HUMAN INTERLEUKIN-1 RECEPTOR ASSOCIATED KINASE-2 (IRAK-2) IS ESSENTIAL FOR TOLL-LIKE RECEPTOR-MEDIATED TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION OF TNFα.

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Running title: Role of IRAK-2 in TLR signaling in primary human cells

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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 in-bred 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 peripheral blood mononuclear cells 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. These data conflict with findings from the in-bred IRAK2$^{-/-}$ mice, but concur with what has been seen in wild-derived mice for TLR2. Moreover, human IRAK-2 was required for regulating MyD88-dependent TNFα mRNA stability via the TNF 3'UTR. Collectively these data demonstrate for the first time an essential role for IRAK-2 in primary human cells for both transcriptional and post-transcriptional TLR responses.

The innate immune system detects the presence of pathogens through a variety of pattern recognition receptors (PRRs[2]), which recognise conserved pathogen-associated molecular patterns (PAMPs) on invading pathogens (1). One important family of such receptors are the Toll-like receptors (TLRs), of which there are 10 members in the human and 13 members in the mouse (2). For example, the PAMP for TLR4 is LPS, TLR9 responds to CpG DNA while TLR7 and TLR8 both recognise viral ssRNA and synthetic imidazoquinoline-like molecules (3). TLRs are transmembrane proteins which contain a cytoplasmic TIR (Toll/IL-1 receptor) domain and leucine-rich repeats (LRRs) located extracellularly (2). Detection of a PAMP by a TLR results in receptor dimerisation, which allows recruitment of the TIR-domain-containing adaptor proteins: MyD88 is required for all TLR signalling pathways except for TLR3 and a MyD88-independent TLR4 pathway (4): MyD88-adaptor-like (Mal) is required for TLR2 and TLR4 signalling to recruit MyD88 to the receptor complex (5); TIR-domain-containing adaptor inducing IFN-β (TRIF) is required for TLR3 signalling and for a MyD88-independent TLR4 pathway (6); while TRIF-related adaptor molecule (TRAM) is also required for the MyD88-independent TLR4 pathway (7). The formation of these receptor-adaptor complexes results in the activation of various signalling pathways of the innate axis of the immune response. These signalling pathways can lead to the activation of MAPKs, such as p38 and JNK, and of transcription factors such as NFκB and IFN-regulatory factors (IRFs) (2). NFκB is required for the transcription of various proinflammatory cytokines such as IL-1 and TNF, and of chemokines such as IL-8. Similarly, p38 activation results in increased expression of cytokines and chemokines due to direct phosphorylation of transcription factors, and through increased stability and enhanced translation of mRNAs (8).

The serine/threonine interleukin-1 receptor associated kinases (IRAKs) are recruited to the TLR-adaptor complexes and control downstream signalling to MAP kinases and transcription factors (9), for example through the activation of the E3 ligase activity of TNF-receptor-associated factor 6 (TRAF6),

which is essential for some signalling events (10). Structurally, IRAK family members share similar domains. They contain an N-terminal death domain (DD), a ProST domain, a central conserved kinase domain, and apart from IRAK-4, a C-terminal domain (9). IRAK-1 and IRAK-4 have been characteristically described as active kinase family members (11, 12), while IRAK-M, a negative regulator, is thought to have no kinase activity (13). IRAK-2, which was always assumed to be a pseudokinase due to the absence of an aspartate residue in the kinase domain, has recently been described as having kinase activity (14). Upon TLR stimulation, IRAK4 interacts with both MyD88 and IRAK-2 via homotypic DD interactions, to form a complex proximal to the receptor termed the Myddosome (15). IRAK-4 is widely regarded as the most critical IRAK in MyD88-dependent TLR pathways as IRAK-4 knockout mice show impaired signalling to MAP kinases and transcription factors for all TLR signalling pathways except TLR3 (16). In addition, patients with an inherited IRAK-4 deficiency have been shown to be susceptible to a range of bacterial infections (17, 18). In contrast to IRAK-4, deletion of IRAK-1 attenuates, but does not eliminate, TLR-induced NFκB, MAPK activation and gene induction (19-21). In addition, IRAK-1 was shown to be required for IRF activation (22-23).

The role of IRAK-2 has been the most enigmatic of the family. Evidence of a role for IRAK-2 in TLR signalling was revealed though studies of the Vaccinia Virus (VACV) protein A52 (24). A52, shown to be important for virus virulence (24) inhibited all TLR pathways to NFκB through interacting with IRAK-2 (25). This viral targeting of IRAK-2 and not IRAK-1 by A52 in order to disrupt TLR-induced NFkB activation (26) suggested a predominant role for IRAK-2 in NFκB activation. Consistent with this, knockdown of IRAK-2 expression by siRNA in a human cell line inhibited TLR3-, TLR4- and TLR8-induced NFκB-dependent reporter gene activity (26). The recent generation of IRAK-2 knockout mice showed that IRAK-$2^{-/-}$ mice were highly resistant to LPS- and CpG- induced septic shock (14), while a previous study showed that the difference in mortality between wild type and IRAK-$1^{-/-}$ mice was only subtle (20). 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 (14). Using independently generated IRAK-$2^{-/-}$mice, another group also showed for TLR7 that only late NFkB activation was impaired, while both early and late NFκB activation was normal for TLR4 stimulation (27). Thus currently there are contradictory findings on the role of IRAK-2 in NFκB and p38 MAPK in a human cell line compared to murine cells from inbred knockout mice (28). Furthermore, nothing is known about the role of human IRAK-2 in post-transcriptional regulation. Of note, IRAK-2 in mice has four splice variants, two of which are inhibitory, while human IRAK-2 has no splice variants (29). Thus IRAK-2 in mice and humans may function differently.

Therefore in order to further elucidate and clarify the role of IRAK-2 in TLR signalling in the human system we investigated the function of IRAK-2 in both transcriptional and post-transcriptional TLR responses in primary human cells. The results show that IRAK-2 is essential for TLR4- and TLR8-induced cytokine and chemokine mRNA and protein production in human peripheral blood mononuclear human cells (PBMCs). In contrast to what was seen in murine cells (14, 27), in human cells IRAK-2 was required for both NFκB and p38 MAPK activation in response to TLR4 or TLR8 stimulation. Furthermore, human IRAK-2 controlled a MyD88-dependent TLR pathway to TNFα mRNA stability via the TNF 3'UTR. Thus we have a revealed an essential role for IRAK-2 for both transcriptional and post-transcriptional TLR responses in primary human cells, while the particular role of IRAK-2 in regulating gene induction of TNFα suggests that IRAK-2 is a critical pro-inflammatory kinase in humans.

EXPERIMENTAL PROCEDURES

Cell Culture - Human PBMCs from healthy volunteers were provided by the Irish Blood Transfusion Service. PBMCs purified from the buffy coat of heparinised whole-blood preparations from healthy volunteers by density centrifugation on low-endotoxin Ficoll-Hypaque. Isolated PBMCs were washed three times in sterile PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM NaH_2PO_4), counted, and seeded at a density of $1X10^6$ cells/ml in complete RPMI 1640 medium. Human embryonic kidney (HEK) 293 cells stably transfected with the IL-1R (HEK293_R1) were provided by Tularik Inc (San Francisco, CA).

Plasmids - WT-IRAK-2-myc and MyD88 expression plasmids were provided by M. Muzio (Mario Negri Institute, Milan, Italy). The TK-renilla construct was purchased from Promega (Madison, WI)). The empty vector control pcDNA 3.1 was purchased from Invitrogen (Carlsbad, CA). The TNF 3'UTR-luciferase reporter construct and the β-actin 3'UTR-luciferase reporter construct were kindly provided by Aihao Ding (Cornell University, New York, NY). The IL-8 3'UTR-luciferase reporter construct was a gift from Peter King (Birmingham Veterans Affairs Medical Centre, Birmingham, AL). 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).

TLR agonists - Ultrapure LPS from Gram-negative bacteria (*Escherichia coli)* (>99.9 pure in respect to contaminating protein, DNA, and TLR2 agonists) was purchased from Alexis Biochemicals (Plymouth Meeting, PA). CL075 (3M002) a thiazoloquinolone derivative, was purchased from Invivogen (San Diego, CA).

Reporter gene assays - HEK293_R1 cells were seeded at $1X10^5$ cells/ml and 24 h later were transfected with either 60ng TNF, IL-8 or β-actin 3'UTR reporter constructs. The TK-renilla construct (20 ng) was included to normalise the data for transfection efficiency. Where indicated, 5-25 ng of expression plasmids for MyD88, IRAK-2 or TRAF6 expression plasmids were co-transfected with reporters. The total amount of DNA transfected was kept constant at 230 ng by the addition of pcDNA3.1. Luciferase activity was measured 24 h following transfection. All transfections were done in triplicate and data are expressed as fold induction (mean ±s.d) relative to control levels for a representative experiment of a minimum of three separate experiments.

SiRNA gene silencing - siRNA duplexes targeting the IRAK-2 gene targeted the sequence 5'-CCAGATCATCCTGAACTGGAA-3', as previously described (26). Non-silencing siRNA was used as a control (Qiagen). PBMCs were seeded at $1X10^6$ cells/ml (200 µl per well) for cytokine analysis in 96 well plates, and transfected with siRNA 1 h later. 5 pmol siRNA diluted in serum free RPMI was used per transfection. Each well received a solution containing 0.2 µl Lipofectamine 2000 (Invitrogen), 0.5 µl siRNA and 49.3 µl serum-free medium per transfection. An identical second transfection of siRNA with Lipofectamine 2000 was carried out 24 h later. After a further 24 h, cells were stimulated with either 100 ng/ml of LPS or 2.5µg/ml of CL075 and finally harvested 24 h later. For detection of IκBα or *p*-p38, PBMCs were seeded at $2X10^6$ cells/ml in 6 well plates (2ml per well). 50 pmol siRNA diluted in serum free RPMI and 2 µl Lipofectamine 2000 were used per transfection, using a protocol identical to that used for the 96-well plates. To examine the effect of IRAK-2 siRNA on MyD88-induced TNFα 3'UTR or the IL-8 3'UTR HEK_293 R1 cells were seeded at $1X10^5$ cells/ml (200 µl per well). Cells were transfected with 5 pmol siRNA using 0.5 µl Lipofectamine 2000 24 h later, followed by transfection with MyD88 and either TNFα 3'UTR, IL-8 3'UTR or β-actin 3'UTR plasmids and a second siRNA transfection 24 h later. After a further 24 h, cells were harvested, lysed and the relative luciferase activity was measured.

Immunoblotting - For detection of IκBα, *p*-p38 and endogenous IRAK-2, cells were harvested and subject to centrifugation, the pellet lysed in 100 µl SDS sample buffer (62.5 mM Tris (pH 6.8), 2 % (w/v) SDS, 10 % glycerol, 0.1 % (w/v) bromophenol blue, 50 mM DTT), lysates boiled for 5 min, and then subject to 2 min sonication. Lysate (20 µl) was loaded onto a SDS-PAGE gel and transferred onto Immobilon polyvinylidene diflouride membrane (Millipore, Bedford, MA). For analysis of IκBα degradation and p38 phosphorylation membranes were probed with either a mouse mAb against IκBα (a gift from Prof. R. Hay, Dundee University, Dundee, U.K) or rabbit anti-p38 phosphospecific Ab (Cell Signalling Technology, Danvers, MA). 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). To control for protein loading, the membranes were reprobed with anti-β-actin Ab (Sigma-Aldrich) or rabbit anti-p38 Ab (Cell Signaling Technology).

Cytokine analysis by ELISA - PBMCs were seeded at $1X10^6$ cells/ml in 96 well plate 1 h prior to siRNA treatment. The supernatants were collected and assayed for IL-8 or TNFα by ELISA (R&D Systems, Minneapolis, MN).

Measurement of mRNA induction and stability - PBMCs were seeded at $1X10^6$ cells/ml in 12 well plates (1 ml per well). Cells were transfected 1 h later with 25 pmol siRNA and Lipofectamine 2000. Each well received a solution containing 1 µl Lipofectamine 2000, 2.5 µl siRNA and 246.5 µl serum-free medium per transfection Cells were transfected with an identical siRNA treatment 24 h later. After another 24 h cells were stimulated with either 100 ng/ml LPS or 2.5 µg/ml CL075 for the indicated times. Cells were harvested and RNA was isolated using High Pure Isolation kits from Roche Applied Science (Burgess Hill, U.K.). 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. 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 h later. After another 24 h cells were stimulated with either 100 ng/ml LPS or 2.5 µg/ml CL075 for 2 h followed by treatment of 5 µg/ml Actinomycin D (Act D, Sigma-Aldrich) and either LPS or CL075. Cells were harvested and RNA analysis was carried out as described above. The primers used were as follows: TNFα, 5'-GAA CCC CGA GTG ACA AGC CTG-3' and 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'; β-actin, 5'-CGC GAG AAG ATG ACC CAG ATC-3' and 5'-GCC AGA GGC GT CAG GGA TA-3'.

Statistical analysis - Statistical analysis was carried out using paired Students *t* test.

RESULTS

IRAK-2 is required for TLR4- and TLR8-mediated cytokine induction in primary human cells - We have previously showed that knockdown of IRAK-2 expression by siRNA inhibited human TLR4- and TLR8-dependent NFκB reporter gene activity in HEK293 cells (26). In contrast to this, reported data from murine IRAK-2 -/- cells has shown that IRAK-2 was dispensable for early NFκB and p38 MAPK activation (14, 27). Thus, here we 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 siRNA oligonucleotides that target IRAK-2 in PBMCs was confirmed by examining their effect on endogenous IRAK-2 protein levels. Figure 1A shows that 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). CL075 is a thiazoloquinolone derivative agonist of TLR7 and TLR8, which is known to preferentially activate TLR8 in PBMC (3). PBMCs are known to respond to both LPS and CL075, via TLR4 and TLR8 respectively, leading to the production of inflammatory cytokines (30). When PBMCs were treated with CL075, the production of IL-8 in response to CL075 was reduced to basal levels in the cells treated with IRAK-2 siRNA relative to those treated with the control siRNA (Fig 1B). CL075-induced TNFα production was also significantly inhibited in PBMCs treated with IRAK-2 siRNA compared to control siRNA treated samples, (Fig 1C) thus demonstrating for the first time to our knowledge a role for IRAK-2 in TLR8 responses in primary human cells. IRAK-2 was also required for LPS/TLR4-mediated IL-8 production in PBMCs (Fig 1D). Furthermore TLR4-mediated TNFα release from PBMCs was also significantly inhibited by treatment of cells with IRAK-2 siRNA (Fig 1E). In addition, both CL075 and LPS-induced IL-6 production was significantly reduced in PBMCs (data not shown). Thus IRAK-2 has a critical role in cytokine production in primary human cells, for both endosomal (TLR8) and non-endosomal (TLR4) TLR pathways.

IRAK-2 is essential for TLR-mediated NFκB and p38 MAP kinase activation in primary human cells - Our 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 in-bred IRAK-$2^{-/-}$ mice was also impaired, although NFκB and MAP kinase activation were largely unaffected (14, 27, 28). In contrast, in macrophages from a wild-derived mouse strain, RNA interference targeting IRAK-2 did inhibit TLR-induced NFκB and p38 MAPK activation (31). 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 we

next explored the role of IRAK-2 in TLR-induced signalling pathways in PBMCs.

Treatment of PBMCs with CL075 led to NFκB activation within 5 minutes of stimulation, as measured by degradation of the inhibitory NFκB subunit, IκBα (Fig 2A). When cells were pretreated with IRAK-2 siRNA, TLR8-dependent degradation of IκBα, was prevented at every time point tested (5-30 min) compared to cells treated with control siRNA (Fig. 2A). Thus this demonstrated 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 (27). A role for IRAK-2 in TLR4-mediated NFκB activation was also observed in PBMCs, since IRAK-2 siRNA caused reduced IκBα degradation after 20 or 30 min LPS stimulation, compared to cell treated with control siRNA (Fig *2*B). Again, this contrasts with what was observed in macrophages from mice lacking IRAK-2, where IRAK-2 had no role in either early or late TLR4-induced NFκB activation (28).

We next determined whether IRAK-2 was involved in p38 MAPK activation in TLR-stimulated primary human cells. Upon stimulation of PBMCs with LPS or CL075 we detected phosphorylation of p38 after 5 min, sustained until at least 20 min (Fig 2C, D). Crucially, when PBMCs were treated with siRNA targeting IRAK-2 prior to stimulation a dramatic reduction of p38 phosphorylation was observed relative to control siRNA-treated cells (Fig 2C, D). Together these results reveal a critical and novel role for IRAK-2 in early signalling events for TLR pathways in primary human cells.

IRAK-2 is required for TLR-mediated TNFα and IL-8 mRNA induction in PBMCs -The role of IRAK-2 in TLR4- and TLR8-dependent mRNA induction was next examined. Since both NFκB and p38 are involved in the regulation of the induction of IL-8 and TNFα mRNA, it was hypothesized that IRAK-2 would have a role in regulating IL-8 and TNFα mRNA levels. A time course of LPS-induced TNFα transcript in PBMCs indicated that TNFα mRNA peaked at 2 h after stimulation (Fig 3A). When cells were treated with IRAK-2 siRNA, there was a significant reduction in the levels of LPS-induced TNFα mRNA at 2 h compared to cells treated with control siRNA (Fig 3A). 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 (27, 28). Furthermore in PBMC, not only was LPS-induced TNFα mRNA induction IRAK-2-dependent, but a 70% reduction of LPS-induced IL-8 mRNA was observed in the presence of IRAK-2 siRNA (Fig 3B). Similarly, CL075-dependent IL-8 and TNFα mRNA were also significantly IRAK-2-dependent (Fig 3B).

IRAK-2 is required for the stabilisation of TNFα but not IL-8 mRNA in primary human cells - IRAK-4 and IRAK-1 have been shown to regulate stability of some transcripts (27, 32, 33), 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 (34-36). Given that 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 stabilisation of these mRNA transcripts was investigated. For this, PBMCs were initially transfected with either control or IRAK-2 siRNA, then stimulated for 2 h with LPS or CL075 followed by treatment with Act D. The effect of reduced IRAK-2 expression on the rate of mRNA decay was then determined. This showed that for both LPS and CL075 induced IL-8 mRNA, there was no difference between the rate of decay of IL-8 mRNA in the control siRNA-treated cells compared to those treated with IRAK-2 siRNA (Fig 4A, B), suggesting that IRAK-2 is not required for the TLR pathway that induces stability of IL-8 mRNA. In contrast to the lack of effect on IL-8 mRNA decay, in the presence of IRAK-2 siRNA, the decay of TNFα mRNA was accelerated compared to cells treated with the control siRNA. This was observed for both TLR8-induced TNFα mRNA (Fig 4C) and TLR4-induced TNFα mRNA (Fig 4D), but was more marked, and statistically significant for TLR4 (Fig. 4D). It is known that p38 is one of the post-transcriptional regulators of TNFα production, and one of the mechanisms whereby p38 regulates LPS-induced TNFα is by stabilising the mRNA (34, 35). Therefore we determined the effect of the p38 inhibitor, SB202190, on LPS-induced TNFα mRNA stability, in comparison to the effect observed with IRAK-2 siRNA. As expected, SB202190 caused accelerated decay of LPS-induced TNFα mRNA (Fig 4E), but actually to a lesser extent than IRAK-2 siRNA (Fig 4D).

Thus IRAK-2 controls a TLR-dependent pathway to TNFα mRNA stability in primary human cells. This can be at least partially explained by the requirement for IRAK-2 for TLR-induced p38 activation. The role of human 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 (27).

IRAK-2 controls a MyD88-dependent pathway to mRNA stability via the TNFa 3'UTR - To further explore mechanistically how IRAK-2 regulates the stability of TNFα mRNA and not IL-8 mRNA we examined the potential role of the 3'UTR of both mRNA transcripts. Some cytokine mRNAs, including TNFα and IL-8, are rendered unstable due to the presence of adenosine- and uridine-rich elements (AREs) in the 3'UTR (37). For TLR signalling, a MyD88-dependent pathway, which would operate for both TLR4 and TLR8, has been shown to mediate an mRNA stabilising signal that converges on some cytokine 3' UTRs (38). In order to assay the role of MyD88 and IRAK2 in mRNA stability pathways converging on cytokine 3'UTRs, luciferase reporters under the control of the TNFα 3'UTR, the IL-8 3'UTR or as a control the β-actin 3'UTR were transfected into HEK293 cells.

As expected, ectopic expression of MyD88 activated mRNA stabilizing pathways leading to increased production of luciferase protein expressed from the reporters under the control of the TNFα and IL-8, but not the β-actin 3'UTR (Fig 5A). Using IRAK-2 siRNA, we examined whether there was a role for IRAK-2 on this TLR-MyD88-3'UTR pathway. In cells that were treated with control siRNA, MyD88-induced 3'UTR reporter activity was barely affected (Fig 5B-D). Similarly, IRAK-2 siRNA did not affect the β-actin 3'UTR (Fig. 5D). However, in cells treated with IRAK-2 siRNA, there was a very significant decrease in the MyD88-dependent activation of TNFα 3'UTR compared to cell treated with control siRNA (Fig 5B). 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 (Fig 5C), confirming the lack of a role for IRAK-2 in IL-8 mRNA stabilisation (Fig. 4).

Consistent with the requirement for IRAK-2 in the ability of MyD88 to stimulate the TNFα 3'UTR, expression of WT IRAK-2 dose dependently induced the TNFα (but not the β-actin) 3'UTR reporter (Fig 5E, F).

Key residues of IRAK-2 and TRAF6 involved in 3'UTR-mediated TNFα mRNA stabilization – Having established that IRAK-2 is required for TNFα mRNA stabilization, in order to begin to ascertain which activities of IRAK-2 are required for this function, we examined the role of key IRAK-2 residues in the regulation of the TNFα 3'UTR using the reporter system. Although long assumed to be a pseudokinase, recently it has been proposed that an invariant lysine residue located in the ATP-binding pocket of murine IRAK-2 (at K237) is necessary for IRAK-2 to act as an active kinase (14, 39). Here we found that a K237A mutant of human IRAK-2 no longer activated NFκB or p38, nor induced cytokine responses (data not shown). Furthermore, this residue was also required for induction of the TNFα 3'UTR (Fig. 5G).

We have previously described another IRAK-2 residue, E528, as critical for the ability of IRAK-2 to stimulate TRAF6-dependent ubiquitination activity, and an E528A IRAK-2 mutant was unable to activate NFκB or p38, or to induce cytokine production (26). Here, we also found that this residue was essential for induction of the TNFα 3'UTR by IRAK-2 (Fig. 5G). This suggested that TRAF6 may also be involved in the MyD88-IRAK2-TNF 3'UTR pathway. Consistent with this notion, WT TRAF6 could strongly induce the TNFα 3'UTR reporter, in comparison to a dominant negative TRAF6 (with residues of 1-289 deleted, Fig 5H). Previously it has been shown that a key cysteine residue (C70) in the RING-domain of TRAF6 is critical for its E3 ligase activity (10). When this cysteine is mutated to an alanine (C70A), the ability of TRAF6 to activate NFκB is impaired (10). Here, C70 was also required for induction of the TNFα 3'UTR pathway as the TRAF6 C70A mutant no longer induced the TNFα 3'UTR reporter (Fig 5H). Finally we sought to identify whether TRAF6 autoubiquitination is required for its function in this pathway. A lysine at residue 124 in TRAF6 has been shown to be the predominant site for TRAF6 autoubiquitination and there have been conflicting reports as to whether TRAF6 autoubiquitination at this site is required for

TRAF6 functions (10, 40). Here, a K124R mutant of TRAF6 activated the TNFα 3'UTR reporter to a level similar to WT TRAF6 (Fig 5H). These data suggest that the ability of IRAK-2 to stimulate TRAF6 E3 ligase activity, but not TRAF6 autoubiquitination, is required for the MyD88-IRAK2-TRAF6-TNFα 3'UTR pathway.

Collectively these data demonstrate for the first time a critical role in primary human cells for IRAK-2 in multiple TLR responses, namely NFκB activation, p38 activation, early cytokine mRNA induction, and stabilisation of TNFα mRNA via the 3'UTR.

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. Prior to this study, we demonstrated that IRAK-2 was required for TLR3-, TLR4- and TLR8-mediated NFκB-dependent reporter gene activation in a human cell line (26). Here for the first time we provide a comprehensive analysis of the role of human IRAK-2 in primary cells, and demonstrate a critical requirement for it in both early signalling events and in post-transcriptional regulation for both a membrane bound TLR (TLR4) and an endosomal TLR (TLR8).

Recent studies using cells from IRAK-2$^{-/-}$ mice, showed 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 (14, 27, 28). However in the primary human cells studied here, reduction of IRAK-2 expression by siRNA led to impaired NFκB activation for both TLR4 and TLR8. Human IRAK-2 was also shown to be more important than the murine form for p38 MAP kinase activation, since in contrast to the lack of a role for IRAK-2 reported in the IRAK-2$^{-/-}$ studies for TLR2, TLR4 and TLR7 (14, 27), in PBMC IRAK-2 was required for p38 MAP kinase activation in both the TLR4 and TLR8 pathways. Further differences in the murine and human system emerged upon examining cytokine induction and post-transcriptional regulation: we showed here that human IRAK-2 regulated TNFα mRNA induction and stability in response to LPS (and to a TLR8 ligand), whereas lack of murine IRAK-2 did not affect TNFα mRNA induction or stability after LPS treatment, but did impair translation of the mRNA (27). Overall then, although IRAK-2 has now 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.

Murine and human IRAK-2 show 67% sequence identity (41). They also share the same domain structure and are highly conserved in their DD and kinase domains (41), and are therefore unlikely to be significantly different structurally (41). However, differences between murine and human IRAK-2 are apparent at the level of splicing. There are four splice variants of murine IRAK-2 (IRAK-2a, - 2b, -2c and -2d) and different functions have been attributed to the different isoforms, since IRAK-2c and IRAK-2d have been proposed as having a negative role in TLR signalling, while IRAK-2a is a positive regulator of signalling (29, 31). In contrast, there is no evidence of splice variants for human IRAK-2, so that the human protein is most like 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 (31). The classical in-bred strain, C57BL/6J, was shown to express high levels of the inhibitory isoform, IRAK-2c, which inhibits the proinflammatory isoform IRAK-2a (29, 31). This may explain the more dominant role of IRAK-1, rather than IRAK-2, in early signalling events in inbred mice (31). However, as time progresses, expression of the inhibitory isoform (IRAK-2c) decreased (31) 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. 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 in-bred 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 (31). Thus TLR-induced p38 and NFκB responses are similarly impaired by IRAK-2 siRNA in MOLF/Ei macrophages and human PBMCs.

Previous studies have revealed a requirement for the IRAK family in post-transcriptional regulation of cytokine induction. Here we show for the first time a critical role for human IRAK-2 in post-transcriptional regulation, in that IRAK-2 was required for TLR-mediated stabilisation of TNFα mRNA in primary human cells. TNFα, a key proinflammatory cytokine and mediator of inflammatory and autoimmune disease, is subject to tightly controlled regulation both at the transcriptional and translational level, and p38 MAP kinase has a key role in this (36, 37). Here, depletion of IRAK-2 by siRNA caused more rapid degradation of TNFα mRNA than that observed for inhibition of p38, thus highlighting the importance of IRAK-2 in the TNFα mRNA stability pathway. Therefore although p38 activation was shown to be IRAK-2-dependent, IRAK-2 may also have further p38-independent mechanisms of stabilising TNFα mRNA.

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 (35, 38). 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. 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 (15). Consistent with this idea, murine IRAK-4 has been shown to be required for both KC and TNFα mRNA stability (33).

IRAK-2 was shown to be required for MyD88-dependent stabilisation of mRNA via the TNFα 3'UTR, but not the IL-8 3'UTR. While both TNFα and IL-8 mRNA are known to be regulated via AU-rich elements (ARE) within their 3'UTRs, these AREs are subject to differential post-transcriptional control depending on which ARE-binding proteins target specific AREs (34). 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 (see http://rna.tbi.univie.ac.at/cgi-bin/AREsite.cgi). 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'UTRs.

How exactly IRAK-2 regulates the mRNA stability pathway converging on the TNFα 3'UTR remains to be determined. IRAK-2, like all other IRAK family members, contains a functional ATP-binding pocket with an invariant lysine residue (K237) in the protein kinase subdomain. It has been recently proposed that this residue is required for IRAK-2 to act as an active kinase (14, 39), although previously IRAK-2 was assumed to be a pseudokinase. Here we found that K237 was required for the ability of IRAK-2 to stimulate the TNFα 3'UTR. Thus similar to the case with IRAK-4 (33), the kinase activity of IRAK-2 may be required for post-transcriptional regulation of the TNFα transcript.

It is known that TRAF6 plays a critical role in TLR-induced NFκB and MAP kinase pathways and in previous work we have demonstrated that IRAK2 is critical for the formation of polyubiquitin chains associated with TRAF6, an activity that required the IRAK-2 E528 residue (26). However the role of TRAF6 in mRNA stabilisation is less well defined, and will require further investigation. It has been previously reported that TRAF6 is not required for IL-1-induced stabilisation of KC and MIP-2 mRNA (32). Here we provided circumstantial evidence that TRAF6 is required for the ability of IRAK-2 to stabilise TNFα mRNA since, IRAK-2 E528A no longer stimulated the TNFα 3'UTR reporter. Furthermore, although WT TRAF6 stimulated the TNFα 3'UTR reporter, a TRAF6 mutant (C70A) impaired in its E3 ligase activity was not active, while a TRAF6 mutant with a mutated autoubiquitination site (K124R) was. Notwithstanding the need for further

experiments to confirm a role for TRAF6, the data suggests the existence of a MyD88-IRAK-2-TRAF6-dependent pathway converging on the TNFα 3'UTR, which is dependent on IRAK-2's kinase activity, and its ability to stimulate TRAF6 E3 ligase activity, but which does not require TRAF6 autoubiquitination.

In conclusion we have revealed a previously unrecognised role for IRAK-2 in early TLR signalling events and in post-transcriptional regulation of TNFα in primary human cells, which differs from its role in in-bred mice. Given that the dysregulation of TLR-mediated NFκB and p38 activity, and of TNFα production, is implicated in many autoimmune and inflammatory diseases, IRAK-2 likely represent an attractive therapeutic target in human disease.

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FOOTNOTES

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[2]**Abbreviations used:** Abbreviations: Act D, Actinomycin D; ARE, Adenine and uridine rich element; DD, Death domain; HEK, Human embryonic kidney; IFN, Interferon; IκB, Inhibitor of NFkB; IKK, IκB kinase; IRAK, Interleukin-1 receptor associated kinase; IRF, Interferon regulatory factor; KO, Knockout; LPS, Lipopolysaccharide; LRR, Leucine rich repeat; MAL, MyD88-adaptor like; MEF, Murine embryonic fibroblast; MyD88, Myeloid differentiation primary response gene 88; NFκB, Nuclear factor kappa B; PAMP, Pathogen associated molecular pattern; PBMC, peripheral blood mononuclear cell; PRR, Pattern recognition receptor; pDC, plasmacytoid dendritic cell; ssRNA, Single stranded RNA; TIR, Toll/IL-1R; 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; VACV, Vaccinia Virus.

FIGURE LEGENDS

Figure 1: TLR4- and TLR8-mediated cytokine production requires IRAK-2 in primary human cells. (A) 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 cell lysates were harvested at time points indicated. Lysates were assayed for IRAK-2 expression by immunoblotting with IRAK-2 Ab (upper panel), or probed with β-actin Ab to confirm equal protein loading (lower panel). (B-E) PBMCs were transfected with Lipofectamine 2000 alone (black bar), or 5 pmol or 10 pmol of either control non-silencing siRNA (grey bar) or siRNA targeting the IRAK-2 gene (white bar). Cells were stimulated with 2.5μg/ml CL075 (B, C) or 100ng/ml LPS (D, E). Supernatants were harvested 24h later and assayed for IL-8 (B, D) and TNFα (C, E). The data are mean ±SD of triplicate samples and are representative of a least three experiments. **p<0.005 or ***p<0.0005 compared to control siRNA.

Figure 2: Role for IRAK-2 in early TLR signalling events in PBMCs. (A-D) 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 2.5μg/ml CL075 (A, C) or 100ng/ml LPS (B, D) and harvested at the time points indicated. Lysates were assayed for IκBα degradation by immunoblotting with IκBα Ab (A, B, upper panel) or probed with β-actin Ab to confirm equal protein levels (A, B, lower panel). Lysates were also assayed for *p*-p38 (C, D, upper panel) or for p38 as a loading control (C, D, lower panel). Each immunoblot is representative of at least three experiments.

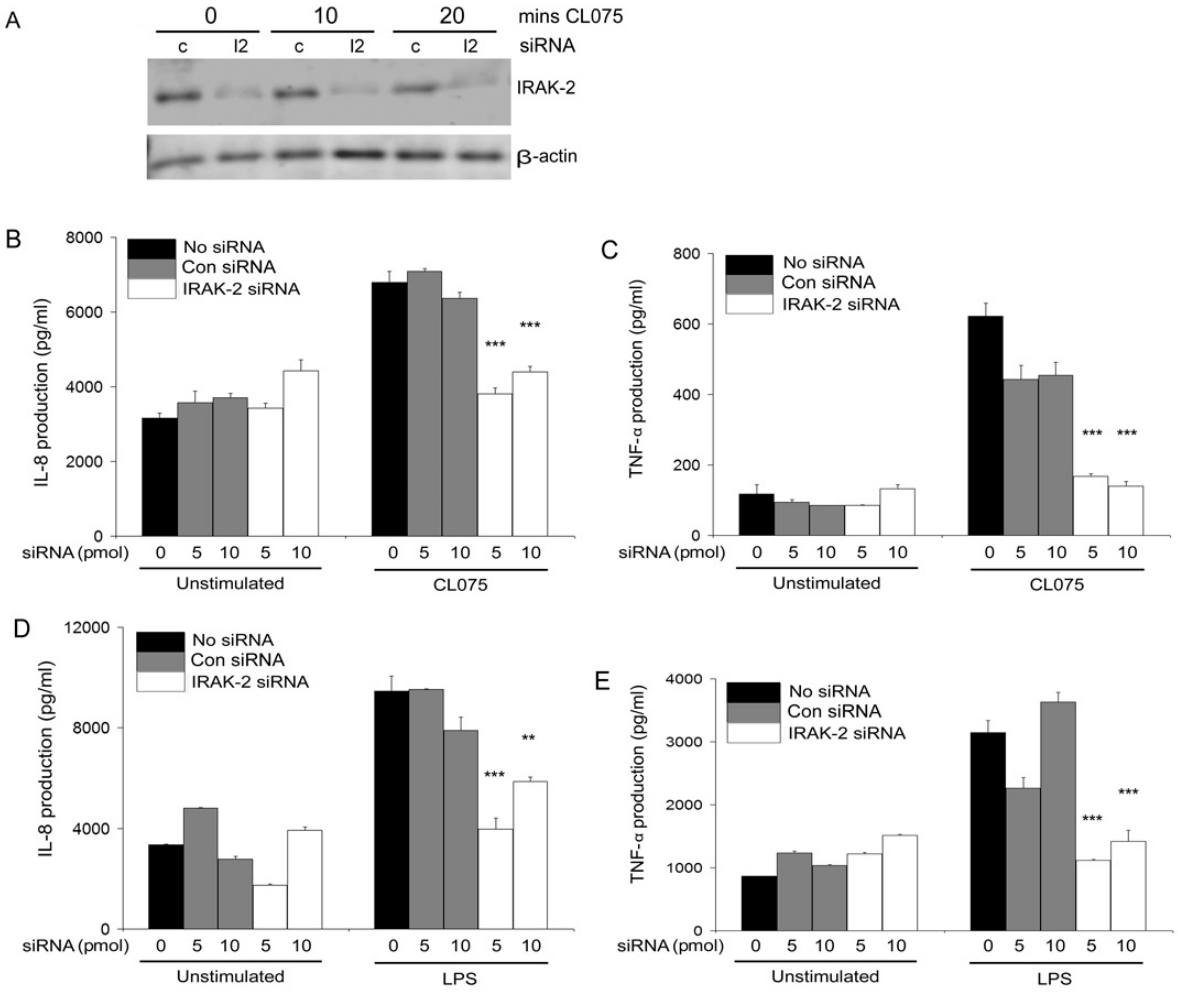
Figure 3: IRAK-2 is required for TLR4- and TLR8-mediated mRNA induction in PBMCs. (A) PBMCs were transfected with 25pmol of either control non-silencing siRNA (black squares) or siRNA targeting the IRAK-2 gene (white squares). Cells were stimulated with 100ng/ml LPS and harvested at the time points indicated. TNFα mRNA expression was assayed by quantitative RT-PCR. (B) PBMC were transfected with siRNA as in (A) and cells were stimulated with either 100ng/ml LPS or 2.5μg/ml CL075 and harvested 2h later. IL-8 and TNFα mRNA expression was assayed by quantitative RT-PCR. The black bar represents the amount of mRNA detected in controls siRNA-treated cells stimulated with LPS or CL075, and is set at 100%. Data is expressed as % mRNA remaining in the presence of IRAK-2 siRNA compared to the non-silencing siRNA control. The data is mean ±SD of triplicate samples and is representative of three experiments. **p<0.005 or ***p<0.0005 compared to control siRNA.

Figure 4: Human IRAK-2 regulates TLR-induced TNFα but not IL-8 mRNA stability.
(A-D) 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 2.5μg/ml CL075 (A, C) or with 100ng/ml LPS (B, D) for 2h and then 5μg/ml actinomycin D (Act D) was added along with 2.5μg/ml CL075 (A, C) or 100ng/ml LPS (B, D). Cells were harvested at the indicated time points and IL-8 (A, B) and TNFα (C, D) mRNA expression was assayed by quantitative RT-PCR. (E) PBMCs were stimulated with 100ng/ml LPS for 2h. Cells were then treated with 5μg/ml Act D and 100ng/ml LPS (black squares) or treated with 5μg/ml Act D, 100ng/ml LPS and 1μM SB 202190 (white squares) and harvested at the indicated time points. 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 5: IRAK-2 regulates TNFα mRNA stability via the TNFα 3'UTR. (A) HEK293_R1 cells were transfected with 10ng MyD88 or empty vector (EV), together with either 60ng of TNFα, IL-8 or β-actin 3'UTR reporter constructs. Results are expressed as fold induction, whereby the luciferase values for each reporter in the presence of EV is set at 1 (black bar). (B-D) Cells 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 25ng MyD88, and 60ng of TNFα (B), IL-8 (C) or β-actin (D) 3'UTR. (E, F) Cells were transfected with EV, or 5, 10 or 25ng WT IRAK-2 with either 60ng of TNFα (E) or β-actin (F) 3'UTR. (G) Cells were transfected with 25 ng pcDNA3.1 (EV), WT IRAK-2, K237A or E528A, and 60ng of TNFα 3'UTR. (H) Cells were transfected with 10 or 25ng WT TRAF6, C70A, K124R or DN TRAF6, and 60ng of TNFα 3'UTR. In all cases activity of the 3'UTRs was measured by luciferase reporter gene assay 24 h later. Data is shown as fold induction. The data is mean ± SD of triplicate samples and is representative of at least three separate experiments. **$p<0.005$ or ***$p<0.0005$ compared to control siRNA.

Figure 1


A
0
10
20
mins CL075
c
I2
c
I2
c
I2
siRNA
IRAK-2
β-actin
B
No siRNA
Con siRNA
IRAK-2 siRNA
IL-8 production (pg/ml)
8000
6000
4000
2000
0
***
***
siRNA (pmol)
0 5 10 5 10
0 5 10 5 10
Unstimulated
CL075
C
No siRNA
Con siRNA
IRAK-2 siRNA
TNF-α production (pg/ml)
800
600
400
200
0
*** ***
siRNA (pmol)
0 5 10 5 10
0 5 10 5 10
Unstimulated
CL075
D
No siRNA
Con siRNA
IRAK-2 siRNA
IL-8 production (pg/ml)
12000
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siRNA (pmol)
0 5 10 5 10
0 5 10 5 10
Unstimulated
LPS
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No siRNA
Con siRNA
IRAK-2 siRNA
TNF-α production (pg/ml)
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3000
2000
1000
0
***
***
siRNA (pmol)
0 5 10 5 10
0 5 10 5 10
Unstimulated
LPS

Figure 2

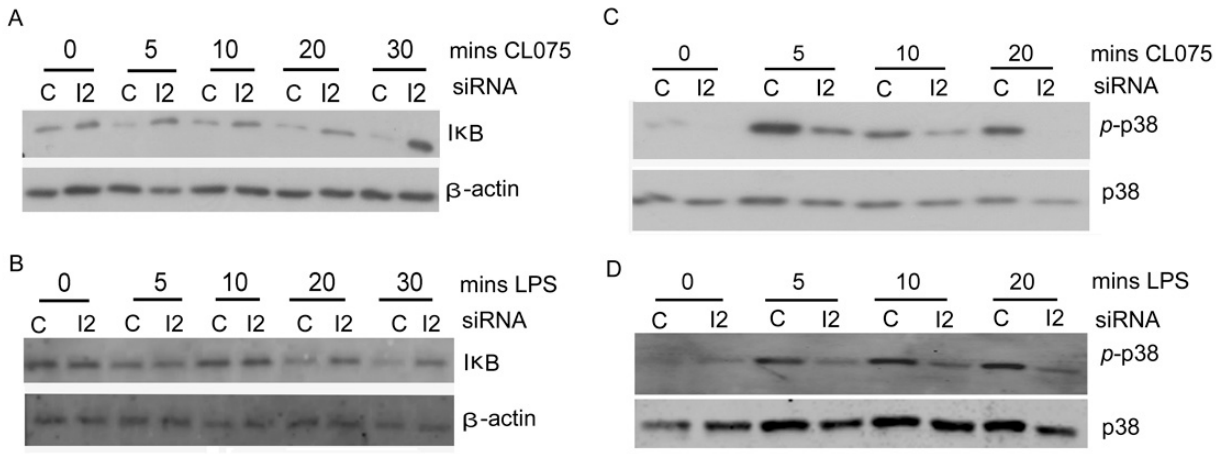

A
0
5
10
20
30
mins CL075
C I2 C I2 C I2 C I2 C I2
siRNA
IκB
β-actin
C
0
5
10
20
mins CL075
C I2 C I2 C I2 C I2
siRNA
p-p38
p38
B
0
5
10
20
30
mins LPS
C I2 C I2 C I2 C I2 C I2
siRNA
IκB
β-actin
D
0
5
10
20
mins LPS
C I2 C I2 C I2 C I2
siRNA
p-p38
p38

Figure 3

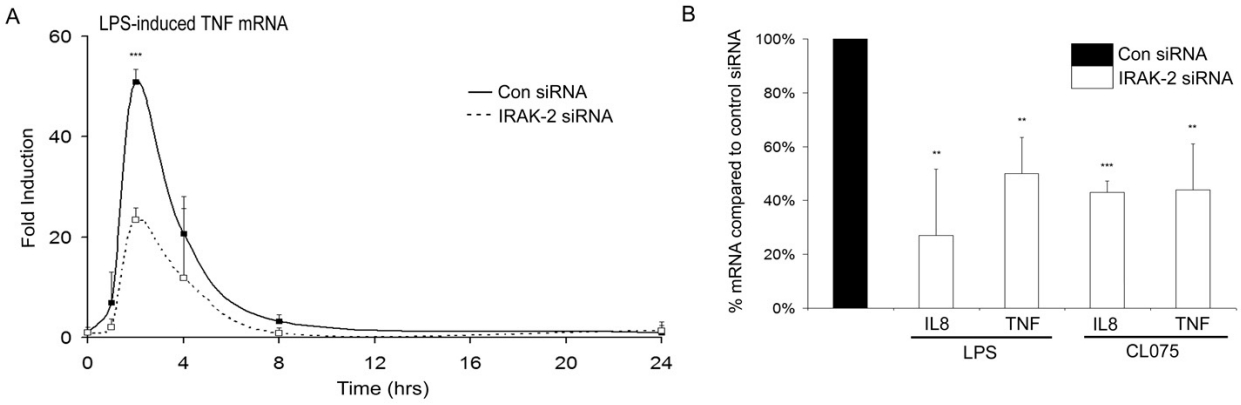

A
LPS-induced TNF mRNA
Fold Induction
Time (hrs)
Con siRNA
IRAK-2 siRNA
B
% mRNA compared to control siRNA
Con siRNA
IRAK-2 siRNA
IL8
TNF
IL8
TNF
LPS
CL075

Figure 4

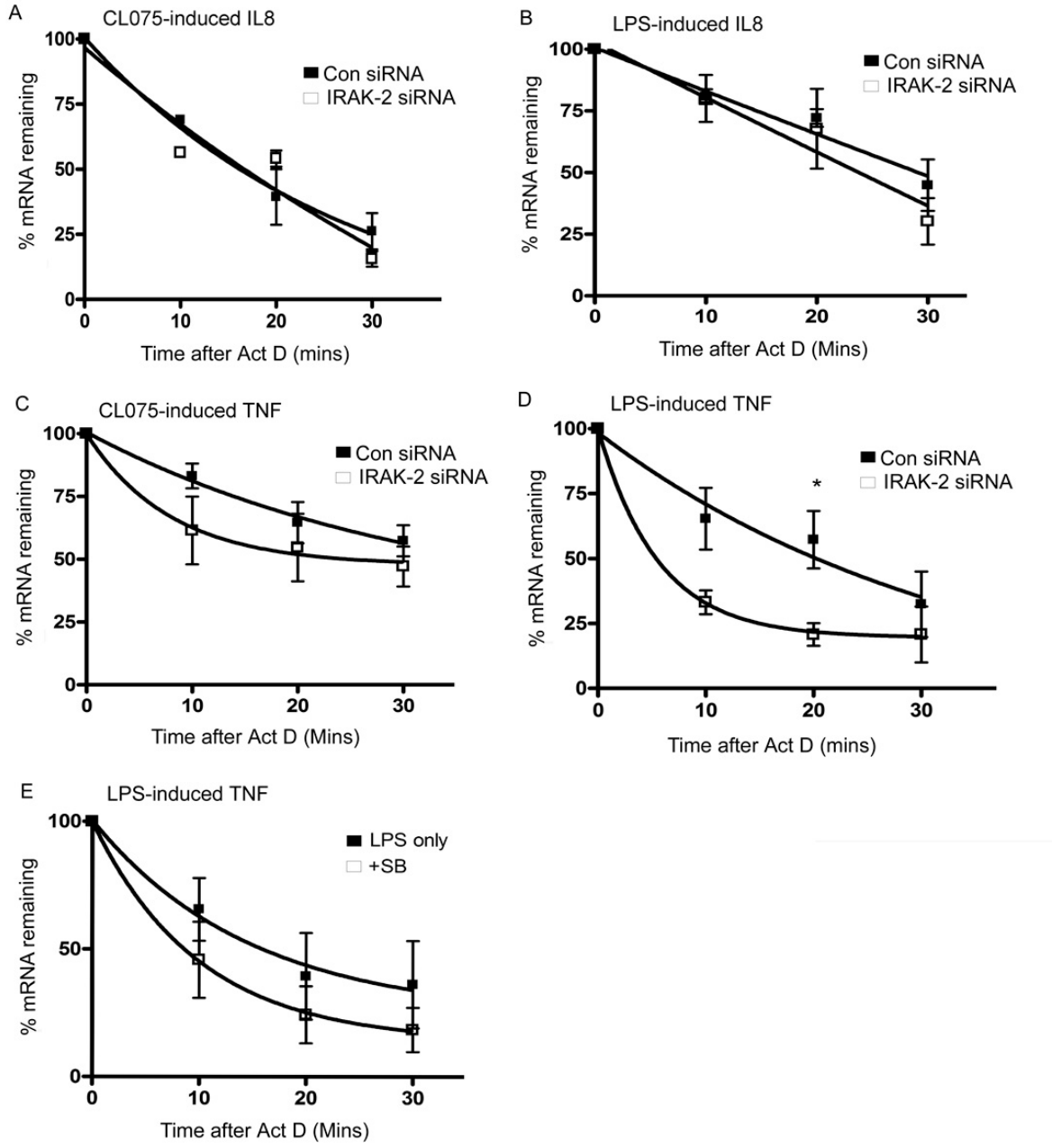
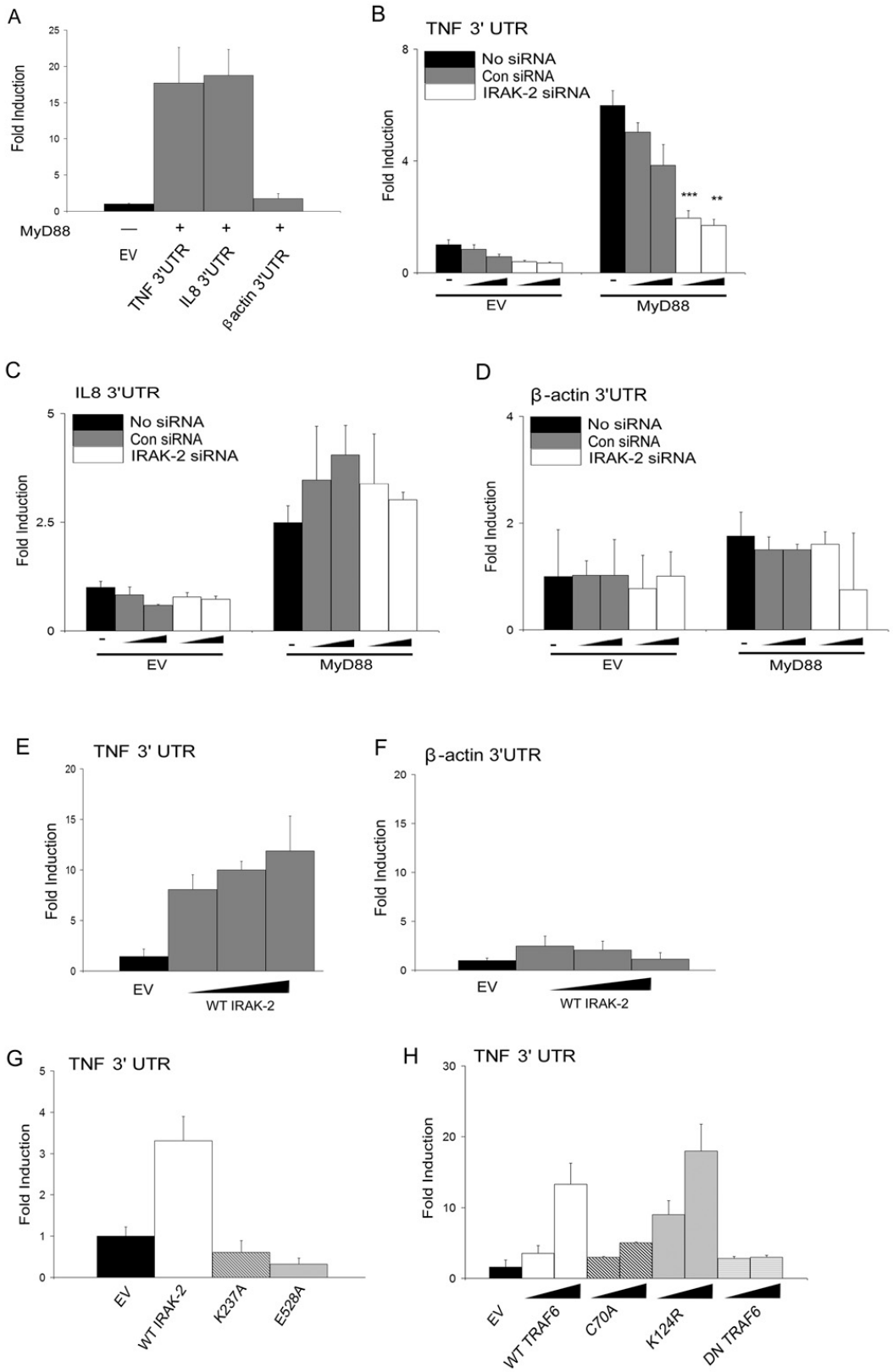

A
CL075-induced IL8
Con siRNA
IRAK-2 siRNA
% mRNA remaining
Time after Act D (mins)
B
LPS-induced IL8
Con siRNA
IRAK-2 siRNA
% mRNA remaining
Time after Act D (Mins)
C
CL075-induced TNF
Con siRNA
IRAK-2 siRNA
% mRNA remaining
Time after Act D (Mins)
D
LPS-induced TNF
Con siRNA
IRAK-2 siRNA
*
% mRNA remaining
Time after Act D (mins)
E
LPS-induced TNF
LPS only
+SB
% mRNA remaining
Time after Act D (Mins)

Figure 5


A
Fold Induction
MyD88
EV
TNF 3'UTR
IL8 3'UTR
βactin 3'UTR
B
TNF 3' UTR
No siRNA
Con siRNA
IRAK-2 siRNA
Fold Induction
***
**
EV
MyD88
C
IL8 3'UTR
No siRNA
Con siRNA
IRAK-2 siRNA
Fold Induction
EV
MyD88
D
β-actin 3'UTR
No siRNA
Con siRNA
IRAK-2 siRNA
Fold Induction
EV
MyD88
E
TNF 3' UTR
Fold Induction
EV
WT IRAK-2
F
β-actin 3'UTR
Fold Induction
EV
WT IRAK-2
G
TNF 3' UTR
Fold Induction
EV
WT IRAK-2
K237A
E528A
H
TNF 3' UTR
Fold Induction
EV
WT TRAF6
C70A
K124R
DN TRAF6