The interleukin-1 receptor-associated kinases: critical regulators of innate immune signalling

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Abstract

The interleukin-receptor-associated kinase (IRAK) family are involved in regulating Toll-like receptor (TLR) and interleukin-1 (IL-1) signalling pathways. TLRs are pattern recognition receptors of the innate immune response that are responsible for sensing pathogens and initiating immunity, while IL-1 is one of the key cytokines that mediates inflammation. As such, IL-1/TLR signalling pathways and the IRAK family are critical in anti-pathogen responses, inflammation and autoimmunity. The family comprises of four members, IRAK-1, IRAK-2, IRAK-M (IRAK-3) and IRAK-4, and has a role in both positive and negative regulation of signal transduction. While it was once thought that the family displayed some redundancy, each member of the family is emerging as a distinct and vital contributor to IL-1/TLR signalling mechanisms. Knockout mouse studies have explored the relative contribution of each of the IRAKs in IL-1/TLR signalling, while the recent generation of kinase-inactive knock-in IRAK-4 mice have revealed which of IRAK-4 functions require its kinase activity. IRAK-2, previously thought of as a pseudokinase, has recently been proposed to have kinase activity that is essential for TLR signalling. Not surprisingly given their critical role in IL-1/TLR signalling, the IRAK family members have been implicated in certain disease models including human immunodeficiencies. Thus the potential targeting of these essential protein kinases therapeutically is also discussed.
Abbreviations: ARE, Adenine and uridine Rich Elements; ATP, Adenosine Triphosphate; BAFF, B-cell activating factor belonging to the TNF family; BMDM, Bone Marrow Derived Macrophages; CpG, Cytidine-phosphate-guanosine; DD, Death Domain; dsDNA Double Stranded DNA; dsRNA, Double Stranded RNA; ID, Intermediary Domain; IFN, Interferon; IκB, Inhibitor of Kappa B; IKK, IκB kinase; IRAK, Interleukin Receptor Associated Kinase; IRF, Interferon Regulatory Factor; JNK, c-jun N terminal Kinase; KD, Kinase Dead; KO, Knockout; LMCV, Lymphocytic Choriomeningitis Virus; LPS, Lipopolysaccharide; LRR, Leucine Rich Repeats; LT, Lymphotoxin β receptor; MAL, MyD88-adaptor like; MEFs, Mouse Embryonic Fibroblasts; MyD88, myeloid differentiation primary response gene 88; NEMO, NFκB Essential Modifier; NIK, NFκB Inducing Kinase; PAMP, Pathogen Associated Molecular Patterns; PBMC, Peripheral Blood Mononuclear Cells; PRR, Pattern Recognition Receptor; Poly IC, Polynosine-polycytidylic acid; RIG-I, Retinoic acid Inducible Gene 1; SAM, sterile α motif; SARM, Sterile α and ARMadillo motif containing protein; SINTBAD, Similar to NAP-1 TBK-1 adaptor; SLE, Systemic Lupus Erythematosus; ssRNA, Single Stranded RNA; TAB,TAK Binding Protein; TAK, TGF-β Activated Kinase; TANK, TRAF-associated NFκB activator; TBK-1, TANK binding kinase-1; TICAM, TIR-containing adaptor molecule; TIR, Toll/IL-1R; TIRAP, TIR-adaptor protein; 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; TTP, Tristetraprolin; UTR, Untranslated Region; VACV, Vaccinia Virus; VSV, Vesicular Stomatitis Virus
1. Introduction

The IRAK family are defined as intracellular kinases that play a significant role in the innate immune system as they participate in signalling networks of the innate axis of the immune response. These signalling networks 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. Innate immune signalling is activated upon detection of pathogens through pattern recognition receptors (PRRs) which recognise pathogen associated molecular patterns (PAMPs) (1). PAMPs are conserved motifs on microorganisms essential for their survival and distinguishable from host structures (2). One central group of PRRs are the Toll-like receptors (TLRs) which have been well characterised since their discovery in the late 1990’s (3). TLRs are defined by having a Toll/IL-1_Receptor (TIR) domain located cytoplasmically and leucine rich repeats (LRRs) located extracellularly. The TLRs are expressed on a variety of cell types and differentially recognise distinct PAMPs (2). They can be broadly divided into two categories: TLRs that are located at the plasma membrane namely TLR1, TLR2 and TLR6 which recognise lipoproteins and TLR4 which recognises LPS, and TLRs located endosomally namely TLR3 (which recognises dsRNA), TLR7 (ssRNA) TLR8 (ssRNA) and TLR9 (CpG motifs in DNA) TLR4 is known to translocate to the endosome and signal from there also (4). TLR7, TLR8 and TLR9 form an evolutionary conserved sub-group within the TLR family. TLR7, 8 and 9 signal through similar signalling mechanisms although they are located on different cell types and are known to induce different cytokine responses (5-7). One major TLR-induced set of
responses is the activation of transcription factors leading to the induction of proinflammatory cytokines and type-I interferons (IFNs).

Engagement of TLRs by PAMPs causes receptor dimerisation leading to the recruitment of one or more of five TIR domain-containing adaptor proteins. They are myeloid differentiation primary-response gene 88 (MyD88), MyD88-adaptor-like (Mal), TIR-domain-containing adaptor inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile-α- and armadillo-motif-containing protein (SARM) (8-12). MyD88 is required for all TLR signalling pathways except for TLR3 and a TLR4/MyD88-independent pathway (13). IL-1R also signals through MyD88. As well as a TIR domain, MyD88 also contains a death domain (DD). Its death domain facilitates its interaction with IRAK proteins (14).

In terms of the four IRAK family members, human IRAK-1, IRAK-2 and IRAK-4 are ubiquitously expressed, whereas human IRAK-M is only detectable in monocytes and macrophages in an inducible manner (15-16). Structurally IRAK family members share similar domains (See Figure 1). 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) (17, 18). 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 (14, 19). The proST region is rich in serines, prolines and threonines. IRAK-1 is reported to undergo hyperphosphorylation in this region (18). 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 (20). 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 (21). Recently
the crystal structure of the kinase domain of IRAK-4 has been reported by
two separate groups (22, 23). 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 (22).
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 (22). The tyrosine residue as a gatekeeper is
exclusive to the IRAK family making them a unique family of kinases (23).
The different IRAK proteins have different residues that undergo
phosphorylation (see Figure 1). Lastly the C-terminal domain is important
for interaction with TRAF6 (24). 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 (24).

The IRAK family contribute to multiple signalling pathways “downstream” of
the TIR adaptors including, but not restricted to, activation of NFκB, MAP
kinases and IFN regulatory factors (IRFs). For IL-1/TLR-induced NFκB
activation, phosphorylation of the IRAKs results in the subsequent activation
of TNF-receptor-associated factor 6 (TRAF6) E3 ligase activity and
polyubiquitination events essential for signalling (see Figure 2). TRAF6 then recruits to a TGF-β Activated Kinase-1 (TAK)/ TAK Binding Protein-2/3 (TAB2/3) complex leading to TAK-1 activation by phosphorylation (25). TAK-1 then activates the IκB kinase (IKK) complex which contains two catalytic subunits which can form homo or heterodimers (IKKα or IKKβ) and a regulatory subunit NFκB-essential modifier (NEMO), also known as IKKγ (26). The IKK complex phosphorylates IκB, an inhibitory subunit of NFκB, thus allowing an active NFκB dimer to translocate into the nucleus.

For MAP kinase activation, the IKK complex also phosphorylates p105 which is a negative regulator of serine/threonine kinase tumour progression locus 2 (Tpl2) (27). Thus upon phosphorylation and subsequent degradation of p105, Tpl2 is activated. Tpl2 then activates MKK1 and MKK2 leading to the phosphorylation of the extracellular signal-regulated kinases ERK-1 and ERK2 (27). p38 and JNK MAP kinases are also activated by TLR signalling, since TAK-1 activates MKK3/6 and MKK4/7 which in turn stimulates p38 and JNK respectively (28).

In addition certain TLRs, namely TLR3, TLR4, TLR7, TLR8 and TLR9 also activate the IRFs in response to viral PAMPs. A broad range of viral infections activate IRF3 and IRF7 while IRF5 activation is more restricted (29). In order to be activated, IRF3 and IRF7 are phosphorylated by two kinases: TBK-1 (TRAF family member associated NFκB activator (TANK)-binding kinase-1) and IKKε (30). These kinases are recruited upon activation of TLR3 and this pathway is thought to be IRAK-independent (see Figure 3).
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 (31). TLR7/8/9 are also known to activate IRF5 and IRF7 in a TBK-1/IKKε-independent, but IRAK-dependent manner (32). IRAK-1 has been shown to stimulate IRF5 ubiquitination via TRAF6 both in mouse and human cells (33). Through the use of IRAK-1 -/- mice, it has been demonstrated that IRAK-1 is important for IRF7 phosphorylation. Furthermore IKKα plays a role in this pathway through the activation of IRF7 (see Figure 3) (33, 34).

2. 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 (15). It is a protein of 712 aa in length giving it a molecular mass of ~85kDa. Human IRAK-1 is ubiquitously expressed while interestingly, murine IRAK-1 has a more restricted expression being primarily expressed in liver, kidneys and testis (15, 35). Human IRAK-1 has three splice variants (36). 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 (34, 37, 38).
2.1 IRAK-1 post-translational modification during IL-1R/TLR signal transduction

Upon ligand binding to the IL-1R or a TLR, MyD88 is rapidly recruited to the receptor via interaction of its TIR domain (40). IRAK-1 interacts with MyD88 through DD interactions (see Figure 2). Thr66 in the DD has been shown to be vital for the formation of homodimers of IRAK-1 but mutation of this residue did not prevent IRAK-1 interaction with IRAK-2 or IRAK-M but prevented activation of NFκB (41).

IRAK-1 has also been shown to undergo phosphorylation upon TLR stimulation and some residues on IRAK-1 have been suggested as phosphorylation targets for IRAK-4 (See Figure 1). 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 (18). This is a critical residue in IRAK-1 as mutation of this residue completely disrupts its kinase activity (18).

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) (18). 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-autophosphorylation. MyD88 only binds non phosphorylated IRAK-1 (42), thus upon phosphorylation, IRAK-1 is released from the receptor complex and binds 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 (37, 43). After phosphorylation and activation of IRAK-1, it has been reported that IRAK-1 undergoes K48-linked polyubiquitination leading to its rapid degradation (42). Until recently it was assumed that IRAK-1 was only targeted by K48 ubiquitination, however recent studies have shown that IRAK-1 undergoes K63-linked polyubiquitination (43-45). K63-linked polyubiquitination of a protein is normally required for signal transduction rather than degradation and upon ubiquitination of IRAK-1 it can interact with NEMO (44). Mutation of the ubiquitination sites on IRAK-1 prevents NEMO binding and subsequent IL-1/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 (43-45). The pellino proteins are a family of E3 ubiquitin ligases that play an important role in IL-1/TLR signalling. One report has shown IRAK-1 undergoes ubiquitin editing and thus is subject to both K63- and K48- linked ubiquitination (46).

2.2 NFκB activation and IRAK-1 kinase activity

IRAK-1-deficient mice were used to examine the role of IRAK-1 in IL-1/TLR-induced activation of NFκB and MAPK pathways (47-49) (See Table 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 (47, 48). Further, IL-1-induced p38 and JNK activation were shown to be reduced in IRAK-1 -/- mouse primary embryonic fibroblast (EF) cells (49). Interestingly at low concentrations of IL-1, IκB degradation was affected in IRAK-1 -/-
fibroblasts, however at higher concentration IkB was completely degraded in wild type and IRAK-1 -/- cells (49). Furthermore, an effect on 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-1/TLR-induced NFkB, MAPK activation and gene induction (47-49). One particular study of interest showed that IRAK-1 was completely dispensable for TLR7/9 mediated NFkB and MAPK cytokine production in plasmacytoid dendritic cells (pDCs) (34). However this study revealed a novel role for IRAK-1 in IRF activation which will be discussed later (see Section 2.3).

Despite the fact that IRAK-1 is a kinase, the function/relevance of its kinase activity in the activation of NFkB by IL-1/TLRs is still uncertain. It has been shown that the kinase activity of IRAK-1 is not essential for NFkB activation as a kinase inactive mutant of IRAK-1 can still activate NFkB (50, 51). In contrast to this IRAK-1 has been shown to phosphorylate the pellino proteins upon overexpression in cells and also directly in vitro (43-45).

Overall, whether the kinase activity of IRAK-1 is directly required for NFkB 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 that its kinase activity may not be essential for TLR-induced NFkB, IRAK-1 may play other roles in innate signalling where its kinase activity is critical.
2.3 IRAK-1 and IRF activation

This holds true for the role for IRAK-1 in IRF activation that 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 (34). 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 using human cells 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 (34). In contrast to this it has also been proposed that IRAK-1 is a negative regulator of the IFN pathway since SHP-1 was shown to promote type I IFN induction by inhibiting IRAK-1 (52).

IRAK-1 was subsequently shown to be important for TLR7- and TLR8-induced IRF5 activation in both mouse and human cells (32,33). 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 (33). It is thought that the explanation for this is that IRAK-1 regulates the TRAF6-mediated ubiquitination of IRF5 (See Figure 3) (33). A single nucleotide polymorphism in IRF5 has been revealed as a risk factor for systemic lupus erythematosus (SLE) and more recently it has also been shown that IRAK-1 has a crucial role in the development of this disease (53, 54). IRAK-1 has also been shown to interact with TRAF3 (55), 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.

2.4 IRAK-1 and STAT activation

IRAK-1 has been shown to have a novel role in TLR4-mediated STAT3-dependent IL-10 expression (38). Surprisingly IRAK-1 -/- mice that were stimulated with LPS in this study were shown to induce multiple NFκB-dependent genes normally. 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 (38). LPS-mediated IL-10 gene expression has been shown to be dependent on STAT3 and phosphorylation of STAT3 on a crucial serine residue was not observed in IRAK-1 -/- splenocytes. Further it was demonstrated that IRAK-1 and STAT3 localise together 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 (56). Furthermore IRAK-1 and STAT1 have been shown to interact in vivo in human glioblastoma cells T98G (56).

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) (19). Initial reports showed that IRAK-2 when overexpressed,
activated NFκB. 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 (19). 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 (8, 19).

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. It was presumed to only have a redundant role with IRAK-1. However the importance of IRAK-2 in TLR-induced NFκB activation was discovered through studies with Vaccinia Virus (VACV). VACV is a member of the poxviridae family, which are large DNA viruses and the proteins they express have diverse ways of evading and subverting the innate immune system (57). A52 is one such protein shown to be important for virus virulence (57). It was found that A52 interacts with IRAK-2 and TRAF6, but not IRAK-1. Subsequently A52 was shown to inhibit all IL-1/TLR pathways to NFκB solely through its interaction with IRAK-2 (58). 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 (59). Of note, knockdown of IRAK-2 in human peripheral blood mononuclear cells (PBMCs) impaired LPS-induced IL8 production. However further studies in primary human cells will be required to fully understand the contribution of IRAK-2 to human TLR
signalling pathways. The role for IRAK-2 in the TLR3 pathway is particularly intriguing as no other IRAK family member has been shown to play a role in this pathway (60, 61). In support of this role for IRAK-2, it has been shown that endogenous IRAK-2 is recruited to the TLR3 receptor (59).

Mechanistically how IRAK-2 functions is also still uncertain. It may act very proximal to the receptor complex for all TLRs as is the case with TLR3. However it has been shown that IRAK-2 can stimulate the formation of polyubiquitin chains associated with TRAF6 (see Figure 2) (59). It had always been assumed that IRAK-1 was the IRAK family member that triggered polyubiquitination of TRAF6, however overexpression of IRAK-2 induced polyubiquitination of TRAF6 while overexpression of IRAK-1 did not (59). This was shown to be independent of IRAK-1 as exogenous IRAK-2 could induce TRAF6 ubiquitination in cells deficient in IRAK-1 (59).

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 (62). These mice were found to be highly resistant to LPS and CpG-induced septic shock (see Table1). 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 (48).

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 (62). Similar results were shown for TLR7 signalling to NFκB by a separate group using independently generated IRAK-2 -/- mice (63). Only when IRAK-2 -/- mice were crossed with IRAK-1 -/- mice was a dramatic impairment of NFκB activation by TLR2 observed.

### 3.3 Differential expression of murine IRAK-2 splice variants in inbred versus wild-derived mice

Mouse and human IRAK-2 show 67% sequence identity (64). They share the same domain structure and are highly conserved in their death domains and kinase domains. Their C-terminal differs slightly as the C-terminal of murine-IRAK-2 is 35 amino acids longer than human IRAK-2 (see Fig 1). Interestingly one study has reported that overexpression of murine IRAK-2 cannot activate NFκB (64). 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) (65). Since overexpression 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 on TLR signalling (65). Consistent with this, IRAK-2c completely lacks a DD which may prevent it from interacting with certain signalling molecules (64, 65).

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 (66). A classical inbred strain of mice, C57BL/6J, expressed high levels of the inhibitory isoform of IRAK-2, IRAK-2c. This isoform inhibits the proinflammatory 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 proinflammatory manner. Thus this isoform (in inbred mouse) is responsible for sustained NFκB activation (66). 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. This results in IRAK-2c 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 human TLR signalling to NFκB (66).

### 3.4 Is IRAK-2 an active kinase?

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 subdomain (21). It has been recently proposed that this residue is sufficient for IRAK-2 to act as an active kinase (62). An in vitro kinase assay showed that IRAK-2 is phosphorylated upon stimulation with a TLR2 ligand (62). It is thought that IRAK-4 phosphorylates IRAK-2 and induces its kinase activity. 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 (62 and Joanna Szymak & Andrew Bowie, unpublished data). Thus this reveals a potential crucial role for the IRAK-2 kinase activity in TLR-mediated NFκB activation.

4. IRAK-M

The third family member to be discovered, IRAK-M, is a protein of 596 amino acids with a molecular mass of 68 kDa (67). 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 (67). 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 (16). Furthermore, given that IRAK-2 is no longer assumed to be a pseudo kinase, IRAK-M is now the only member of the family to lack kinase activity. Its most distinct feature is its function as a negative regulator of TLR signalling (68).

Initial reports on IRAK-M showed that upon overexpression it was able to activate NFκB (67). 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 (67). However IRAK-M -/- mice showed an increased inflammatory response (but not increased susceptibility) to the bacterium *Salmonella typhimurium* (68). IRAK-M -/- mice were shown to have reduced survival upon influenza infection *in vivo* (69). Furthermore, macrophages derived from IRAK-M knockout mice displayed enhanced activation of IL-1/TLR signalling, thus suggesting a negative role for this family member (68, 69) (see Table 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 (68). A novel role for IRAK-M recently has recently been shown in specifically negatively regulating TLR2-induced p38, but not JNK or ERK, activation (70). The regulation of p38 by IRAK-M was found to be completely IRAK-1 independent but rather occurred through IRAK-M-mediated stabilisation of the phosphatase MKP-1. MKP-1 had been previously shown to negatively regulate p38 phosphorylation (71). Thus in contrast to the proposal that IRAK-M may interact with and regulate IRAK-1 (68), this study suggests that IRAK-M does not contribute to IRAK-1 regulation and function.

IRAK-M was also shown to negatively regulate the alternative NFκB pathway in a TLR2-specific manner (72). 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 mechanism. 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) (73). However this study shows that upon stimulation of cells by the TLR2 agonist Pam$_3$Cys$_k$elevated levels of NIK protein and altered distribution of the NFkB subunit RelB were observed in IRAK-M -/- cells (72). This hinted at the possible regulation of the alternative NFkB pathway by IRAK-M. It appeared that IRAK-M did not regulate the TLR-2 induced classical NFkB pathway as p65/RelA phosphorylation and nuclear translocation were unchanged in wild-type and IRAK-M -/- cells (72). Thus it seems that the role for IRAK-M may be more vital in the alternative NFkB pathway, and for TLR2-induced p38 regulation, rather than in the classical NFkB pathway as originally thought.

5. IRAK-4

IRAK-4 is the most recent member of the IRAK family to be discovered (74). It was discovered through a database search as a human cDNA sequence that encodes a polypeptide sharing significant but previously unrecognised homolog with IRAK-1. It is the closest human homolog to the $Drosophila$ Pelle protein. Pelle, the only IRAK in the 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 (75).

5.1 Role of IRAK-4 in TLR signalling

The essential role of IRAK-4 was revealed through knockout 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 (75). 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 (75). 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 the IL-1R (75).

IRAK-4 plays a critical role proximal to the receptor and MyD88 and mediates NFκB activation through initially interacting with MyD88 (76). It has recently been described that the DDs of IRAK-4 and MyD88 form large oligomeric structures termed the myddosome (77). The DDs were shown to assemble in a complex with two layers containing 7/8 MyD88 subunits and 4 IRAK-4 subunits altogether. This complex may allow for the recruitment of other molecules such as IRAK-2 or IRAK-M (77).

MyD88 acts as a scaffold protein for the interaction between IRAK-1 and IRAK-4 (77). 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 (78), thus acting in a negative feedback loop to regulate the MyD88-dependent pathway.

IRAK-4 has also been shown to have an important role in type I IFN induction by TLR7/8/9 (61). IRAK-4 was also shown to interact with IRAK-2 (79 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 (61).

5.2 Differential requirements for IRAK-4 kinase activity in signalling

While it was apparent from knockout studies that IRAK-4 has a vital role in IL-
1/TLR signalling, whether the kinase activity of IRAK-4 was essential for its
function was unknown. Initial studies using IRAK-4 deficient murine embryonic
fibroblasts (MEFs) that were reconstituted with an IRAK-4 kinase inactive
mutant showed that the kinase activity of IRAK-4 was required for the optimal
induction of IL-1 induced NFκB, JNK activation and proinflammatory cytokines
(80). Then 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 (81, 82). In
vivo, these mice were highly resistant to TLR-induced shock (81, 82).

However macrophages from these mice revealed that the kinase activity of
IRAK-4 was dispensable for the activation of IL-1-, TLR2-, TLR4- and TLR7-
induced NFκB (81, 82). Interestingly in one particular study it was observed
that TLR2-induced NFκB DNA binding still occurred in IRAK-4 knockout
macrophages, indicating the existence of an IRAK-4 independent TLR2
pathway (81).

While IL-1/TLR-induced NFκB was not greatly affected in IRAK-4 kinase
inactive knock-in mice, there was a dramatic impairment of IL-1/TLR induced
cytokine and chemokine production (82-84).
Furthermore MAP kinase activation appears to have a higher dependency on IRAK-4 kinase activity than NFκB. IL-1-, TLR2-, TLR4- and TLR7-induced JNK activation was shown to be strongly dependent on IRAK-4 kinase activity (81–84). Whether the kinase activity of IRAK-4 is required for IL-1/TLR-induced p38 activation is still controversial (81–84). Two groups have shown that the kinase activity is required for IL-1/TLR-induced p38 activation in macrophages and fibroblasts (83,84), while others showed that IRAK-4 kinase activity is not required for TLR4/TLR7-induced p38 activation (82).

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 (85, 86). 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 IL8 gene expression to a similar degree as the wild-type IRAK-4 (85). 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 (86). 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 expression. Thus in the human system it appears that the kinase activity of IRAK-4 is not required, perhaps being redundant with IRAK-1 or IRAK-2.
5.3 IRAK-4 and human disease

IRAK-4 is one of the few IL-1/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 (87-90). 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 (87). Furthermore for these patients, infections began early in life but decreased with age, presumably due to the help of the adaptive immune system (88). In contrast, the susceptibility of the immune system of IRAK-4 -/- mice does not decrease with age (88). This suggests a rather specific role for IRAK-4 in terms of which human pathogens the human immune system requires it for.

Even though IRAK-4 is required for all MyD88-dependent signalling pathways to NFkB 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/TLR4. PBMCs that were deficient in IRAK-4 failed to respond to stimulation with TLR7/8 agonists, and no IFNα, IFNβ and IFN λ mRNA or protein was induced in these cells (61). In contrast, normal p38, JNK, IRF3 and NFkB 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. 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 (61).

6. IRAKs and post-transcriptional regulation

Although much of the research into IL-1/TLR signalling focuses on transcriptional regulation many short-lived inflammatory mRNAs induced by TLRs are regulated at the post-transcriptional level. These mRNAs contain AU-rich elements (ARE) in their 3’ untranslated (UTR) region (91). ARE-binding proteins (ARE-BP), such as tristetraprolin (TTP), HuR and AUF1 bind to these ARE regions and positively and negatively regulate mRNA decay (91-94). The MAPK pathways mediate the regulation of these ARE-BPs (91). Some of the ARE-BPs regulate both mRNA decay and the translational pathway (95).

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 (63, 82, 96). IRAK-1 has been shown to be essential for IL-1-induced stabilisation of the mRNAs of two chemokines MIP2 and KC, via a signalling pathway that did not require TRAF6 (96). IRAK-2 has been shown to have a role in LPS-induced post-transcriptional and translational regulation of cytokine and chemokine expression (63). While it was shown, using IRAK-2 -/- macrophages that murine IRAK-2 has no role in TLR4-induced early or
sustained NFκB activation, TLR-4 induced cytokines and chemokine production was greatly affected since TLR4-induced stability of IL-6 and KC mRNA was shown to be regulated by IRAK-2 (63). It was found that the role of IRAK-2 in mRNA stability was ligand specific as there was no role for murine IRAK-2 in TLR7-induced post-transcriptional control (63). In addition to its role in post-transcriptional regulation, IRAK-2 was also shown to regulate LPS-induced TNFα mRNA 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 (62).

It was also reported that IRAK4 regulates mRNA stability of various cytokines (82). As with murine IRAK-2, the absence of murine IRAK-4 did not significantly reduce IL-1/TLR-induced NFκB activation, but 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 essential for IL-1/TLR-induced mRNA stability (82).

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 and miR146a has been shown to regulate IRAK-1 and IRAK-2 (97).

**7. Future Perspectives**
While it is apparent that the IRAK family is essential for IL-1/TLR signalling, the intricate details of how each family member is involved are still emerging. Additionally more and more studies are showing that the role for IRAKs in IL-1/TLR signalling is ligand- and cell type-dependent. This adds a further level of complexity to IL-1/TLR signalling and suggests that signalling pathways are more complex than initially imagined.

Moreover signalling these studies have revealed an emerging potential difference between mouse and human IRAKs. Increasing evidence suggests that the IRAK members function differently in mouse and human. Whether this is due to the experimental systems used rather than a clear species difference remains to be proven. When IRAK-4 is depleted in mice, this dramatically affects responses to a wide range of microorganisms. In contrast, humans with an IRAK-4 deficiency are only susceptible to a narrow range of bacteria. Similarly IRAK-2 in the murine system is thought to be somewhat redundant with IRAK-1 for early post-receptor signalling events, whereas there is increasing evidence for a potential significant role for IRAK-2 in these events in both human cell lines (59) and primary cells (Sinéad Flannery & Andrew Bowie unpublished data). These emerging species differences suggest caution is advised on extrapolating data from experimental murine studies to the human system.
As new research on the IRAK family emerges it is clear that each member plays a vital but non-redundant role in the IL-1/TLR signalling. Thus the IRAK family may be prospective candidates as therapeutic targets in disease (98). Drugs/inhibitors targeting individual IRAKs could be used specifically for a certain IL-1/TLR pathway in certain contexts, while not affecting the entire IL-1/TLR system. This would prevent a treated individual from being susceptible to a wide range of infections. For example inhibition of IRAK-2 would be predicted to block inflammation via NFκB-dependent genes, while leaving the TLR-IFN arm unaffected. Similarly blocking the kinase activity of IRAK-4 would block certain proinflammatory responses by multiple TLR pathways but would still allow the IFNα/β responses. Alternatively targeting the kinase activity of IRAK-1 may block TLR7/9 induction of IFN (an underlying trigger of some autoimmune diseases) while not affecting activation of NFκB.

Interestingly, it has also become clear that the role of the IRAK family is now not restricted to only the IL-1/TLR pathways. Studies now show a role for IRAK-4 in the adaptive immune response and a critical role in T-cell receptor signalling (99, 100). In addition it has been shown that IRAK-4 is required for Th17 differentiation (101). IRAK-1 and IRAK-2 have also been shown to participate in the RIG-1 antiviral pathway upon VSV infection (97). Thus, further ongoing investigations into the roles of the IRAK family members are likely to reveal further surprises, not only in the IL-1/TLR pathway but also in the broader immune response.
Acknowledgments

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Figure Legends

Figure 1. 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 a critical residue at Thr66 which is important for signalling. The proST domain has been shown to be vital for autophosphorylation. The kinase domain contains Thr209 and Thr387, located in the activation loop, which are potential phosphorylation 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 an 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 2. The role of IRAKs in IL-1/TLR—induced MyD88-dependent activation of NFκB and MAPK.

1) Stimulation of IL-1/TLR, by TLR ligand or IL-1, 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 Pellino which can ubiquitinate IRAK-1. IRAK-1 and Pellino form a complex with TRAF6. 5) NEMO binds to ubiquitinated IRAK-1. 6) IRAK-2 induces the polyubiquitination of TRAF6. The polyubiquitination of TRAF6 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 arrows indicate less defined pathways).

Figure 3. IRAK-dependent and IRAK-independent pathways in TLR-induced IRF activation.
1) Recognition of ds RNA (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. The 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 both in mouse and human cells. 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 phosphorylation and its kinase activity is vital for IRF7 transcriptional regulation both in mice and human cells. 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).
Table 1: Knockout studies showing the effect of absence of IRAKs on IL-1/TLR signalling.

<table>
<thead>
<tr>
<th>IRAK</th>
<th>Phenotype in vivo</th>
<th>Effect on NFκB activation</th>
<th>Effect on MAPK</th>
<th>Effect on IRF activation</th>
<th>Effect on mRNA stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRAK-1</td>
<td>Partially resistant to LPS-induced septic shock (47)</td>
<td>Partial impairment for IL-1/TLR 4 (47-49)</td>
<td>Impairment for IL-1/TLR4 (47-49)</td>
<td>No impairment of TLR2-induced p38 (70)</td>
<td>Dramatic impairment of TLR2-induced ERK and JNK (70)</td>
</tr>
<tr>
<td>IRAK-2</td>
<td>Mice completely resistant to LPS and CpG-induced septic shock (62)</td>
<td>Impairment of late TLR2/TLR7-activation (62)</td>
<td>Impairment of late TLR2-activation (62)</td>
<td>N/D</td>
<td>Required for TLR4-induced mRNA stability (63)</td>
</tr>
<tr>
<td>IRAK-M</td>
<td>Reduced survival upon viral infection (69)</td>
<td>Enhanced activation of TLR4 and TLR9 (68)</td>
<td>Enhanced activation TLR4 and TLR9 (68)</td>
<td>Enhanced TLR2-induced p38 (70)</td>
<td>No effect on TLR2-induced ERK and JNK (70)</td>
</tr>
<tr>
<td>IRAK-4</td>
<td>Mice completely resistant to LPS and CpG-induced septic shock (75)</td>
<td>Impairment of IL-1 and MyD88-dependent TLR (61,75)</td>
<td>Impairment of TLR4-induced JNK (75)</td>
<td>Impairment of IL-1-induced p38 (75)</td>
<td>Effect on TLR7/8/9-induced IFN (61)</td>
</tr>
</tbody>
</table>
**Figure 1**

**IRAK-1**

1 aa 103 aa 198 aa 522 aa 618 aa 712 aa

N-term  |  |  |  |  |  | C-term

**IRAK-2**

1 aa 94 aa 196 aa 489 aa 590 aa

N-term  |  |  |  |  | C-term

**IRAK-M**

1 aa 106 aa 157 aa 469 aa 596 aa

N-term  |  |  |  |  | C-term

**IRAK-4**

1 aa 95 aa 166 aa 460 aa

N-term  |  |  |  | C-term

---

**Death Domain**

**ProST Domain**

**Kinase Domain**

**C-terminus Domain**

- **Important for dimerisation**
- **Important for kinase activity**
- **Invariant lysine residue**
- **Phosphorylated by IRAK-4**
- **Critical aspartate residue**
- **TRAF6 binding motif**
Figure 2
Figure 3
Graphical Abstract