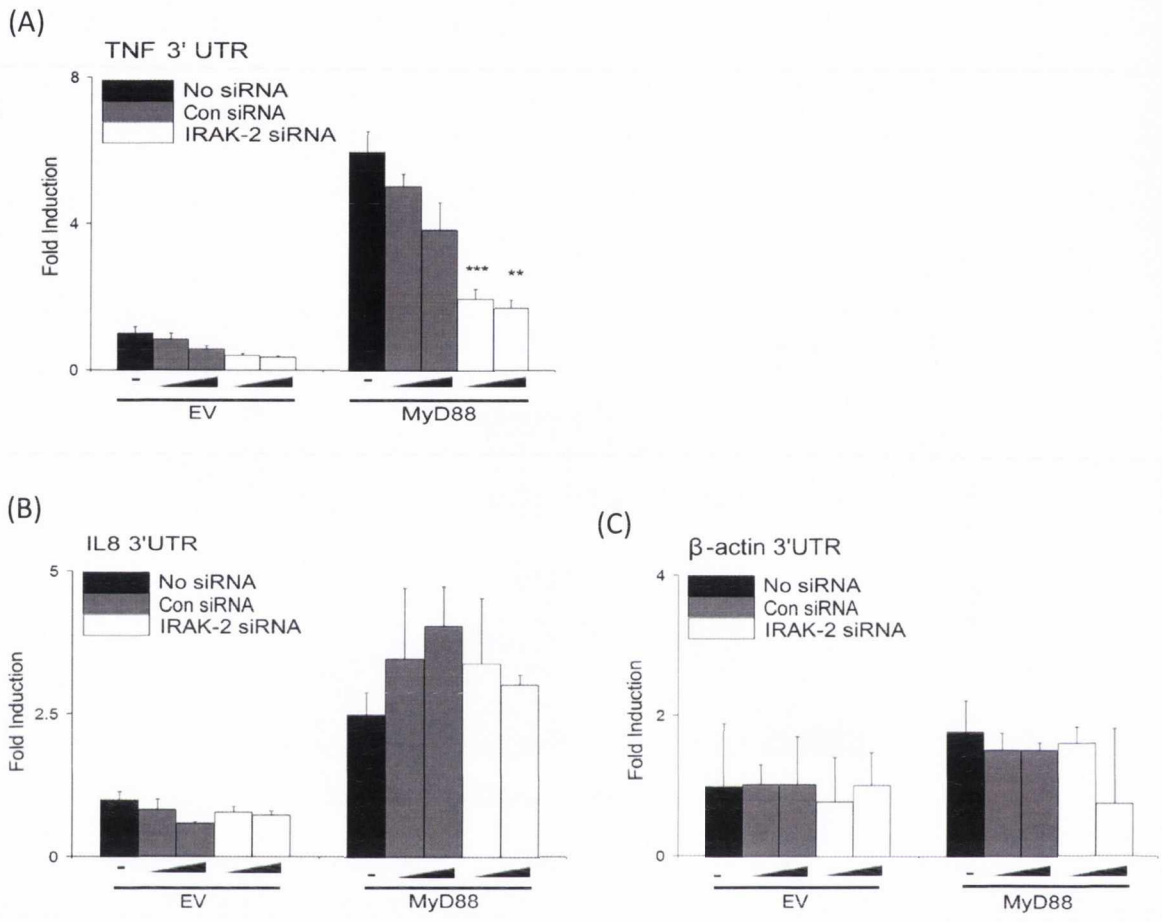


Figure 4.18 IRAK-2 regulates TNFα mRNA stability via the TNFα 3'UTR. HEK293_R1s were transfected with a Lipofectamine 2000 (black bar), control non-silencing siRNA (grey bars) or siRNA targeting the IRAK-2 gene (white bar), and where indicated pcDNA3.1 (EV) or 25 ng MyD88, and 60 ng of TNFα (A), IL-8 (B) or β-actin (C) 3'UTR. In all cases activity of the 3'UTRs was measured by luciferase reporter gene assay 24 h later. Data is shown as mean fold induction. The data is mean ± SD of triplicate samples and is representative of three separate experiments. **p<0.005 or ***p<0.0005 compared to control siRNA.

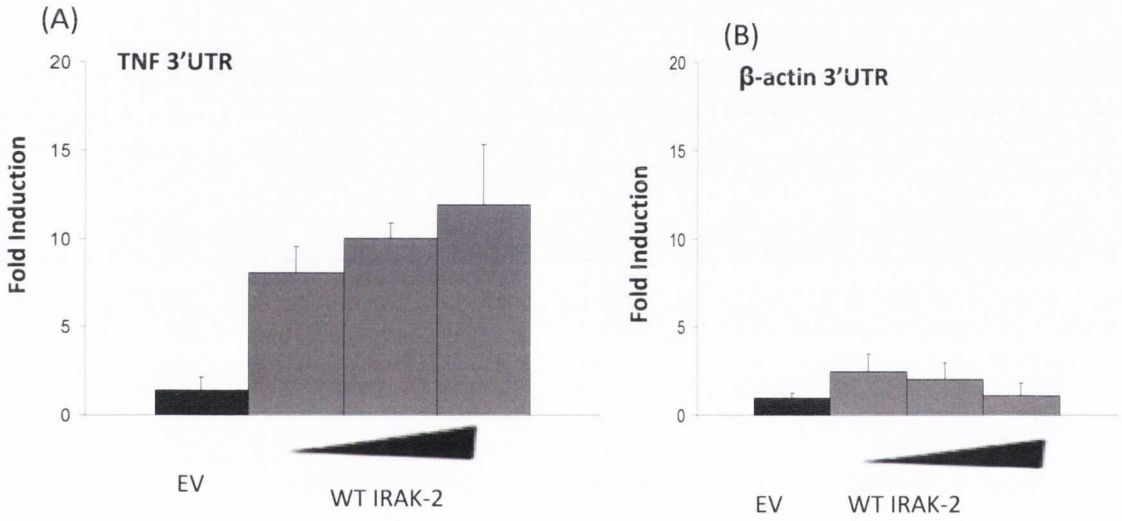


Figure 4.19 Expression of IRAK-2 induces the activation of the TNF 3'UTR (A) HEK293_R1 cells were transfected with EV, or 5, 10 or 25 ng WT IRAK-2 with either 60 ng of TNFα (A) or β-actin (B) 3'UTR. In all cases activity of the 3'UTRs was measured by luciferase reporter gene assay 24 h later. Data is shown as mean fold induction. The data is mean ± SD of triplicate samples and is representative of three separate experiments.

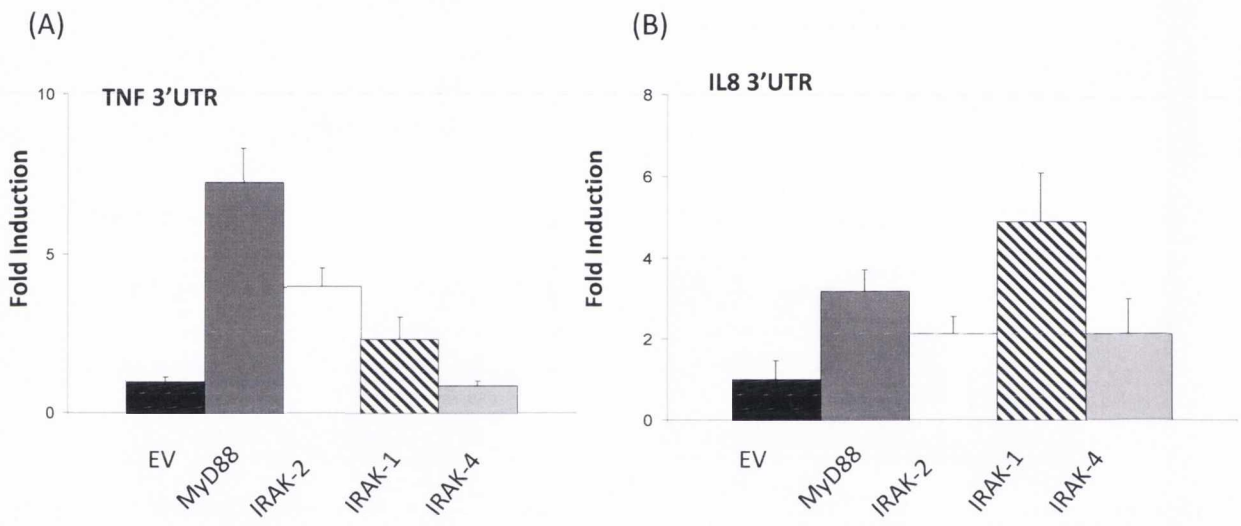


Figure 4.20. Role for IRAK-1 and IRAK-4 in the activation of the TNF and IL-8 3'UTR. HEK293_R1 cells were transfected with 60 ng of TNFα (A) or IL8 3'UTR reporter constructs (B). Where indicated, 25ng of MyD88, WT IRAK-2, WT IRAK-1 or WT IRAK-4 were cotransfected with reporters. In all cases activity of the 3'UTRs was measured by luciferase reporter gene assay 24 h later. Data is shown as mean fold induction. The data is mean ± SD of triplicate samples and is representative of three separate experiments.

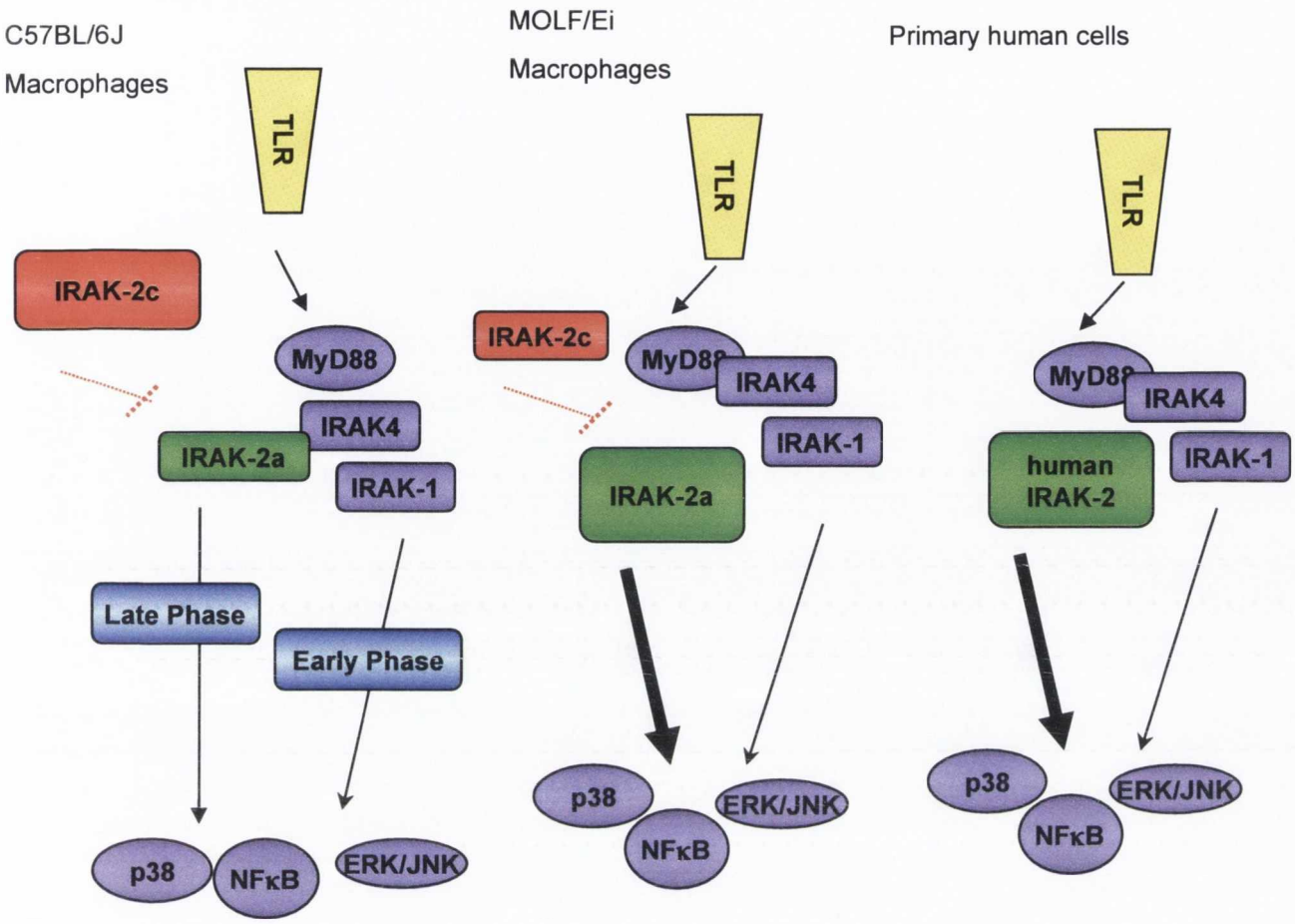


Figure 4.21 Differential expression of IRAK-2 splice variants in wild derived mice versus inbred strains versus human IRAK-2. The classical inbred strain C57BL/6J, was shown to express high levels of the inhibitory isoform, IRAK-2c. This isoform was shown to inhibit the proinflammatory isoform IRAK-2a (green) in early TLR signalling.This may explain why IRAK-1 has a more dominant role in early signalling events. As time progresses the expression of the inhibitory isoform (IRAK-2c) decreases which likely allows IRAK-2a, which is no longer being inhibited, to function in a proinflammatory manner. IRAK-2a is responsible for late/sustained NFκB. In wild derived mice (MOLF/Ei), a natural mutation occurs in the promoter of IRAK-2c, which results in it being expressed significantly less. Thus the proinflammatory isoform (IRAK-2a) functions in a dominant manner. In the human system, there is no evidence of splice variants and thus human IRAK-2 acts most like IRAK-2a, the positive regulator.

Chapter 5:

The Role of TRAF6 in IRAK-2 function

5.1 Introduction

Previously we have shown that IRAK-2 is essential for early signalling events in primary human cells and has a key role in post-transcriptional regulation of TNFα Mechanistically, the involvement of IRAK-2 in TLR signalling to NFκB and its regulation of TNFα still remains unclear.

Previous work from Keating *et al.* demonstrated that expression of IRAK-2 (and not IRAK-1) could trigger the formation of polyubiquitin chains associated with TRAF6 [106]. This was intriguing as it had always been assumed that IRAK-1 was critical for TRAF6 activation. Furthermore IRAK-2 was shown to contain two TRAF6 binding motifs and mutation of a key residue in one of these motifs prevented IRAK-2 from activating NFκB or p38 [106]. Therefore it appeared that TRAF6 was an important effector of IRAK-2 function in TLR signalling to NFκB.

For TLR signalling, TRAF6 is the seminal E3 ligase that is critical for all TLR pathways to NFκB. TRAF6 together with 2 E2 enyzmes, Ubc13 and UeV1a, catalyse the synthesis of K63-linked polyubiquitin chains on target proteins in the TLR pathway including TRAF6 itself [249]. Until recently, the autoubiquitination of TRAF6 was always assumed to be a critical step in the activation of the TLR-NFκB pathway. These ubiquitin chains serve as a recruitment platform for downstream substrate proteins. Recent studies have dissected the role of TRAF6 E3 ligase activity and its ubiquitin substrate function leading to a re-evaluation as to whether the autoubiquitination of TRAF6 is necessary for its function [263].

In addition, it is known that polyubiquitin chains of different topologies direct distinct outcomes for target proteins. There are two main types of ubiquitination that occur in TLR signalling, K48-linked chains that target a protein for degradation and K63-linked chains that target a protein for signalling. Interestingly a recent study has shown that unanchored ubiquitin chains can be formed to directly activate a protein kinase [257].

Thus importance of the polyubiquitin chains associated with TRAF6 to IRAK-2 function remains to be determined as does whether such chains are covalently attached to TRAF6. IRAK-2 is critical for early TLR signalling to NFκB and for p38 activation. Mutation of a key residue in the TRAF6 binding motif of IRAK-2 prevents IRAK-2 from activating NFκB and p38 [106]. Furthermore, this IRAK-2 mutant could no longer induce the formation of polyubiquitin chains associated with TRAF6. This suggested that the ability of IRAK-2 to activate NFκB and p38 correlates with its ability to trigger the formation of polyubiquitin chains associated with TRAF6.

Mechanistically, how IRAK-2 might trigger the formation of polyubiquitin chains is still unclear. A key residue in the ATP-binding pocket of IRAK-2 has been previously described [197]. IRAK-2, like other IRAK family members contain a functional ATP-binding pocket with an invariant lysine residue (K237) in the protein kinase domain. A recent study has proposed that this residue in IRAK-2 is sufficient for IRAK-2 to act as an active kinase although whether IRAK-2 has kinase activity is still controversial. This residue, when mutated, was shown to be important for IRAK-2-induced cytokine gene expression. Whether this "ATP-binding activity" of IRAK-2 is required for its

function has not been determined. Furthermore, whether this residue in IRAK-2 is required for the formation of polyubiquitin chains is still unknown.

In addition to its critical role in early TLR signalling events, it is now known that IRAK-2 is essential in the regulation of TNFα mRNA stability via the TNF 3'UTR. However, it is unknown whether TRAF6 is required for this pathway. A previous study examining KC mRNA stabilisation revealed that while TRAF6 is essential for IL-1 induced NFκB activation, it was not required for IL-1-induced mRNA stabilisation in the murine system [238]. Thus, the role for TRAF6 in mRNA stabilisation pathways is less clear than its role in NFκB activation.

Therefore in this chapter I explore the nature of the polyubiquitin chains associated with TRAF6 triggered by IRAK-2. Furthermore I examine the importance of these chains relative to IRAK-2's function in NFκB signalling and TNFα regulation.

5.2 Results

5.2.1 IRAK-2 triggers the formation of polyubiquitin chains associated with TRAF6

5.2.1.1 Expression of IRAK-2 induces the formation of endogenous ubiquitin chains associated with TRAF6

Previous work had showed that when ubiquitin was over expressed, expression of IRAK-2 could trigger the appearance of polyubiquitin chains associated with TRAF6. Those findings were extended here by examining whether IRAK-2 could induce endogenous ubiquitination of TRAF6. HEK293_T cells were transfected with 0.5μg of IRAK-2 and harvested 24 hour later. TRAF6 was immunoprecipitated from cell lysates, subject to SDS-PAGE and immunoblots were probed with an antibody specific for detecting endogenous ubiquitin. The appearance of higher molecular weight bands detected by the ubiquitin antibody in TRAF6 immunoprecipitates was induced in the presence of IRAK-2 (Figure 5.1, upper panel, lane 2). This pattern represents polyubiquitin chains associated with TRAF6 and this demonstrates for the first time that IRAK-2 can induce the formation of endogenous ubiquitin chains associated with TRAF6.

5.2.1.2. IRAK-2 interacts with endogenous TRAF6

Thus since we had confirmed that IRAK-2 could induce ubiquitination associated with TRAF6, we next examined whether IRAK-2 could interact with endogenous TRAF6. HEK293_T cells were transfected with either 0.5 μg or 1 μg of myc-tagged IRAK-2.

Cells were harvested and endogenous TRAF6 was immunoprecipitated from cell lysates. Immunoprecipitates were probed with a myc antibody to detect an interaction between endogenous TRAF6 and myc-IRAK-2. It was clearly shown that at either dose of IRAK-2 (0.5 μg or 1 μg of DNA), there was an interaction of myc-IRAK-2 and endogenous TRAF6 (Figure 5.2, upper panel, lane 2 and 3).

5.2.1.3 Absence of murine IRAK-2 and IRAK-1 prevents IL-1-induced TRAF6 ubiquitination

Expression of IRAK-2 can trigger the formation of ubiquitin chains associated with TRAF6 in human HEK293 cells. I next examined whether IRAK-2 was required for TRAF6 ubiquitination in the murine system. Given that there was a redundancy between IRAK-1 and IRAK-2 in early NFκB signalling in the murine system, I first examined in double knockout (DKO) MEFs whether the absence of both IRAK-1 and IRAK-2 prevents TRAF6 ubiquitination.

IL-1 was used as a stimulus firstly as IL-1 is known to be a potent inducer of NFκB. Therefore endogenous ubiquitination of TRAF6 in response to IL-1 was examined. WT MEFs and DKO MEFs were seeded in 100 mm dishes and stimulated with IL-1 (100 ng/ml). Cells were subsequently harvested at time points up to 5 mins. TRAF6 was immunoprecipitated and then immunoblots were probed with an antibody specific for detecting endogenous ubiquitin. It was found that IL-1-stimulated endogenous ubiquitination of TRAF6 in WT MEFs was detectable and peaked after 1 minute (Figure 5.3, lane 3). In the absence of IRAK-1 and IRAK-2 there was a substantial decrease of endogenous ubiquitination of TRAF6 (Figure 5.3, compare lane 7 to lane 8 or lane 3 to lane 4)). IL-1-induced ubiquitination of TRAF6 is not

completely absent in the DKO MEFs but is much decreased, thus revealing that IRAK-1 and/or IRAK-2 are required for IL-1-induced TRAF6 ubiquitination.

Given that in the presence of DKO MEFs, reduced endogenous ubiquitination of TRAF6 was detected, what effect the absence of IRAK-2 alone would have was next examined. WT MEFs and IRAK-2 KO MEFS were set up in a similar experiment as described above. These cells were stimulated with 100 ng/ml of IL-1 and subsequently harvested at the time points indicated. The cells were then subject to IP of TRAF6. The IP samples were immunoblotted for endogenous ubiquitin (see section 2.3.9) and TRAF6. The samples were blotted for TRAF6 to ensure equal IP of TRAF6 occurred between samples (Figure 5.4, middle panel). In the blot that was blotted for ubiquitin, with stimulation of WT MEFs with IL-1, ubiquitination of TRAF6 was detected (Figure 5.4, upper panel 3 and 7). However in the IRAK-2 KO cells that were stimulated with IL-1 polyubiquitin TRAF6 was barely detectable. This difference can be seen most strikingly at 5 mins (Figure 5.4, upper panel, compare lanes 7 and 8). Thus endogenous ubiquitination of TRAF6 is detected in the WT MEFs but is much decreased in the IRAK-2 KO MEFs.

5.2.2. Exploring the nature of the polyubiquitin chains induced by IRAK-2 expression

5.2.2.1 TRAF6 E3 ligase activity is required for the ubiquitination events associated with TRAF6 autoactivation

It is clear that IRAK-2 triggers the formation of polyubiquitin chains associated with TRAF6. Therefore IRAK-2 may have a role in turning on the E3 ligase activity of TRAF6. It is well known that TRAF6 is a critical E3 ligase in NFκB activation. TRAF6 subsequently undergoes autoubiquitination, which was always assumed to be a critical step in the activation of NFκB. This autoubiquitination was assumed to act as a scaffold for downstream signalling molecules, allowing conformational changes or phosphorylation events to occur. Recent studies have explored the relative contribution of the autoubiquitination of TRAF6 to TRAF6 function [263]. Thus, I wanted to explore whether the E3 ligase and the autoubiquitination activity of TRAF6 were essential for its function. For this, two TRAF6 mutants were used. A TRAF6 mutant with a mutation of a key cysteine residue in the RING-domain of TRAF6 was used to test the role of the E3 ligase activity of TRAF6. The RING finger domain of TRAF6 functions as an ubiquitin E3 ligase capable of generating non-degradative K63-linked ubiquitin chains [249]. In order to explore the contribution of the autoubiquitination of TRAF6, a TRAF6 mutant with residue K124 mutated to an arginine was used. This residue has been shown to be the predominant site for TRAF6 autoubiquitination. This site is absolutely conserved in TRAF6 between species and is known to be exposed on the TRAF6 surface [278].

Thus to test these mutants, over expression of TRAF6 was used. Over expression of TRAF6 results in the autoactivation of TRAF6 itself. For this reason it was not sensible to co express IRAK-2 to trigger TRAF6-associated ubiquitination. HEK293_T cells were transfected with either 1 μg of FLAG-WT TRAF6, FLAG-C70A-TRAF6, FLAG-K124R-TRAF6 or FLAG-DN-TRAF6 (dominant negative TRAF6 with residues 1-289 removed) and 1 μg of HA-ubiquitin. Cells were harvested and subject to immunoprecipitation with a FLAG antibody. Association of HA-ubiquitin to WT-TRAF6 occurred in the presence of WT TRAF6 (Figure 5.5, upper panel, lane 5) while DN-TRAF6 failed to induce the ubiquitination of TRAF6 (Figure 5.5, upper panel, lane 6). Upon expression of the C70A TRAF6, this mutant failed to induce polyubiquitination of TRAF6 (Figure 5.5, upper panel, lane 7). This demonstrates that the E3 ligase activity of TRAF6 is required for the formation of polyubiquitin chains associated with TRAF6. Interestingly, upon expression of K124R TRAF6 in this ubiquitination assay, this mutant could induce the formation of polyubiquitin chains associated with TRAF6, comparable with expression of WT TRAF6 (Figure 5.5, upper panel compare lanes 5 and lane 8). Expression of C70A TRAF6 was shown to be slightly higher than WT TRAF6 and K124R TRAF6 (Figure 5.5, lower panel, compare lane 7 to lane 5 and lane 8) therefore the lack of polyubiquitin chains formed associated with TRAF6 could not be explained by reduced C70A TRAF6 expression.

5.2.2.2 The TRAF6 E3 ligase activity, but not K124 autoubiquitination, is essential for TRAF6 function

Thus mutation of TRAF6 at a key residue (C70) in its RING-finger domain has revealed that the E3 ligase activity is required for the formation of polyubiquitin chains

associated with TRAF6. The ubiquitin substrate activity of TRAF6 at lysine 124 is dispensable for the formation of these chains. We next examined whether the E3 ligase and autoubiquitination functions of TRAF6 are required for TRAF6 to activate downstream signalling pathways.

A NFκB reporter gene assay was used to examine whether the E3 ligase activity and the autoubiquitination of TRAF6 at K124 were required for TRAF6 to activate NFκB. The results clearly show that WT TRAF6 could induce the activation of NFκB (Figure 5.6 (A)). C70A-TRAF6 could no longer activate NFκB, revealing that the E3 ligase activity of TRAF6 is essential for NFκB activation. The autoubiquitination of TRAF6 was dispensable for activation of NFκB as the K124R-TRAF6 mutant could still induce the NFκB-reporter (Figure 5.6, (A)).

It was next examined whether the E3 ligase activity and the autoubiquitination of TRAF6 were required for the activation of p38 MAP kinase. For this, the p38 CHOP assay was used. This assay is based on the ability of p38 MAP kinase to phosphorylate and activate the transcription factor CHOP. This is assayed by an increase in the ability of the GAL4-CHOP fusion protein to transactivate the pFR-luciferase reporter, which contains GAL4 binding sites in its promoter. As is the case with NFκB, expression of WT TRAF6 could activate p38 MAP kinase (Figure 5.6 (B). However, expression of C70A-TRAF6 failed to activate p38 MAP kinase, demonstrating that the E3 ligase activity of TRAF6 is also required for p38 MAP kinase activation. Expression of K124R-TRAF6 induced activation of p38 MAP kinase to comparable levels as the WT TRAF6, indicating that the autoubiquitination of TRAF6 is not required to activate p38 MAP kinase (Figure 5.6 (B)).

The role for TRAF6 in mRNA stability is unknown, therefore we examined the possible role for TRAF6 in the MyD88-IRAK-2-TNF 3'UTR pathway. Interestingly, WT TRAF6 could strongly induce the TNFα 3'UTR reporter, in comparison to DN TRAF6 (Figure 5.7). Furthermore the RING finger of TRAF6 was also required for the induction of the TNF 3'UTR pathway as the TRAF6 C70A mutant no longer induced the TNF 3'UTR reporter (Figure 5.7). The autoubiquitination activity of TRAF6 was shown to be dispensable for the activation of the TNF 3'UTR as the K124R mutant was able to induce the TNF 3'UTR to a similar degree as the WT TRAF6 (Figure 5.7). The results show that TRAF6 E3 ligase activity, but not K124-linked autoubiquitination was essential for NFκB, p38 and TNF 3'UTR activation, three signalling events shown to also require IRAK-2.

5.2.2.3 IRAK-2 stimulates the formation of K63-linked chains associated with TRAF6

Previous results indicate that TRAF6 is the E3 ligase that is required for the formation of polyubiquitin chains associated with TRAF6 and that the requirement of TRAF6 E3 ligase activity correlates with the requirement for IRAK-2 for NFκB, p38 and TNF 3'UTR activation. However the nature of the TRAF6-associated polyubiquitin chains is still unknown. As mentioned previously, polyubiquitin chains of various lengths and conformations can attach to substrate proteins resulting in differential signalling outcomes. There are at least seven different types of polyubiquitin chains that can be formed. Of these both K48 and K63 linked chains have been well characterised to play vital roles in TLR signalling.

Here we wanted to examine whether the polyubiquitin chains associated with TRAF6 induced in the presence of IRAK-2 were K48- and/or K63–linked chains. HEK293_T

cells were transfected with increasing amounts of WT IRAK-2 and harvested. Cell lysates were divided in two and subject to immunoprecipitation with an endogenous TRAF6 antibody. Immunoprecipitated samples were subject to SDS-PAGE and immunoblotted with antibodies specific for K63- or K48-linked polyubiquitin chains (Figure 5.8, upper panel, lanes 1-4 probed with K63-linked antibody and lanes 5-8 probed with K48-linked antibody).

Increasing doses of IRAK-2 increased the appearance of K63-linked polyubiquitin chains (Figure 5.8, upper panel lanes 3 and 4). In contrast, expression of IRAK-2 did not cause the appearance of K48-linked polyubiquitin chains. This suggests that the polyubiquitin chains associated with TRAF6 that are induced upon expression of IRAK-2 are K63-linked polyubiquitin chains. The detection of immunoprecipitated TRAF6 was reduced with increasing amounts of IRAK-2 (Figure 5.8, second panel). This may be due to the presence of TRAF6-associated ubiquitin blocking antibody recognition of TRAF6 epitopes. This was not due to degradation of TRAF6, as expression of TRAF6 was not affected in the lysates (Figure 5.8, lower panel).

Thus the IRAK-2 dependent activation of TRAF6 E3 ligase activity results in the generation of (non-degradative K63-linked) ubiquitin chains that are critical for NFκB activation. This experiment was only performed once as the detection of K63- and K48-linked chains were difficult to detect so this experiment would have to be repeated.

5.2.2.4 Polyubiquitin chains induced by IRAK-2 are not covalently attached to TRAF6

A recent study by Xia *et al.* has shown that free polyubiquitin chains play an important role in NFκB signalling. These chains are free K63-linked chains that are not covalently associated with a protein and can directly activate protein kinases [257]. Therefore it is possible that the IRAK-2-dependent ubiquitin chains formed by TRAF6 are not covalently attached to TRAF6. To examine whether the polyubiquitin chains were directly attached to TRAF6, immunoprecipitates could be treated with SDS-precipitation to remove any non-covalently bound interactions.

HEK293_T cells were transfected with 1 μg of HA-ubiquitin and 0.5 μg of WT IRAK-2. Cells were harvested and immunoprecipitated with an endogenous antibody that was specific for TRAF6. The TRAF6 immunoprecipitates were resuspended in 1% SDS without any reducing agent, followed by incubation for 5 mins at 70˚C to disrupt, by denaturation, the interaction with TRAF6 with other proteins. After dilution to 0.1% SDS, TRAF6 was again immunoprecipitated and released from the beads by denaturation in 1% SDS, subject to SDS-PAGE, transferred to PVDF and immunoblotted with an anti HA-antibody to detect HA-ubiquitin covalently attached chains.

When HEK293_T cells are subject to SDS precipitation, IRAK-2-dependent induction of polyubiquitin chains were significantly reduced and almost undetectable (Figure 5.9, upper panel compare lane 4 and lane 8). Importantly, the detection of TRAF6 after IP is still detectable after SDS treatment (Figure 5.9, middle panel, lanes 5-8). This indicates that the majority of polyubiquitin chains induced by IRAK-2 are not covalently attached to TRAF6.

Recently it has been proposed that TLRs triggers the formation of unanchored Lys63 polyubiquitin chains, which are not conjugated to any target protein, activate TAK-1. Association with these polyubiquitin chains promotes TAB-2-mediated TAK-1 transphopshorylation and activation [157]. Using the enzyme isopeptidase T (IsoT) which specifically degrades free but not covalently attached polyubiquitin chains, it was shown that IsoT completely blocked the activation of TAK-1 (ref). Other signalling pathways have also been shown to be activated by unanchored polyubiquitin chains; It has been shown that the IKK complex is activated by NEMO-bound free ubiquitin chains [157] while RIG-I requires binding of unanchored polyubiquitin for full activation [14].

The SDS precipitation IP suggested that IRAK-2-induced the TRAF6-dependent formation of unanchored polyubiquitin chains. In order to gain further evidence for this we next examined whether WT IRAK-2 induced polyubiquitin chains associated with TRAF6 were affected after treatment with IsoT. Thus, cells that were transfected with 0.5 μg WT IRAK-2 were subject to immunoprecipitation with an endogenous TRAF6 antibody. These immunoprecipitated samples were then incubated with or without IsoT for 1 hour at 30˚C. As shown previously, IRAK-2 triggered the formation of endogenous polyubiquitin chains associated with TRAF6 (Figure 5.10) However in the presence of IsoT these chains were somewhat reduced indicating that the chains associated with TRAF6 may be free polyubiquitin chains. Thus, consistent with the critical role of IRAK-2 in TRAF6 activation and recent

reports of the importance of free polyubiquitin chains in TLR signalling, evidence suggests that IRAK-2 can stimulate the formation of such chains.

5.2.3 Important residues of IRAK-2 required for its function

5.2.3.1 Residues in IRAK-2 required for the formation of polyubiquitin chains

Recently it has been proposed that a residue in the ATP-binding pocket of IRAK-2 may be important for its function [197]. The invariant lysine residue at K237 has been proposed to be necessary for IRAK-2 to act as an active kinase although whether IRAK-2 has actual kinase activity is still controversial [162]. Therefore we wanted to determine whether this residue of IRAK-2 was required for IRAK-2-induced polyubiquitination associated with TRAF6. HEK293T cells were transfected with HA-ubiquitin and either WT IRAK-2, E528A-IRAK-2 or K237A IRAK-2. E528A-IRAK-2 is a mutation in the TRAF6 binding motif of IRAK-2 and it has previously been shown by mutation that this residue is critical for IRAK-2 function [106]. A TRAF6 antibody was used to immunoprecipitate endogenous TRAF6 from HEK293_T cells. The immunoprecipitated proteins were then immunoblotted with an antibody against HA or TRAF6. In this ubiquitination assay it was observed that conjugation of HA-ubiquitin to endogenous TRAF6 was clearly induced in the presence of WT IRAK-2 (Figure 5.11, lane 3, upper panel). Significantly, in the presence of E528A-IRAK-2, this mutant could no longer induce the polyubiquitination associated with TRAF6 (Figure 5.11, lane 4, upper panel). Expression of K237A-IRAK-2 could induce the formation of polyubiquitin chains associated with TRAF6 comparable to the WT-IRAK-2 (Figure

5.11, lane 5, upper panel). This reveals that this key residue in the ATP-binding pocket of IRAK-2 is not required for the formation of polyubiquitin chains associated with TRAF6.

5.2.3.2 Residues in IRAK-2 required for IRAK-2 function

Next, I examined whether these residues in IRAK-2 were required for downstream signalling events that previously have been shown to require IRAK-2. A reporter gene assay was used to examine whether these IRAK-2 mutants could still activate NFκB. HEK293_R1 cells were transfected with WT IRAK-2, E528A-WT IRAK-2 or K237A-IRAK-2 and NFκB activity was measured. As expected, WT IRAK-2 could activate NFκB in a dose-dependent manner (Figure 5.12 (A)). Expression of E528A-IRAK-2 could no longer activate NFκB confirming that a single amino acid change in the first TRAF6 binding motif in IRAK-2 is essential for IRAK-2 function. Interestingly, while expression of K237A-IRAK-2 could still induce the formation of polyubiquitin chains associated with TRAF6, it could no longer activate NFκB (Figure 5.12 (A)). Next I examined whether expression of E528A-IRAK-2 and K237A-IRAK-2 could activate p38 MAP kinase. Using the CHOP assay, I next showed that expression of WT IRAK-2 could activate p38 MAP kinase while expression of either E528A-IRAK-2 or K237A-IRAK-2 could no longer activate p38 MAP kinase (Figure 5.12, (B)).

Thus here we demonstrate for the first time that this residue in human IRAK-2 (K237) is critical for the activation of NFκB and p38 MAP kinase but dispensable for the induction of polyubiquitin chains associated with TRAF6. In contrast to this mutation of the E528 residue in the TRAF6 binding domain of IRAK-2 could no longer activate NFκB and p38 and this correlated with its ability to stimulate the polyubiquitination

of TRAF6. Therefore the polyubiquitin chains that are associated with TRAF6 and triggered by IRAK-2 are necessary but not sufficient for IRAK-2 to activate downstream signalling.

In the previous chapter I have shown that IRAK-2 is required for TNFα mRNA stabilisation via the TNF 3'UTR. Thus, I examined the role of key IRAK-2 residues in the regulation of the TNF 3'UTR using the reporter system. The previous experiment showed that when the residues K237 and E528 were mutated IRAK-2 could no longer activate NFκB nor p38 MAPK. Expression of E528A IRAK-2 failed to induce the activity of the TNF 3'UTR thus demonstrating for the first time that this residue in IRAK-2 is required for the activation of TNF 3'UTR (Figure 5.13). Similarly, here the K237A IRAK-2 mutant could no longer induce the activity of the TNF 3'UTR (Figure 5.13). Thus this reveals that the TRAF6-binding activity and the "ATP-binding activity" of IRAK-2 are required for the induction of the TNF 3'UTR.

We next examined other residues of IRAK-2 that may be important for IRAK-2 function. Some Single nucleotide polymorphisms (SNPs) in TLRs and their adaptor molecules have been shown to influence susceptibility to various infections [279]. In the human population 12 natural occurring variants in the IRAK-2 gene were selected as potentially affecting the function of IRAK-2 (L503I, L392V, L78M, L358M, E501I, D431E, L439V, R43Q, S47Y, A498G, R214G, I99V) (A. Weber pers. comm.) Thus, we wanted to examine whether these IRAK-2 SNPs affected IRAK-2 function. Firstly a reporter gene assay was used to screen which of these SNPs altered NFκB activation compared to WT IRAK-2. HEK293_T cells were transfected with 25 ng of WT IRAK-2

or 25ng of 12 different IRAK-2 SNPs. As expected, expression of WT IRAK-2 stimulated activation of NFκB. Most of the IRAK-2 SNPs were unable to activate NFκB, however for two IRAK-2 SNPs, L78M and S47Y (both located in the death domain of IRAK-2), their ability to activate NFκB was not impaired. (Figure 5.14 (A)). Of the 12 SNPs examined, 5 of these IRAK-2 SNPs impaired NFκB robustly (Figure 5.14 (B)). Therefore it was next examined whether these naturally occurring mutations in IRAK-2 impaired its ability to form polyubiquitin chains associated with TRAF6. Firstly, expression of the IRAK-2 SNPs was examined. HEK293_T cells were transfected with 1μg of either HA-WT IRAK-2 or the HA-IRAK-2 SNPs. Cells were harvested and cell lysates were subject to SDS-PAGE and immunoblots were probed with a HA antibody to detect IRAK-2 expression. As shown in Figure 5.15 (A), the expression of IRAK-2 SNPs were comparable. Therefore we next examined what effect the expression of these IRAK-2 SNPs would have on the formation of polyubiquitin chains associated with TRAF6. Both WT IRAK-2 (myc tagged C terminus) and WT IRAK-2 (HA-tagged C terminus) were able to trigger the formation of endogenous polyubiquitin chains associated with TRAF6 (Figure 5.15 (B), upper panel, lanes 2 and 4). Interestingly, the ability of WT IRAK-2, with a HA-tagged on the N-terminus, to trigger formation of these chains was somewhat impaired (upper panel, lane 3) Two of the IRAK-2 SNPs (L503I and L392V) displayed impaired ability to trigger polyubiquitin chains associated with TRAF6 (Figure 5.15, (B), upper panel, lanes 5 and 6) while three of the IRAK-2 SNPs (R43Q, A498G and R214G) induced the formation of these chains comparable to the WT IRAK-2 (Figure 5.15, (B) upper panel, lanes 7, 8 and 9). Therefore, as was the case with K237A-IRAK-2, even though these three IRAK-2 SNPs were no longer able to activate NFκB, this did not correlate with a lack of ability to

trigger the formation of polyubiquitin chains. This further confirms that the formation of polyubiquitin chains associated with TRAF6 induced by IRAK-2 are necessary but not sufficient for IRAK-2 function.

5.3 Discussion

IRAK-2 has a critical role in early signalling events in primary human cells. Furthermore, IRAK-2 is now known to be essential for post transcriptional regulation of TNFα. Mechanistically, how IRAK-2 regulates these processes is still unknown. An important effector downstream of IRAK-2 in TLR signalling is TRAF6. Thus the role of TRAF6 in IRAK-2 function was explored in this chapter.

Previous work had shown that upon expression of IRAK-2 with over expressed ubiquitin, IRAK-2 could induce the formation of polyubiquitin chains associated with TRAF6 [106]. I extended those findings here by demonstrating for the first time that the expression of IRAK-2 triggered the formation of endogenous polyubiquitin chains associated with TRAF6.

Since the previous publication which showed that IRAK-2 could trigger TRAF6 polyubquitination [106], it is now known that the physiological relevant ubiquitin chains for NFκB signalling are TRAF6-catalysed unanchored K63-linked polyubiquitin chains. Unanchored polyubiquitin chains are required for the activation of TAK-1 in the NFκB pathway. These are free K63-linked polyubiquitin chains that are not attached to their protein targets. Thus given that IRAK-2 has a critical role in the formation of polyubiquitin chains associated with TRAF6, it was hypothesised that

IRAK-2 may contribute to the formation of TRAF6-catalysed free polyubiquitin chains. TRAF6 contains a RING-finger domain which functions as an ubiquitin E3 ligase which can form K63-linked ubiquitin chains required for signalling. I have shown here that the E3 ligase activity of TRAF6 is essential for the formation of these chains. Furthermore the E3 ligase activity of TRAF6 is required for TRAF6 to activate NFκB and MAP kinase pathways. This is in agreement with two other reports which have shown that upon mutation of the C70 residue in the RING finger domain of TRAF6, the ligase activity of TRAF6 was abolished and consequently the ability of TRAF6 to activate TLR signalling pathways. Thus TRAF6 is the E3 ligase responsible for its own ubiquitination and it was previously assumed that this autoubiquitination of TRAF6 was a key step in the activation of NFκB. A key lysine residue, K124, has previously been reported to be essential for both TRAF6 autoubiquitination and signalling [278]. However I have shown here that when this lysine residue is mutated to an arginine, TRAF6 autoubiquitination is not impaired and the polyubiquitin chains that are triggered are comparable to the wild type TRAF6. Thus even though one of the targets of TRAF6 ubiquitin ligase activity is TRAF6 itself, since the K124 residue has been suggested to be the predominant site for autoubiquitination of TRAF6, this suggests that TRAF6 autoubiquitination is dispensable for TRAF6 to activate NFκB and p38 MAPK. TRAF6 is known to have seven conserved lysine residues located in the RING domain and first zinc finger of TRAF6 which are subject to autoubiquitination [278]. However another group who mutated all lysine residues in TRAF6 demonstrated that this did not impair TRAF6 function [263]. Thus the autoubiquitination of TRAF6 is dispensable for its function and may only be required in the absence of alternative ubiquitin substrates.

IRAK-2 can trigger the formation of endogenous polyubiquitin chains associated with TRAF6 and this event requires the E3 ligase activity of TRAF6 to form these polyubiquitin chains. Therefore, to examine what type of ubiquitin chains were formed by IRAK-2, linkage specific antibodies were used which recognise polyubiquitin chains through lysine 63 (K63) or lysine 48 (K48). Use of these antibodies to detect specific lysine polyubiquitin chains is thought to be more beneficial than previous methods of detecting polyubiquitin chains such as ubiquitin-specific mutants or mass spectrometry [179]. Here I show that increasing doses of IRAK-2 increased the appearance of K63-linked polyubiquitin chains but did not increase the appearance of K48-linked chains. This experiment was only preformed once however as it was difficult to detect K63- and K48-linked chains. It is possible that IRAK-2 could trigger the formation of both K63- and K48-linked chains. It is known that ubiquitin targets are often ubiquitinated with both K48-linked and K63-linked chains at multiple locations [179]. For example RIP-1 and IRAK-1 both undergo K63-linked polyubiquitination initially, while at later times are subject to K48-linked polyubiquitin which targets them for proteasomal degradation [179].

As mentioned previously, a recent study has demonstrated that unanchored polyubiquitin chains are required for the activation of TAK-1 in the NFκB pathway. These are free K63-linked polyubiquitin chains that are not attached to their protein targets. Therefore the TRAF6-associated polyubiquitin chains triggered by IRAK-2 may not be covalently linked to TRAF6. When immunoprecipitates were treated with SDS precipitation, the formation of these polyubiquitin chains by IRAK-2 were

significantly reduced. This demonstrates that the polyubiquitin chains formed by IRAK-2 are not directly attached to TRAF6. It is possible that these polyubiquitin chains are not unanchored chains but rather could be attached to a TRAF6 binding protein such as TAK-1. However to further clarify whether the polyubiquitin chains triggered by IRAK-2 may be unanchored chains, cells were treated with Isopeptidase T (IsoT). IsoT is an enzyme which specifically cleaves unanchored polyubiquitin chains. Here upon treatment with IsoT, the formation of IRAK-2-dependent chains were somewhat reduced. This further confirmed that IRAK-2 triggers the formation of free polyubiquitin chains associated with TRAF6. Given that IsoT treatment did not entirely remove the polyubiquitin chains associated with TRAF6, it is possible that IRAK-2 can trigger the formation of both unanchored and anchored chains associated with TRAF6.

Overall then, our findings regarding the role for IRAK-2 in TRAF6-associated polyubiquitination in NFκB signalling, lead to an updated model of the current understanding of TRAF6 ubiquitination in this pathway (Figure 1.8). Upon receptor engagement, MyD88 is recruited to the receptor complex. The Myddosome complex is formed which contains 6-8 MyD88, 4 IRAK-4 and 4 IRAK-2/IRAK-1 [105, 143]. IRAK-4 is thought to phosphorylate IRAK-2 and IRAK-1 in this complex. IRAK-2 then triggers the E3 ligase activity of TRAF6, perhaps due to the oligomerisation of TRAF6. TRAF6 then forms *unanchored* K63-linked polyubiquitin chains, TRAF6 itself is autoubiquitinated which we now know is dispensable for TRAF6 function. These unanchored polyubiquitin chains are required for the activation of downstream signalling mediators such as TAK-1 and NEMO, ultimately leading to NFκB activation.

TRAF6 is unique from other TRAF family members in that it utilises a distinct interaction motif found in its upstream activators, for example IRAK-2 is known to contain two TRAF6 binding motifs [106].Previously it has been shown that E528, a residue in one of these TRAF6 binding motifs of IRAK-2, was critical for IRAK-2 to induce the formation of polyubiquitin chains associated with TRAF6. It was also shown to be critical for IRAK-2 activation of NFκB and p38 MAP kinase. Another residue in IRAK-2, in the ATP-binding pocket of IRAK-2 (at K237), has recently been proposed to be important for IRAK-2 function. Therefore I wanted to examine whether this residue, like E528, was required for the formation of polyubiquitin chains associated with TRAF6. When this residue at K237 was mutated, IRAK-2 could still trigger the formation of polyubiquitin chains associated with TRAF6, thus revealing that the "ATP binding activity" of IRAK-2 was not required for the formation of TRAF6-associated polyubiquitin chains. However, this residue in IRAK-2 was essential for activation of NFκB, p38 MAP kinase and TNF 3'UTR by IRAK-2. Thus while the ability of the E528A mutant to activate downstream signalling correlated with its ability to stimulate TRAF6-associated polyubiquitination; this was not the case for the K237 mutant. Therefore this suggests that the polyubiquitination of TRAF6 by IRAK-2 is necessary but not sufficient for IRAK-2 to activate downstream signalling.

Interestingly IRAK-2 was also required for the formation of TRAF-6 associated polyubiquitin chains in the murine system. With the use of IRAK-2 $^{-/-}$ MEFs, I revealed that IRAK-2 was required for early IL-1-induced polyubiquitin chains associated with TRAF6. Therefore even though it has been reported that IRAK-2 is not essential for

early TLR events in the murine system, I demonstrate here that IRAK-2 is required for the formation of IL-1-induced polyubiquitin chains associated with TRAF6. This is further evidence that TRAF6-associated polyubiquitination is necessary but not sufficient for IRAK-2 function.

Recent studies have described single nucleotide polymorphisms (SNPs) of various TLR signalling molecules that can influence the susceptibility of individuals to infection. For example a SNP that was discovered in the MAL gene at position 180 converting a serine to a leucine revealed a protective effect between MAL and a number of diseases including malaria [149]. Hetereozygotes for the gene showed a near halving of disease risk [149]. In the human population 12 natural occurring variants in the IRAK-2 gene were selected as potentially affecting the function of IRAK-2 (L503I, L392V, L78M, L358M, E501I, D431E, L439V, R43Q, S47Y, A498G, R214G, I99V) (A. Weber. *pers. comm.*).

For two of these SNPs, located in the DD of IRAK-2 (S47Y and L78M), the ability to activate NFκB was not impaired (Figure 5.16). In contrast, the R43Q SNP, also located in the DD of IRAK-2, this residue was shown to be essential for IRAK-2 to activate NFκB. This residue has been shown to be important for Myddosome formation [105]. Five of these SNPs ability to activate NFκB was robustly impaired (R43Q, R214G, L392V, A498G and L503I). Interestingly, 3 of these SNPs (R43Q, R214G and A498G) could still trigger the formation of polyubiquitin chains comparable to WT IRAK-2. Thus as is the case with K237, these residues are dispensable for IRAK-2 to trigger the formation of polyubiquitin chains associated with TRAF6 (see Table 1.2).

Two of the SNPs (L392V and L503I) impaired NFκB activation and these residues were also required for the induction of TRAF6-associated polyubiquitin chains. Thus these SNPs are similar to E528, when this residue is mutated, IRAK-2 could no longer activate NFκB and this correlated to its lack of ability to trigger TRAF6-associated polyubiquitin chains.

Thus it appears that the IRAK-2 induction of polyubiquitin chains associated with TRAF6 is necessary but not sufficient for NFκB activation. It is possible that there are some functions of IRAK-2 that require only the formation of polyubiquitin chains associated with TRAF6, whereas there are other functions of IRAK-2 including NFκB, p38 MAPK and TNF 3'UTR activation, that the formation of the polyubiquitin chains are required but not sufficient. Other activities of IRAK-2, such as its ATP binding activity, are also required for NFκB, p38 MAPK and TNF 3'UTR but is dispensable for TRAF6-associated polyubiquitin chain formation.

While the role for various IRAK family members in post transcriptional regulation has been described, the role of TRAF6 in mRNA stabilisation is less well defined. For example, it has been previously reported that IL-1-induced stabilisation of KC and MIP-2 mRNA was IRAK-1-dependent, but TRAF6-independent [238]. Here we provided evidence that TRAF6 is required in the MyD88-IRAK-2-TNF 3'UTR pathway as WT TRAF6 stimulated the TNFα 3'UTR reporter. Furthermore although WT TRAF6 induced the activity of the TNF 3'UTR, a TRAF6 mutant (C70A) impaired in its E3 ligase activity was not active, while a TRAF6 mutant with a mutated autoubiquitination site (K124R) was. Thus, the E3 ligase activity of TRAF6 is also

essential for activation of the TNF 3'UTR. This data suggests an existence of a MyD88-IRAK-2-TRAF6-dependent pathway converging on the TNF 3'UTR.

Thus in conclusion, IRAK-2 is required for triggering the E3 ligase activity of TRAF6 to catalyse the formation of K63-linked free polyubiquitin chains. These polyubiquitin chains are necessary but not sufficient for IRAK-2 ability to activate NFκB. Finally, there is emerging preliminary evidence for a role of TRAF6 in mRNA stabilisation.

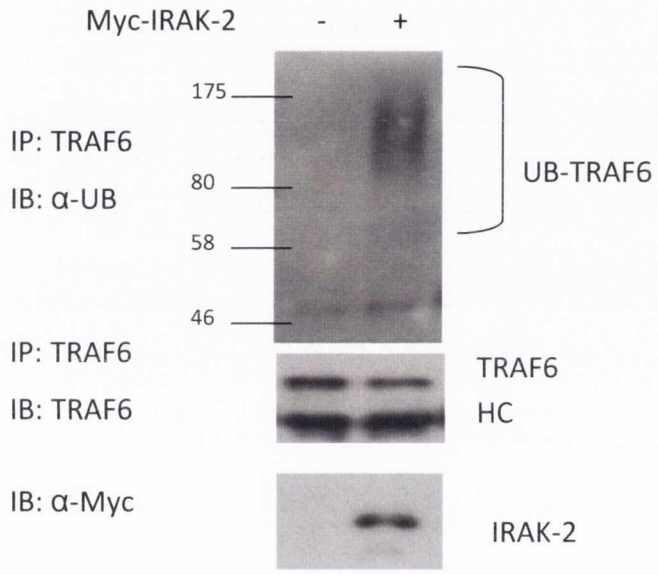


Figure 5.1 IRAK-2 induces endogenous ubiquitination associated with TRAF6.

HEK293T cells were transfected with either 0.5μg myc-IRAK-2 for 24 hrs. Endogenous TRAF6 was immunoprecipitated from cell lysates and endogenous ubiquitination of TRAF6 was assessed by immunoblotting with an antibody specific for detecting endogenous ubiquitin (upper panel, UB TRAF6; ubiquitin chains associated with TRAF6). The second panel shows anti-TRAF6 immunoprecipitates immunoblotted for endogenous TRAF6 (second panel HC; heavy chain of anti-TRAF6 antibody used in IP). The third panel shows expression of myc-IRAK-2 in lysates. The experiment is representative of three individual experiments.

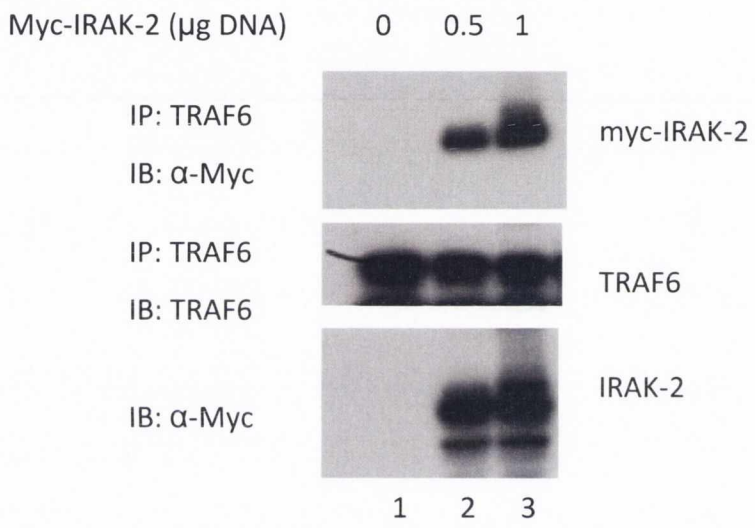


Figure 5.2 myc-IRAK-2 interacts with endogenous TRAF6.

HEK293T cells were transfected with either 0.5μg or 1μg myc-IRAK-2 for 24 hrs. Endogenous TRAF6 was immunoprecipitated from cell lysates and interactions between endogenous TRAF6 and myc-IRAK-2 were assessed by immunoblotting with anti-myc Ab (upper panel). The second panel shows anti-TRAF6 immunoprecipitates immunoblotted for endogenous TRAF6. The third panel shows expression of myc-IRAK-2 in lysates. The experiment is representative of three individual experiments.

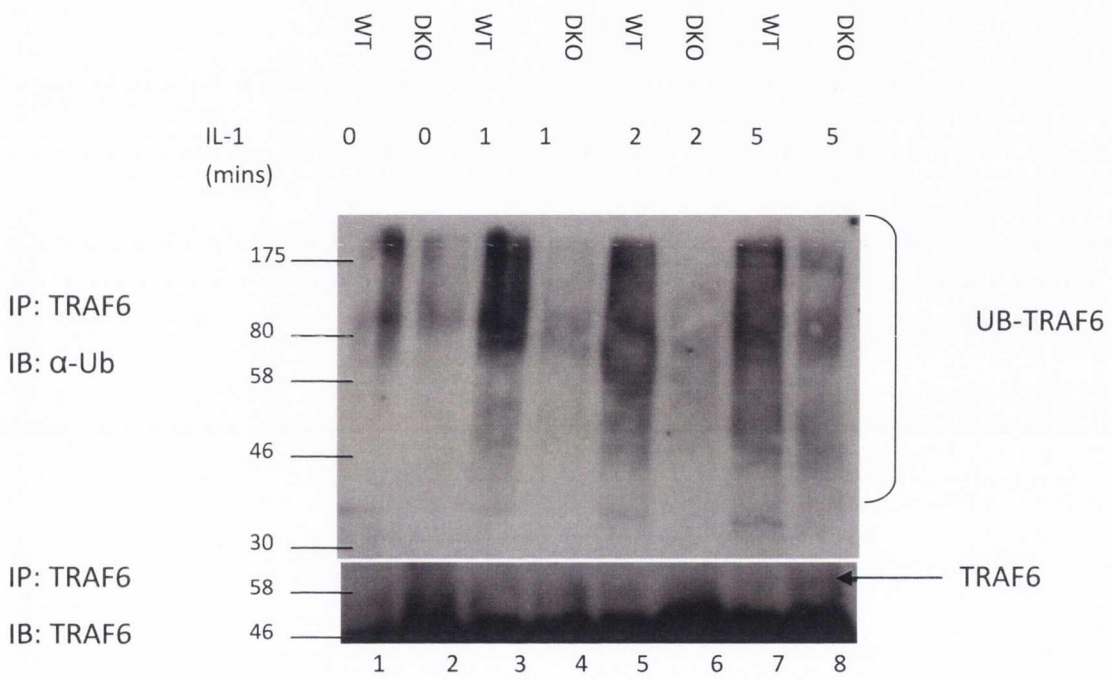


Figure 5.3 Absence of murine IRAK-1 and IRAK-2 inhibit TRAF6 ubiquitination. Wild-type (WT) and IRAK-1/IRAK-2 double knock-out (DKO) murine embryonic fibroblasts (MEFs) were seeded in 100mm dishes and 24h later stimulated with 100ng/ml IL-1 for the times indicated. The cells were harvested and subjected to immunoprecipitation. Samples immunoprecipitated with an anti-TRAF6 antibody were resolved by SDS-PAGE and were immunoblotted with an antibody specific for endogenous ubiquitin (upper panel). The samples were also immunoblotted for endogenous TRAF6 (lower panel). The experiment is representative of two independent experiments.

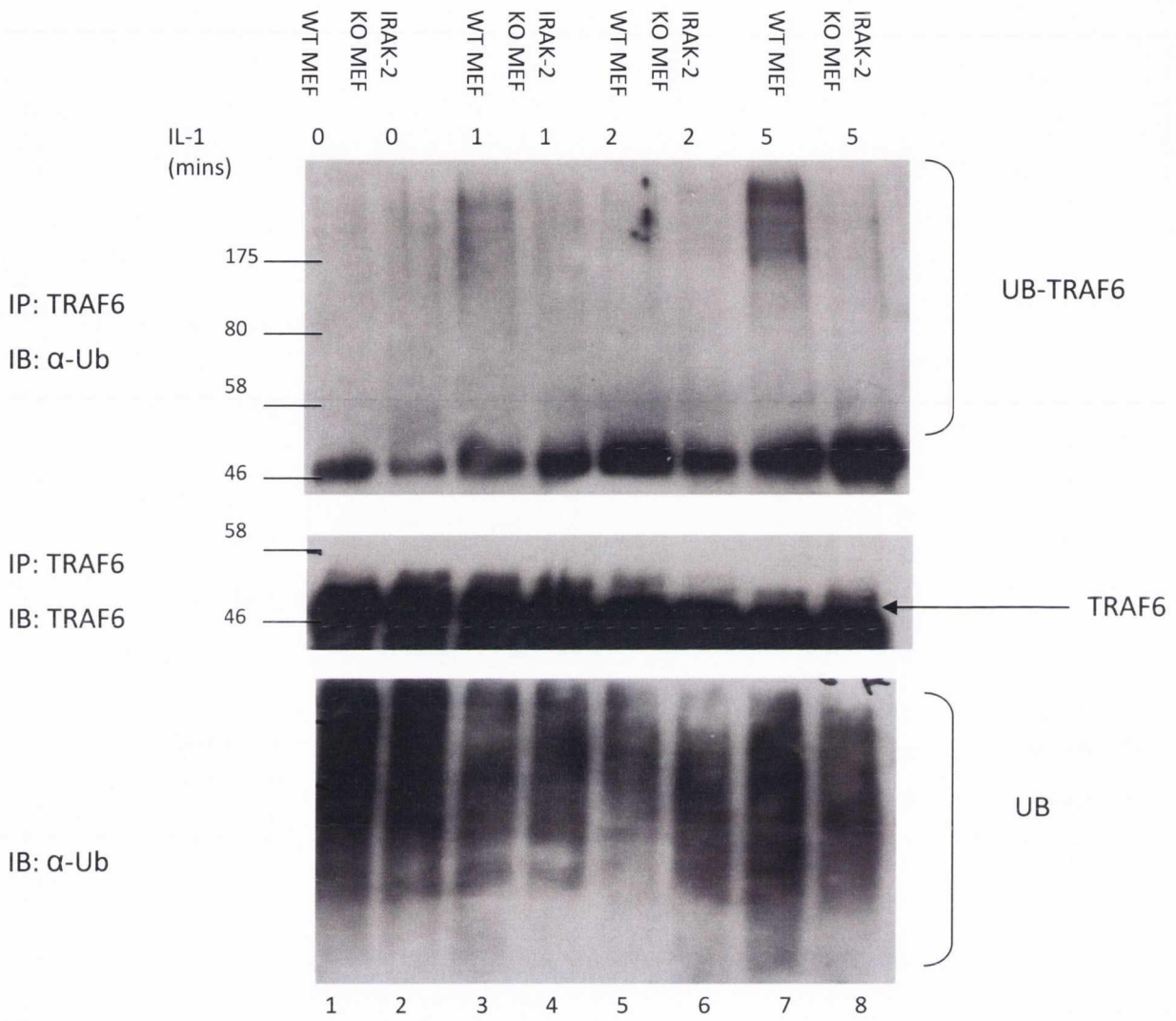


Figure 5.4 Examining TRAF6 ubiquitination in IRAK-2 KO MEFs. Cells were seeded at $1x10^5$/ml and stimulated 24 hours later with 100ng/ml of IL-1. Cells were harvested at times indicated in table above and then subject to immunprecipitation with a TRAF6 antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted with either an antibody specific for endogenous ubiquitin (upper panel) or a TRAF6-specific antibody (middle blot) Cell lysates were also immunoblotted for endogenous ubiquitin (lower panel). The experiment is representative of three independent experiments.

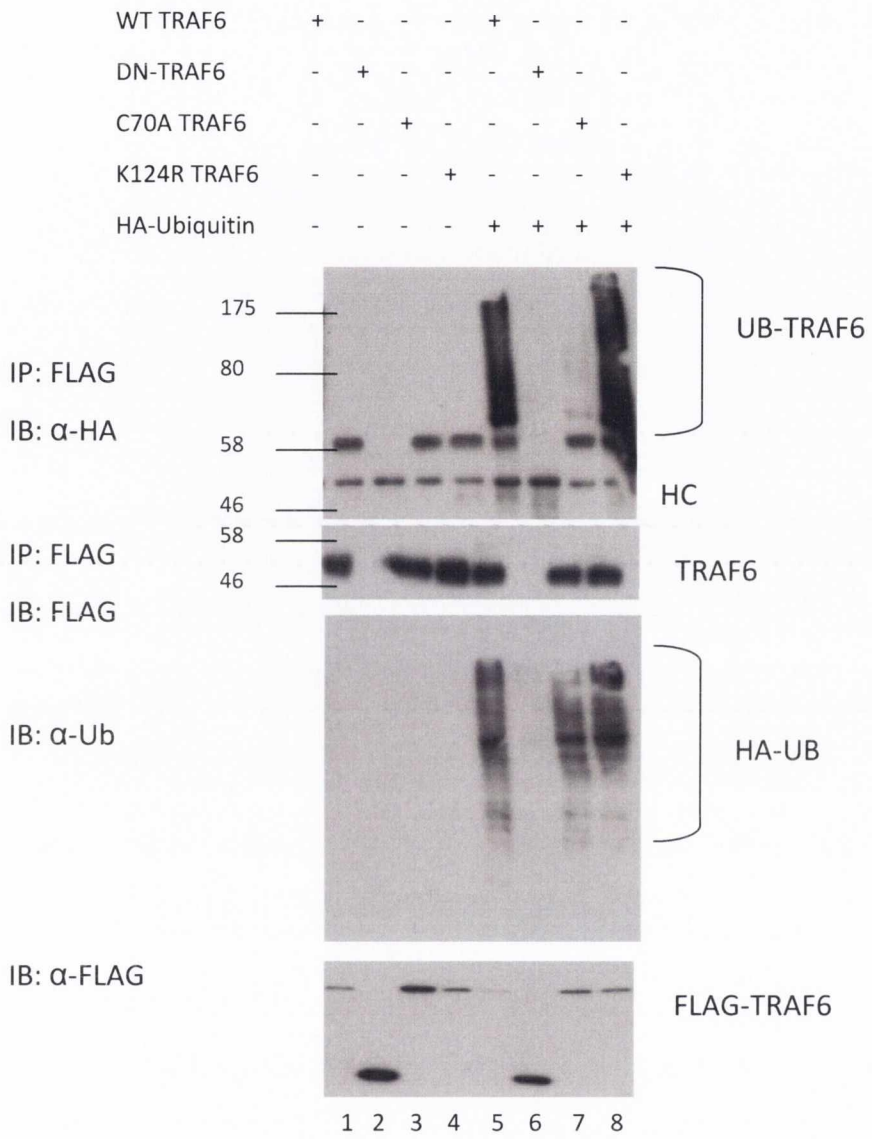


Figure 5.5 The E3 ligase activity of TRAF6 is essential for TRAF6-associated Ubiquitination. HEK293T cells were transfected with either 1μg FLAG-WT TRAF6, FLAG-C70A TRAF6, FLAG-K124R TRAF6 or FLAG-DN TRAF6 and 1μg HA-ubiquitin for 24 hrs. FLAG-TRAF6 was immunoprecipitated from cell lysates and levels of ubiquitinated TRAF6 were assessed by immunoblotting with anti-HA Ab (upper panel HC; heavy chain of anti-FLAG antibody used in IP). The second panel shows anti-FLAG immunoprecipitates immunoblotted for FLAG-TRAF6. The third panel shows HA-ubiquitin expression in lysates. The lower panel shows expression of FLAG TRAF6 by immunoblotting with anti-FLAG antibody. The experiment is representative of three independent experiments.

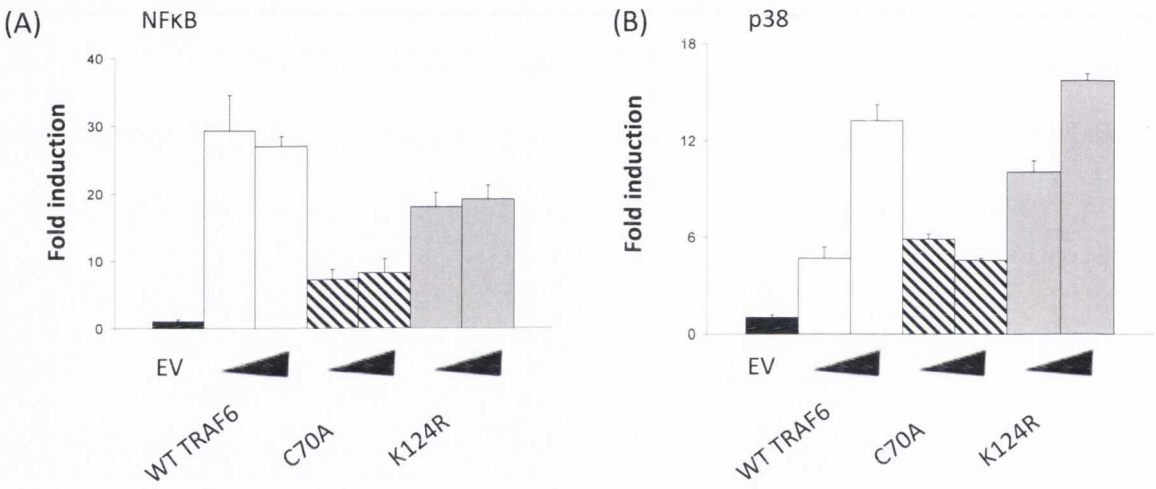


Figure 5.6. The E3 ligase activity of TRAF6 is required for NFκB and p38 activation. HEK293_R1 cells were transfected with either 10 or 25ng or WT TRAF6, C70A TRAF6, K124R TRAF6 or pcDNA 3.1 empty vector (EV), together with either (A) 60ng NFκB-luc reporter plasmid or (B) CHOP-GAL4 expression plasmid and pFR-luciferase reporter construct. In both cases activity was measured by luciferase reporter gene assay 24 h later. Data is shown as mean fold induction. The data is mean ± SD of triplicate samples and is representative of three separate experiments.

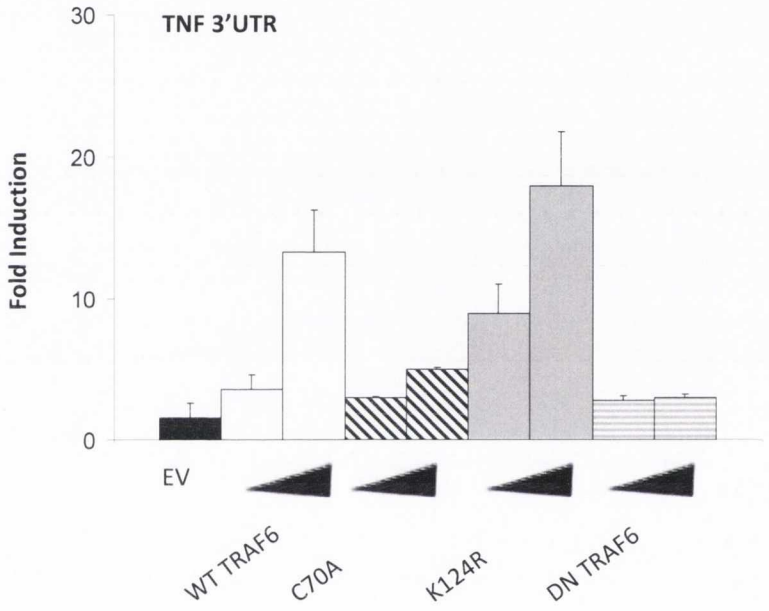


Figure 5.7. E3 ligase activity of TRAF6 is required for TNF 3'UTR activation.
HEK293_R1 cells were transfected with either 10 or 25ng or WT TRAF6, C70A TRAF6, K124R TRAF6, DN TRAF6 or pcDNA 3.1 empty vector (EV), together with 60ng TNFα reporter construct. TNFα 3'UTR activity was measured by luciferase reporter gene assay 24 h later. Data is shown as mean fold induction. The data is mean ± SD of triplicate samples and is representative of three separate experiments.

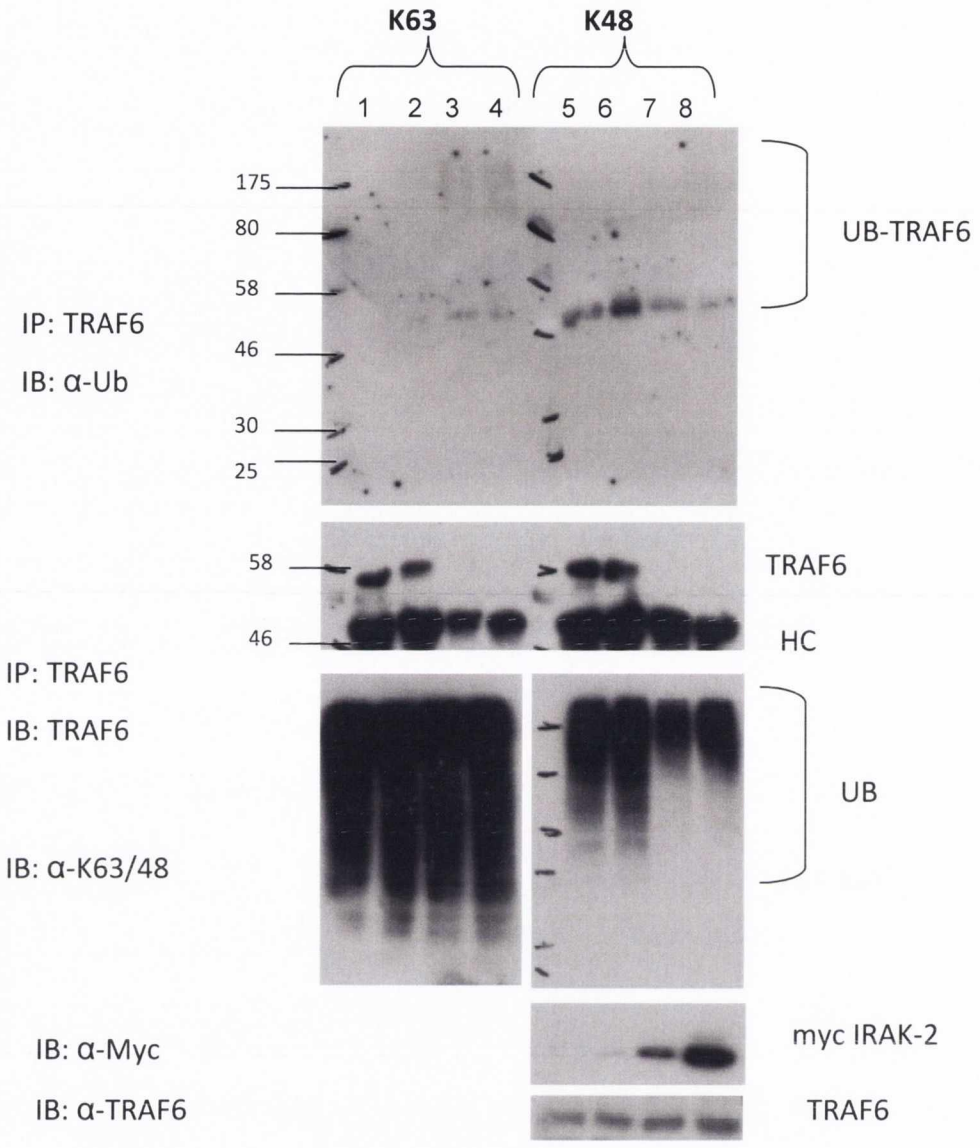


Figure 5.8. Detection of K63/K48-linked polyubiquitin chains associated with TRAF6 in presence of IRAK-2. HEK293T cells were transfected with 0.5μg,1μg or 2μg WT IRAK-2 and harvested 24 hours later. Cells were immunoprecipitated with a TRAF6 antibody and immunblots were probed with an antibody specific for K63-linked chains (upper panel, lanes 1-4) or a K48-linked chains (upper panel, lanes 5-8). The second panel shows anti-TRAF6 immunoprecipitates immunoblotted for TRAF6 (HC;heavy chain). The third panel shows K63-linked or K48-linked ubiquitin chains in lysates. The fourth panel shows expression of WT IRAK-2 by immunoblotting with an anti-myc antibody. The lower panel shows expression of endogenous TRAF6 in the lysates. The experiment was only done once.

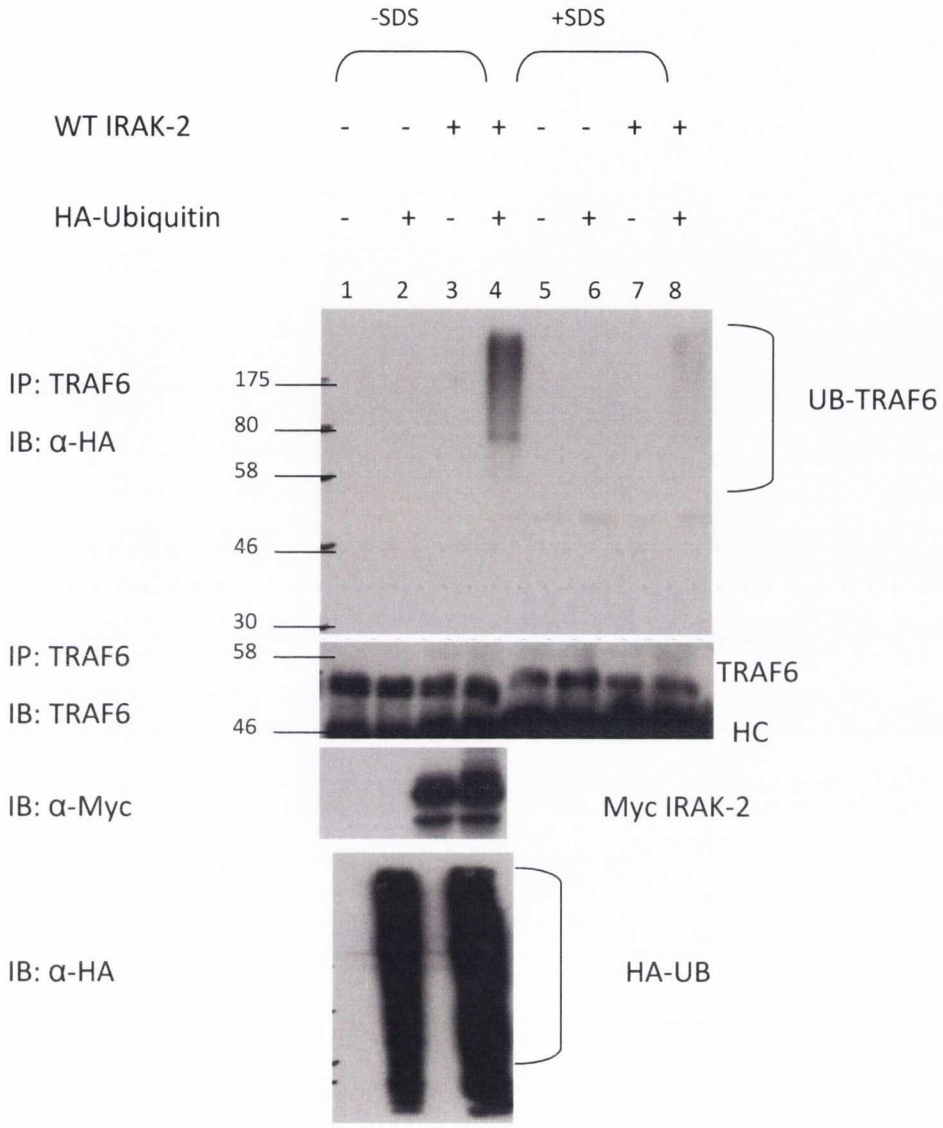


Figure 5.9 IRAK-2 induced polyubiquitin chains are not covalently attached to TRAF6. HEK293T cells were seeded at $1x10^5$/ml and transfected with 0.5μg WT IRAK-2 and 1μg HA-ubiquitin. Endogenous TRAF6 was immunoprecipitated from cell lysates and the immunoprecipitated TRAF6 protein was re-suspended in 1% SDS and incubated for 5 min at 70˚C. After dilution to 0.1% SDS with lysis buffer, the TRAF6 was reimmunoprecipitated and levels of ubiquitinated TRAF6 were assessed by immunoblotting with anti-Ha Ab (upper panel). The second panel shows anti-TRAF6 immunoprecipitates immunoblotted for TRAF6 (HC;heavy chain). The third panel shows expression of WT IRAK-2 by immunoblotting with an anti-myc antibody. The lower panel shows HA-ubiquitin expression in lysates.

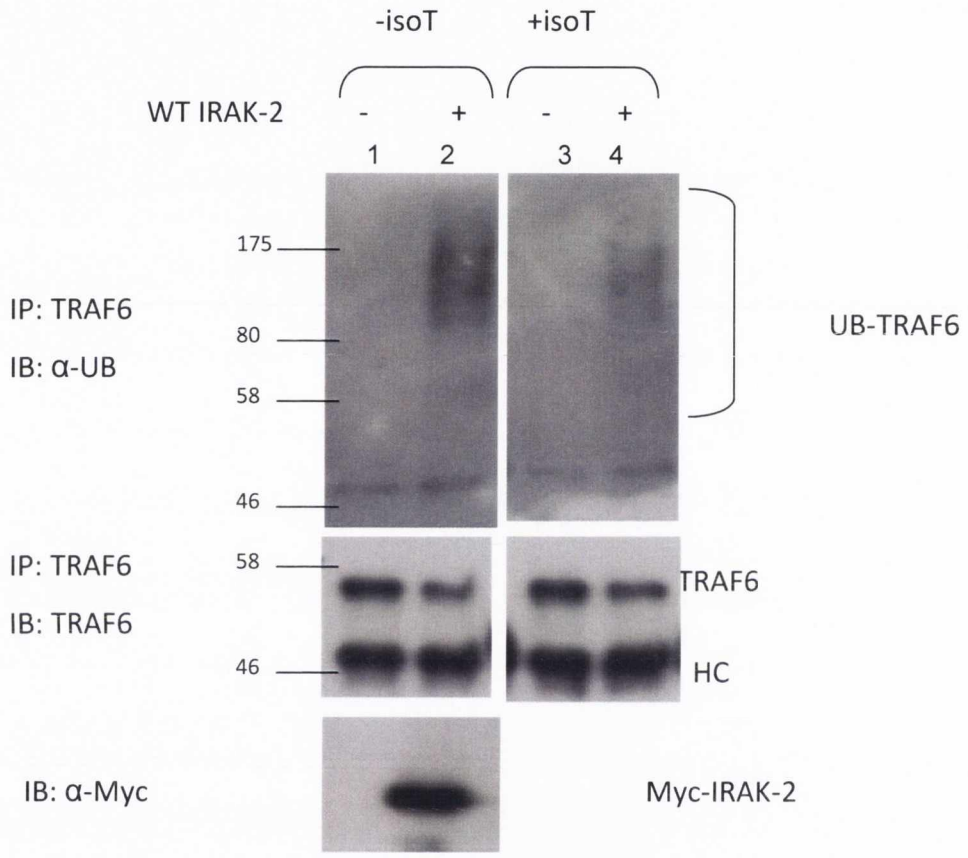


Figure 5.10 WT IRAK-2 induced polyubiquitin chains associated with TRAF6 are reduced in the presence of IsoT. HEK293T cells were transfected with 0.5μg WT IRAK-2 and 7.5μg of pcDNA 3.1 and harvested 24 hours later. Cells were immunoprecipitated with a TRAF6 antibody. The precipitated samples were incubated at 30˚C either with (+IsoT) or without IsoT (-IsoT) for 1 hour. Levels of ubiquitinated TRAF6 were assessed by immunoblotting with anti-Ubiquitin antibody (upper panel). The second panel shows anti-TRAF6 immunoprecipitates immunoblotted for TRAF6 (HC;heavy chain). The third panel shows expression of WT IRAK-2 by immunoblotting with an anti-myc antibody. The experiment is representative of two individual experiments.

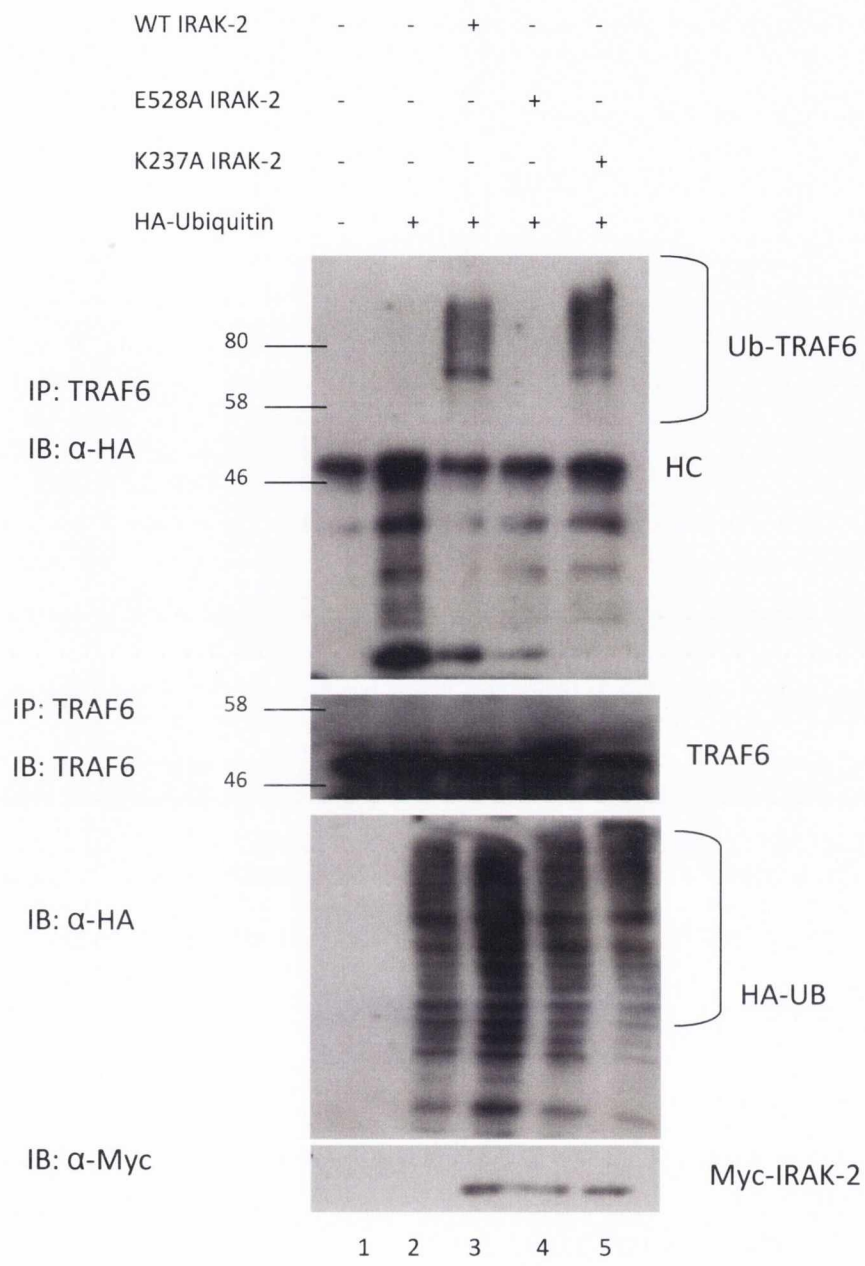


Figure 5.11 Residues in IRAK-2 required for TRAF6 ubiquitination. HEK293T cells were transfected with 0.5μg or either WT IRAK-2-myc, E528A IRAK-2 or K237A IRAK-2 and 1μg HA-ubiquitin for 24 hrs. Endogenous TRAF6 was immunoprecipitated from cell lysates and levels of ubiquitinated TRAF6 were assessed by immunoblotting with anti-HA Ab (upper panel HC; heavy chain of anti-TRAF6 antibody used in IP). The second panel shows anti-TRAF6 immunoprecipitates immunoblotted for TRAF6. The third panel shows HA-ubiquitin expression in lysates. The lower panel shows expression of WT IRAK-2 and IRAK-2 mutants by immunoblotting with anti-myc antibody. The experiment is representative of three independent experiments.

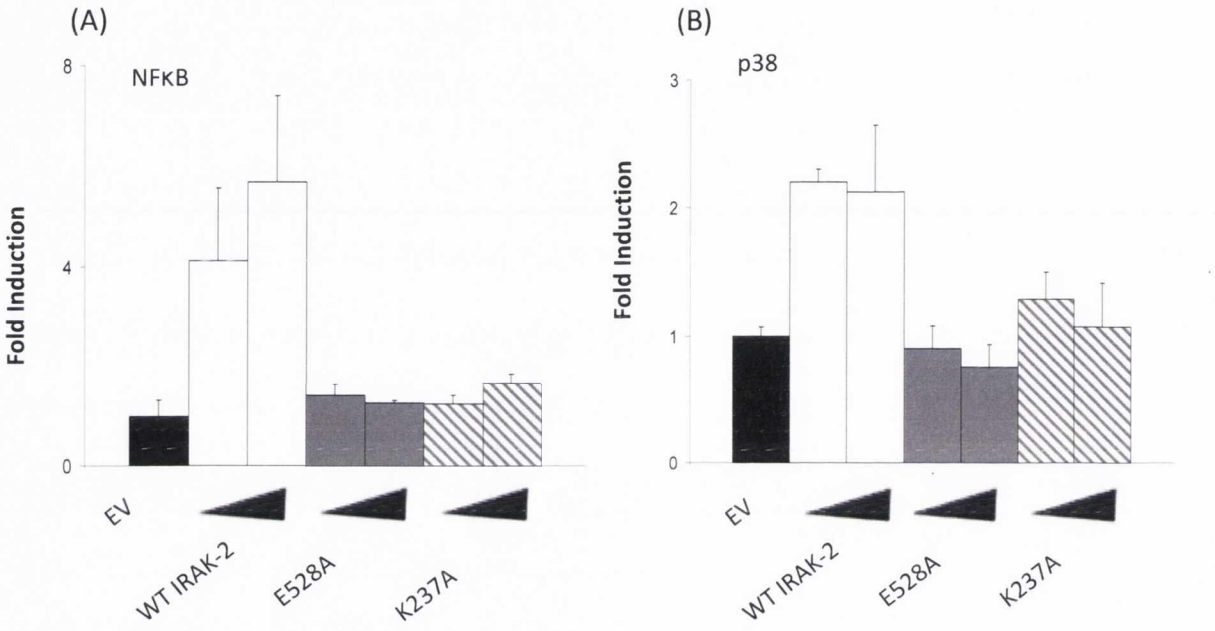


Figure 5.12 Residues of IRAK-2 required for activation of NFκB and p38 activation. HEK293 R1 cells were seeded at a density of IX10^5/ml and transfected 16-18 hrs later with either (A) NFκB_luc plasmid or (B) chop and pFR-luciferase plasmid. Where indicated, cells were transfected with pcDNA 3.1 (EV) or 10 or 25 ng of WT IRAK-2, E528A IRAK-2 or K237A IRAK-2. The data is expressed as the mean fold induction ± SD for a single experiment performed in triplicate representative of three experiments.

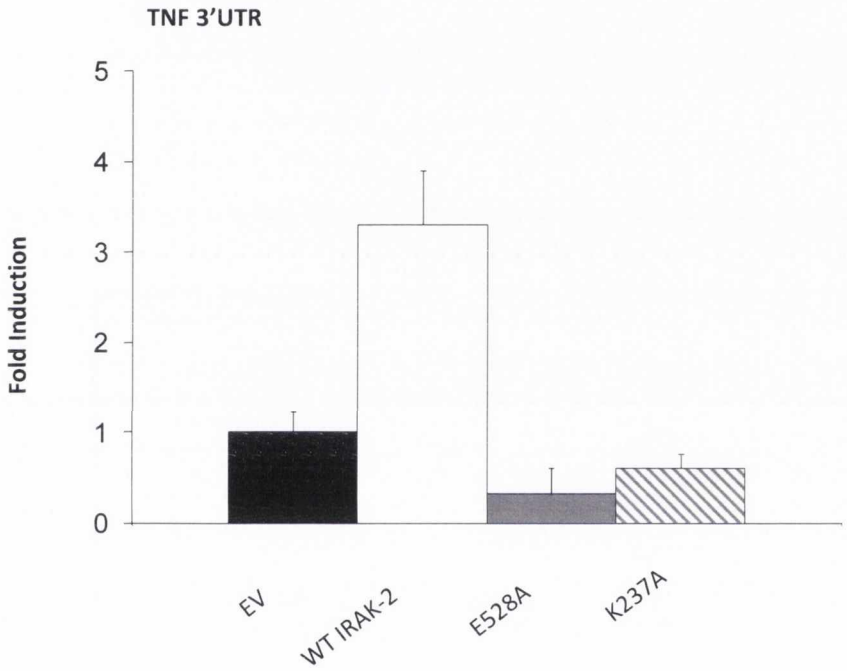


Figure 5.13. Residues of IRAK-2 that are required for TNF 3'UTR activation. HEK293_R1 cells were transfected with either 25 ng of WT IRAK-2, E528A IRAK-2, K237A IRAK-2 or pcDNA 3.1 empty vector (EV), together with either 60 ng of TNFα 3'UTR reporter construct. In all cases activity of the 3'UTRs was measured by luciferase reporter gene assay 24 hrs later. Data is shown as mean fold induction. The data is mean ± SD of triplicate samples and is representative of three separate experiments.

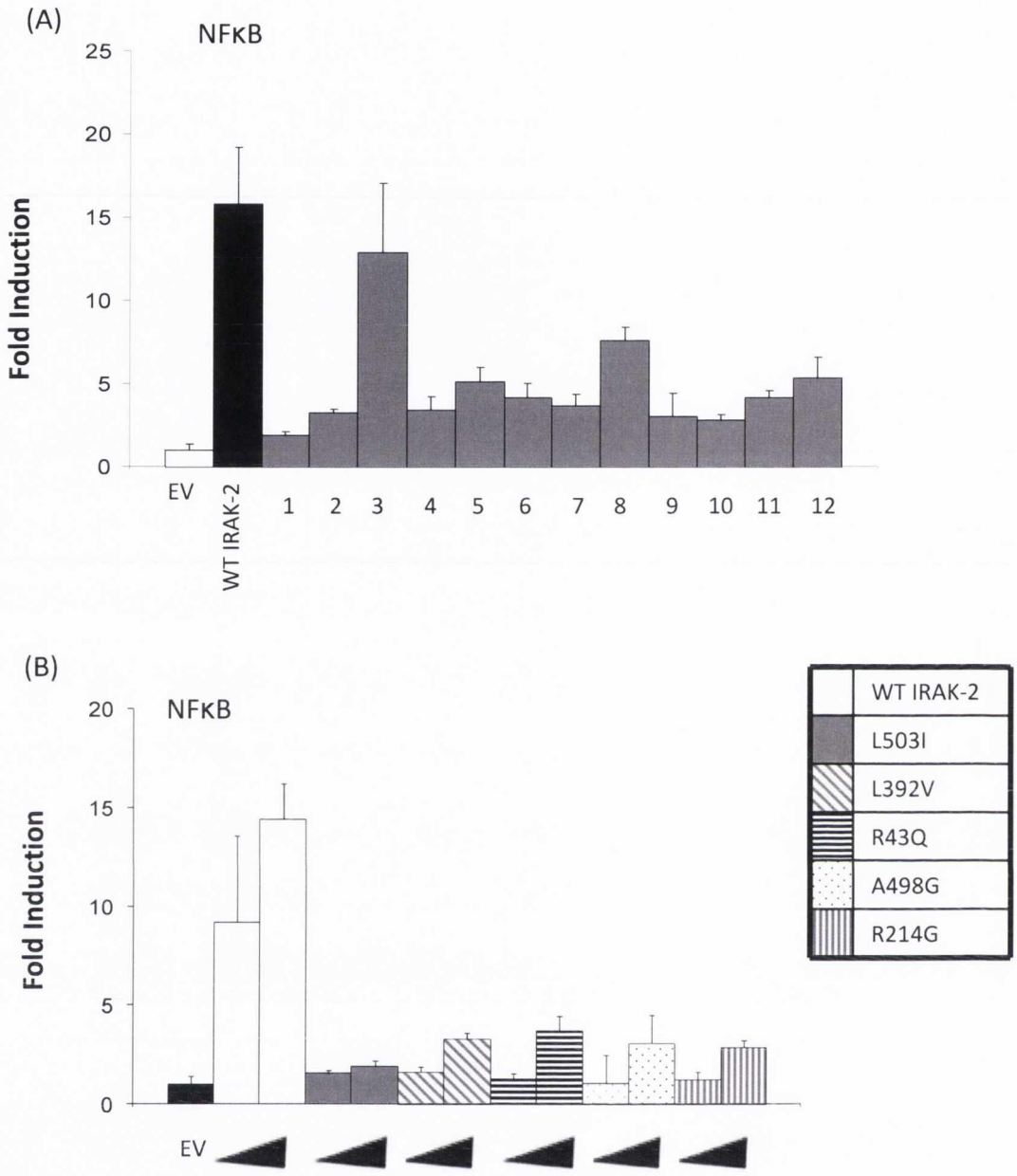
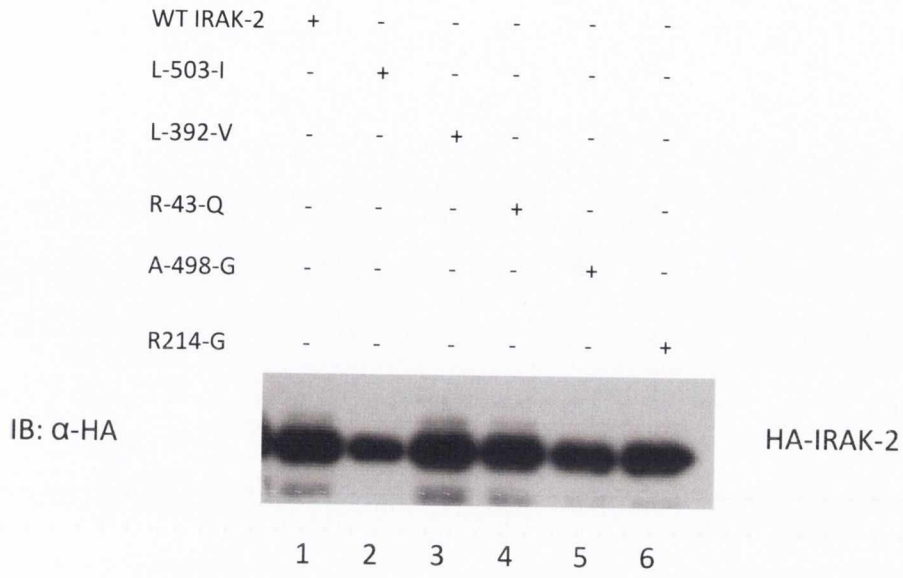


Figure 5.14 Human IRAK-2 single nucleotide polymorphisms inhibit NFκB activation. (A) HEK293T cells were seeded at a density of Ix10^5/ml and transfected 16-18 hrs later with either 25 ng of WT IRAK-2 or 25 ng IRAK-2 SNPs (1) L503I, (2) L392V, (3) L78M, (4) E501I (5)D431E, (6) L439V, (7) R43Q, (8) S47Y, (9) A498G, (10) R214G, (11) I99V ,(12) L358M or EV control. (B) HEK293_T cells were seeded at a density of Ix10^5/ml and transfected 16-18 hrs later with either 10 or 25 ng of WT IRAK-2, 10 or 25 ng IRAK-2 SNPs or pcDNA 3.1 (EV) together with 60 ng of NFκB_luc reporter construct. Data in (A) and (B) is shown as mean fold induction. The data is mean ± SD of triplicate samples and is representative of three separate experiments.

(A)


WT IRAK-2 + - - - - -
L-503-I - + - - - -
L-392-V - - + - - -
R-43-Q - - - + - -
A-498-G - - - - + -
R214-G - - - - - +
IB: α-HA
HA-IRAK-2
1 2 3 4 5 6

(B)

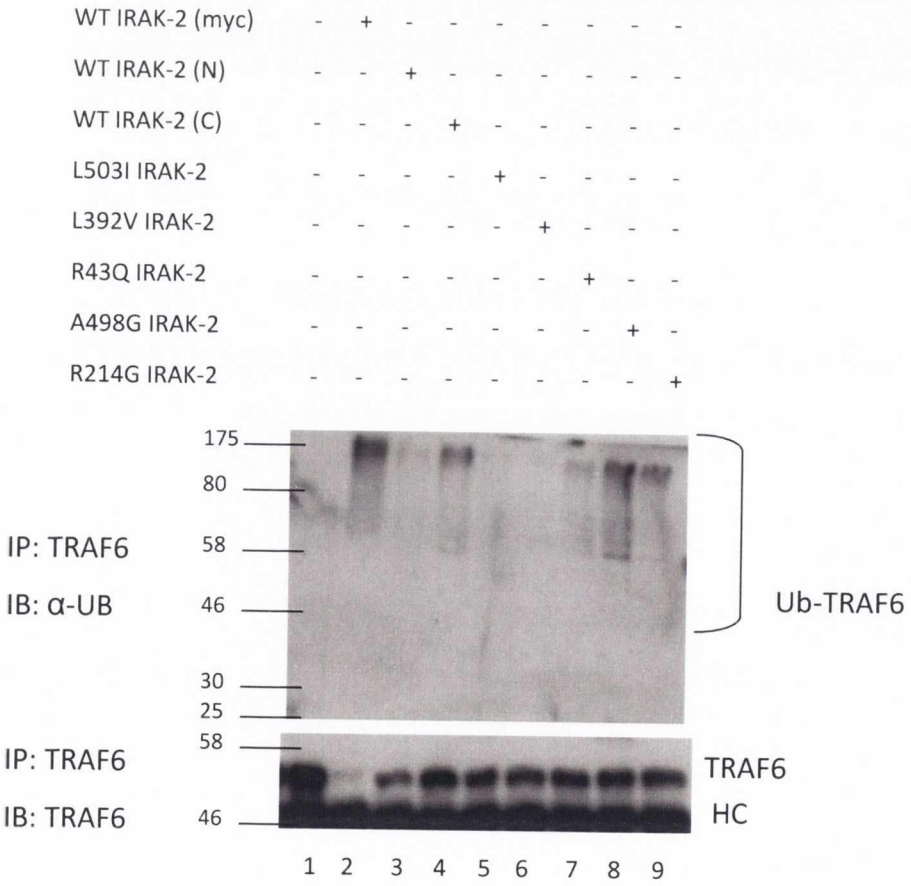

WT IRAK-2 (myc) - + - - - - - - -
WT IRAK-2 (N) - - + - - - - - -
WT IRAK-2 (C) - - - + - - - - -
L503I IRAK-2 - - - - + - - - -
L392V IRAK-2 - - - - - + - - -
R43Q IRAK-2 - - - - - - + - -
A498G IRAK-2 - - - - - - - + -
R214G IRAK-2 - - - - - - - - +
175
80
58
46
30
25
IP: TRAF6
IB: α-UB
Ub-TRAF6
58
46
IP: TRAF6
IB: TRAF6
TRAF6
HC
1 2 3 4 5 6 7 8 9

Figure 5.15 IRAK-2 SNPs that are required for polyubiquitin chains associated with TRAF6. (A) HEK293T cells were seeded at a density of Ix10^5/ml and transfected 16-18 hrs later with 1µg HA-WT IRAK-2 or HA-IRAK-2 SNPs. Cells were harvested and cell lysates were immunoblotted with HA antibody to detect HA-IRAK-2. The blot is representative of one independent experiment. (B) HEK293T cells were transfected with 1µg or either WT IRAK-2-myc,HA-WT IRAK-2 (N-terminal tag), WT IRAK-2-Ha (C-terminal tag) or the indicated IRAK-2 SNP for 24 hrs. Endogenous TRAF6 was immunoprecipitated from cell lysates and levels of ubiquitinated TRAF6 were assessed by immunoblotting with anti-Ub Ab (upper panel HC; heavy chain of anti-TRAF6 antibody used in IP). The second panel shows anti-TRAF6 immunoprecipitates immunoblotted for TRAF6. The experiment is representative of two independent experiments.



Figure 5.16 Residues in IRAK-2 required for its function. In the human population 12 naturally occurring variants in the IRAK-2 gene were selected as potentially affecting the function of IRAK-2. 10 of these were shown to be required for NFκB activation (white stars). 2 of the SNPs were not impaired in their ability to activate NFκB (red stars). 5 of these SNPs impaired NFκB robustly and therefore their effect on the formation of polyubiquitin chain formation associated with TRAF6 was examined. L503I and L392V were required for TRAF6-associated chain formation (grey stars), while R43Q, R214G and A498G were dispensable for TRAF6-associated polyubiquitin chain formation. Two other residues required for IRAK-2 function, K237 and E528, when mutated IRAK-2 could no longer activate NFκB (yellow stars). E528 was required for TRAF6-associated polyubiquitin chain formation while K237 was dispensable for IRAK-2 to form polyubiquitin chains associated with TRAF6 (see Table (B))

Residue in IRAK-2	Location	Required for NFκB Activation	Required for TRAF6-associated polyubiquitin chain formation
R-43-Q	DD	+	-
S-47-Y	DD	-	n/d
L-78-M	DD	-	n/d
I-99-V	UD	+	n/d
R-214-G	Kinase D	+	-
K237	Kinase D	+	-
L-358-M	Kinase D	+	n/d
L-392-V	Kinase D	+	+
D-431-E	Kinase D	+	n/d
L-439-V	Kinase D	+	n/d
A-498-G	C-term	+	-
E-501-I	C-term	+	n/d
L-503-I	C-term	+	+
E528	C-term	+	+

TABLE 1.3: Residues in IRAK-2 required for NFκB activation and its ability to trigger TRAF-associated polyubiquitin chains. DD (death domain), UD (undetermined domain), Kinase D (kinase domain) and C-term (C-terminus domain) n/d not determined.

Chapter 6: Final Discussion

Elucidating the roles of IRAK family members in IL-1 and TLR signalling has been an intense subject of research in the past decade; however IRAK-2 has remained the most enigmatic family member. It was presumed to play a redundant role with IRAK-1 in TLR signalling. Through examination of how VACV proteins participate in evading and subverting the innate immune response, it was shown that the poxvirus protein A52 could inhibit multiple TLR pathways via its interaction with IRAK-2 [195]. Thus IRAK-2 was implicated in having a central role in TLR signalling to NFκB. A central role for IRAK-2 in TLR signalling was further confirmed with the generation of IRAK-2 $^{-/-}$ mice [197]. *In vivo* IRAK-2 $^{-/-}$ mice were shown to be highly resistant to LPS- and CpG-induced challenge whereas a previous study showed that the difference in mortality between wild type and IRAK-1 $^{-/-}$ mice was only subtle [180]. However while the IRAK-2 $^{-/-}$ mice were resistant to TLR-induced septic shock, in cells derived from these mice early TLR2 signalling events were intact, with IRAK-2 being only required for late and sustained NFκB activation [197]. Thus the role of IRAK-2 relative to IRAK-1 in TLR signalling was unclear, and furthermore there may have been differences between the mouse and human system.

Here for the first time this study provides a comprehensive analysis of the role of human IRAK-2 in primary cells and demonstrates a critical requirement for it in early TLR signalling events. IRAK-2 was shown to be essential for TLR4- and TLR8-induced NFκB and p38 MAPK activation. This was in contrast to the murine system which showed that early NFκB activation was normal for TLR2, TLR7 and TLR9, although delayed activation was impaired, whereas for TLR4, there was no defect in NFκB [197, 198]. Furthermore in the murine system IRAK-2 was not required for TLR2, TLR4 and

TLR7 p38 MAPK activation. The difference between the mouse and the human system was likely due to expression of a negative splice variant in the murine system which inhibited early IRAK-2 function [201, 202]. In the human system, there is no evidence of splice variants, therefore there is only one isoform of IRAK-2 which functions as a positive mediator of TLR signalling. This study has revealed an emerging potential difference between mouse and human IRAKs. Further evidence for this can also be found when examining IRAK-4. When IRAK-4 is depleted in mice, this dramatically affects responses to a wide range of microorganisms [211]. In contrast, humans with an IRAK-4 deficiency are only susceptible to a narrow range of bacteria [221]. Thus these studies reveals that caution must be exercised in extrapolating data from experimental murine studies to the human system.

A critical requirement for IRAK-2 in post-transcriptional regulation has also been demonstrated. While it has previously been shown that other IRAK family members are required for post-transcriptional regulation of cytokine induction, this is the first time a critical role for human IRAK-2 in post-transcriptional regulation has been demonstrated. IRAK-2 was shown to be critical for TLR-mediated TNFα mRNA stability in primary human cells. TNFα, a key pro-inflammatory cytokine and mediator of inflammatory and autoimmune disease is subject to tightly controlled regulation both at the transcriptional and translational level. The full characterisation of how exactly IRAK-2 regulates the TNFα mRNA stability pathway remains to be determined although it occurs via the TNFα 3'UTR. Multiple ARE-BPs can regulate TNFα mRNA via binding to the TNF 3'UTR via AU-rich elements. The best characterised AU-BP that regulates TNFα mRNA stability is TTP [232]. TTP binds

specifically to a nonamer sequence UUAUUUA(U/A)(U/A) that is located in multiple cytokine 3'UTRs including TNF [232]. TTP is regulated via the p38 pathway and IRAK-2 has been shown to regulate p38 phosphorylation, thus it is possible that IRAK-2 may regulate TNFα mRNA stability via TTP.

Another possible mechanism of regulation of TNFα mRNA stability may be via the Zc3h12a protein. Zc3h12a has been shown to regulate TLR-induced IL-6 mRNA stability in the murine system [280]. This regulation was shown to be both IRAK-1 and IRAK-2 dependent. Zc3h12a does not regulate TNFα mRNA stability in the murine system although regulation of TNF mRNA stability has been shown to be different in the mouse and human system. Therefore TNF mRNA stability may be regulated via an IRAK-2-Zc3h12a-dependent pathway in the human system. It has recently been shown that IRAK-2 interacts with Zc3h12a in HEK cells [166]. In addition, IRAK-2 may regulate IL-6 mRNA stability in the human system.

IRAK-2 regulates TNFα mRNA stability and this pathway is MyD88-dependent. IRAK-2 does not have a role in the IL-8 mRNA stability pathway. It is possible that IRAK-1 has a role in the regulation of IL-8 mRNA stability as IRAK-1 in the murine system was shown to regulate KC (the murine equivalent of IL-8) [238]. In addition, over expression of IRAK-1 induced the activation of the IL-8 3'UTR more potently than IRAK-2. Thus a MyD88-IRAK-4-IRAK-2 pathway may regulate some cytokine mRNA stability such as TNFα, whereas a MyD88-IRAK-4-IRAK-1 pathway may regulate other

cytokines such as IL-8. Consistent with this idea, it has been shown that IRAK-4 is required for both KC and TNFα mRNA stability [216].

While it is clear that IRAK-2 is required for the regulation of a LPS-TLR4-MyD88-dependent TNFα mRNA stability pathway, it would be interesting to examine whether IRAK-2 is required for other MyD88-dependent TNFα mRNA stability pathways. It has also been described that MyD88 is required for an IFN-γ-dependent pathway that converges on the TNF 3'UTR [142]. This pathway was shown to be IRAK-1 independent.

It is known that TRAF6 plays a critical role in TLR-induced NFκB and MAPK pathways. However the role for TRAF6 in mRNA stability is less well defined. Here, TRAF6 has been implicated in the MyD88-IRAK-2-TNF 3'UTR pathway as over expression of TRAF6 could induce the activation of the TNF 3'UTR. In contrast to this, the regulation of KC mRNA stability by IRAK-1 was shown to be TRAF6-independent [238]. Therefore the regulation of cytokine mRNA stability pathways by IRAK-2 may be TRAF6-dependent, whereas IRAK-1 may not require TRAF6. Consistent with this idea, it has recently been shown that IRAK-2 can interact with TRAF6 in a ligand-induced manner in human cells, whereas IRAK-1 failed to do so [166].

TRAF6 is critical mediator in TLR signalling to NFκB and previous work has shown that IRAK-2 could trigger the formation of polyubiquitin chains associated with TRAF6, while IRAK-1 failed to do so. Here I have shown a role for IRAK-2 in the formation of physiologically relevant unanchored polyubiquitin chains associated with TRAF6.

Thus the current model for TRAF6-associated polyubiquitination in NFκB signalling is as follows; For TLR signalling, receptor dimerisation results in recruitment of adaptor molecules such as MyD88. The Myddosome is formed which contains 6-8 MyD88 DDs, 4 IRAK-4 DDs and 4 IRAK-2 or IRAK-1 DDs. IRAK-4 then phosphorylates IRAK-2 and IRAK-1. IRAK-2 (and not IRAK-1) can trigger the E3 ligase activity of TRAF6, perhaps due to the oligomerisation of TRAF6. Thus TRAF6 is the E3 ligase that forms the physiologically relevant unanchored K63-linked polyubiquitin chains that are important for TLR-induced NFκB signalling. The formation of these chains requires IRAK-2.

Given that it is only a recent discovery that unanchored polyubiquitin chains can activate signalling molecules such as TAK-1 the exact mechanism of how these free polyubiquitin chains can directly activate protein kinases is still unknown. It has been suggested that the free polyubiquitin chains act as second messengers, similar to cyclic AMP, in that they are synthesized in cells to activate protein kinases [257]. Thus the binding of unanchored chains may lead to the dimerisation or oligomerisation of the TAK-1 kinase complex.

The mechanism of how exactly IRAK-2 triggers E3 ligase activity is still unknown although one possibility is through ligand-induced oligomerisation of TRAF6. It has been shown that TIFA could induce the oligomerisation and polyubiquitination of TRAF6. The oligomerisation of TRAF6 has been shown to be important for its RING finger E3 ligase activity and to activate the IKK complex [264]. Thus, other proteins

such as IRAK-2 may function in a similar manner and induce the oligomerisation of TRAF6 which is a prerequisite for its E3 ligase activity.

Another possibility of how IRAK-2 may trigger the E3 ligase activity of TRAF6 is via the phosphorylation of TRAF6. IRAK-2 can interact with TRAF6 and recently it has been shown that IRAK-2 can interact with TRAF6 in a ligand-dependent manner [166]. Thus IRAK-2 may bring TRAF6 in close proximity to another kinase for phosphorylation. It is well known that phosphorylation is often shown to precede ubiquitination. A well known example of this is IκB [241]. IκB is initially phosphorylated in order for NFκB activation, IκB is then ubiquitinated and degraded by the proteasome. It is unlikely that IRAK-2 itself phosphorylates TRAF6 as an IRAK-2 mutant (K237A, with a key lysine residue mutated in the ATP-binding pocket of IRAK-2) could still trigger the formation of polyubiquitin chains associated with TRAF6.

It had been historically established that IRAK-1 and IRAK-4 were the only active kinases from the IRAK family. It has been recently proposed that the lysine residue in the ATP binding pocket of IRAK-2 is sufficient for IRAK-2 to act as an active kinase [197]. An *in vitro* kinase assay showed that IRAK-2 is phosphorylated upon stimulation with a TLR2 ligand [197]. It is thought that IRAK-4 phosphorylates IRAK-2 and induces its kinase activity. I have shown here that this IRAK-2 mutant with the key lysine mutated (K237) can no longer activate NFκB nor p38 MAPK. It is of interest to note that overexpression of IRAK-1 and IRAK-4 kinase dead mutants can still activate NFκB whereas the kinase mutant of IRAK-2 fails to do so.

According to well defined rules, the analysis of the primary amino acid sequence of a kinase domain is highly predictive of the activity or inactivity of the corresponding kinase. The kinase domain of a protein generally consists of 250-300 amino acids of which there are 12 conserved subdomains [281]. Within these subdomains, there are certain crucial amino acids that are invariant in active kinases. As mentioned, there is a lysine in subdomain II and this is required for ATP binding. In subdomain VIb, there is a HRD motif in which the aspartic acid acts as a proton acceptor for the substrate's hydroxyl group during phosphotransfer. Lastly a DFG motif in subdomain VII, the aspartic acid in this motif is required for chelating Mg^{2+} for correct positioning of the ATP γ-phosphate. For IRAK-2 both the DFG and HRD motifs are missing, therefore it is very likely that IRAK-2 is not catalytically active.

It has been suggested that the IRAK-2 K237 residue, rather than being essential for IRAK-2 kinase activity, it may be important for interactions between IRAK-2 and another signalling molecule. It is possible that mutation of this residue results in a conformational change in IRAK-2 which prevents it from interacting with its signalling partners [162]. In addition it may reveal the "ATP binding activity" of IRAK-2 is required for signalling, rather than IRAK-2 being an actual kinase.

Overall then, it is clear that there is a critical, non redundant role for IRAK-2 in early TLR signalling events in primary human cells. Furthermore IRAK-2 is essential for TNFα post-transcriptional regulation in primary human cells. Given that the dysregulation of TLR-mediated NFκB and p38, and of TNFα production, is implicated

in many autoimmune and inflammatory diseases, IRAK-2 likely represents an attractive therapeutic target in human disease.

Chapter 7: References

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Appendix

List of Abbreviations:

AIM-2	Absent In Melanoma-2
APS	Ammonium Persulphate
ARE	Adenine and uridine Rich Elements
ASC	Apoptosis-associated speck-like protein containing a CARD
BAFF	B-cell activating factor belonging to the TNF family
BMDC	Bone Marrow Derived Dendritic Cells
BMDM	Bone Marrow Derived Macrophages
CARD	Caspase Recruitment Domain
CARDIF	CARD-Adaptor Inducing IFN-β
CDE	Constitutive Decay Element
CpG	Cytidine-phosphate-guanosine
CTLD	C-type lectin domains
CYLD	The tumour suppressor cylindromatosis protein
DAI	DNA-dependent activator of IRF
DC	Dendritic Cell
DMEM	Dulbecco's modified Eagle's medium
dsDNA	Double Stranded DNA
DTT	Dithiothreitol
dsRNA	Double Stranded RNA
DUB	Deubiquitinase
DKO	Double Knockout
EBV	Epstein-Barr Virus

ECD	Ectodomain
ECL	Enhanced Chemiluminescence
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinases
FCAS	Familial Cold Autoinflammatory Syndrome
FCS	Foetal Calf Serum
GPI	GlycosylPhosphatidylInositol
HECT	Homologus to E6-associated protein carboxyl terminus
HMGB1	High Mobility Group Box-1
HOIL-1	longer isoform of hem-oxidised iron-regulatory protein 2 ubiquitin ligase-1
HOIP	HOIL-1L interacting protein
ID	Intermediary Domain
IFI16	Interferon Inducible protein 16
IFN	Interferon
IκB	Inhibitor of Kappa B
IKK	IκB kinase
IM	Inflammatory monocytes
IP-10	Interferon gamma inducible protein 10
IPD	Invasive Pneumonoccal Disease
ISRE	Interferon Stimulated Response Element
IPS-1	IFNβ promoter stimulator-1
IRAK	Interleukin Receptor Associated Kinase
IRF	Interferon Regulatory Factor

I-TAC	Inducible T-cell α chemoattractant
ITAM	Immunoreceptor Tyrosine Activation Motif
JNK	c-jun N terminal Kinase
KD	Kinase Dead
KO	Knockout
KSRP	KH-type splicing regulatory protein
LGP2	Laboratory of Genetics and Physiology 2
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeats
LT	Lymphotoxin β receptor
LUBAC	Linear Ubiquitin chain Assembly Complex
MAVS	Mitochondrial Antiviral Signalling Protein
MAL	MyD88 Adaptor-Like protein
mCMV	mouse cytomegalovirus
MD-2	myeloid differentiation factor 2
MDA-5	Melanoma differentiation associated gene 5
MEFs	Mouse Embryonic Fibroblasts
MK-2	p38/MAPK-activated protein kinase 2
MMTV	Mouse Mammary Tumour Virus
MyD88	Myeloid differentiation primary response gene 88
NACHT	domain present in NAIP, CIITA, HET-E and TP-1
NALP3	NACHT-LRR-PYD-containing protein 3
NBD	Nucleotide Binding Domain
NDV	Newcastle Disease Virus

NEMO	NFκB Essential Modifier
NFAT	Nuclear Factor of Activated T cells
NIK	NFκB Inducing Kinase
NLR	Nod-Like Receptor
ODN	oligodeoxynucleotide
PBMC	Peripheral Blood Mononuclear Cells
PRR	Pattern Recognition Receptor
Poly IC	Polyinosine-polycytidylic acid
PP2α	protein phosphatase 2α
PVDF	PolyVinyidene DiFluoride
PYN	Pyrin Domain
PYHIN	Pyrin and HIM domain-Containing Protein
RA	Rheumatoid Arthritis
RAGE	Receptor for Advanced Glycation End-products
RANTES	Regulated on Activation, normal T cell and Secreted
RHD	Rel Homology Domain
RHIM	RIP-Homotypic Interaction Motif
RIG-I	Retinoic acid Inducible Gene 1
RING	Really Interesting New Gene
RIP	Receptor-Interacting Protein
RLR	RIG-Like-Receptor
RSV	Respiratory Syncytical Virus
SAM	sterile α motif
SARM	Sterile α and ARMadillo motif containing protein

SDS	Sodium Dodecyl Sulphate
SHARPIN	SHANK-associated RH domain interacting protein
SINTBAD	Similar to NAP-1 TBK-1 adaptor
SLE	Systemic Lupus Erythematosus
ssRNA	Single Stranded RNA
STAT	Signal Transducers and Activators of Transcription
STING	Stimulator of Interferon Genes
Syk	Spleen Tyrosine Kinase
TAB	TAK Binding Protein
TAG	TRAM adaptor with Golgi Dynamics (GOLD) domain
TAK	TGF-β Activated Kinase
TANK	TRAF-associated NFκB activator
TBK-1	TANK binding kinase-1
TEMED	N,N,N',N'-tetramethyldiamine
TICAM	TIR-containing adaptor molecule
TIFA	TRAF-interacting protein with a Forkhead-Associated domain
TIR	Toll/IL-1R
TIRAP	TIR-adaptor protein
Tpl-2	Tumour Progression Locus-2
TLR	Toll-Like-Receptor
TNF	Tumour Necrosis Factor
TRAF	TNFR-associated factor
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR domain containing adaptor protein inducing IFN-β

TRIKA	TRAF6-regulated IKK activator
Tris	Trizma Base
TTP	Tristetraprolin
UBAN	Ubiquitin binding domain in ABIN and NEMO proteins
Ubc13	ubiquitin conjugating enzyme 13
Uev1a	ubiquitin E2 variant 1a
VACV	Vaccinia Virus
VISA	Virus-Induced Signalling Adaptor
VSV	Vesicular Stomatitis Virus
WNV	West Nile Virus
5'ppp	5' triphosphate