IRAK-M mediates Toll-like receptor/IL-1R-induced NF κ B activation and cytokine production

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Toll-like receptors transduce their signals through the adaptor molecule MyD88 and members of the IL-1R-associated kinase family (IRAK-1, 2, M and 4). IRAK-1 and IRAK-2, known to form Myddosomes with MyD88–IRAK-4, mediate TLR7-induced TAK1-dependent NF κ B activation. IRAK-M was previously known to function as a negative regulator that prevents the dissociation of IRAKs from MyD88, thereby inhibiting downstream signalling. However, we now found that IRAK-M was also able to interact with MyD88–IRAK-4 to form IRAK-M Myddosome to mediate TLR7-induced MEKK3-dependent second wave NF κ B activation, which is uncoupled from post-transcriptional regulation. As a result, the IRAK-M-dependent pathway only induced expression of genes that are not regulated at the post-transcriptional levels (including inhibitory molecules SOCS1, SHIP1, A20 and I κ B α), exerting an overall inhibitory effect on inflammatory response. On the other hand, through interaction with IRAK-2, IRAK-M inhibited TLR7-mediated production of cytokines and chemokines at translational levels. Taken together, IRAK-M mediates TLR7-induced MEKK3-dependent second wave NF κ B activation to produce inhibitory molecules as a negative feedback for the pathway, while exerting inhibitory effect on translational control of cytokines and chemokines.

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Introduction

Toll-like receptors (TLRs) detect microorganisms and protect multicellular organisms from infection by inducing the production of pro-inflammatory cytokines and chemokines (Medzhitov *et al*, 1997; Rock *et al*, 1998; Hemmi *et al*, 2000; Diebold *et al*, 2004; Heil *et al*, 2004; Zhang *et al*, 2004). TLRs transduce their signals through the adaptor molecule MyD88 and members of the IL-1R-associated kinase (IRAK) family, which consists of four members: IRAK-1, IRAK-2, IRAK-M and IRAK-4 (Cao *et al*, 1996; Muzio *et al*, 1997; Wesche *et al*, 1997, 1999; Li *et al*, 2002). The crystal structure of the MyD88–IRAK-4–IRAK-2 death domain (DD) complex, referred as Myddosome complex, demonstrated their sequential assembly, in which MyD88 recruits IRAK-4 and the MyD88–IRAK-4 complex recruits the IRAK-4 substrates IRAK-2 or the related IRAK-1 (Lin *et al*, 2010). Subsequently, the IRAK-1/2 form complex with TRAF6 and dissociate from the receptor complex to activate cascades of downstream kinases, leading to the activation of transcription factor NF κ B (Deng *et al*, 2000). On the other hand, IRAK-M is believed to function as a negative regulator that prevents the dissociation of IRAK-1/2 from receptor complex, thereby inhibiting downstream signalling (Kobayashi *et al*, 2002).

We previously reported the co-existence of the two parallel TLR/IL-1R-mediated NF κ B activation: TAK1 dependent and MEKK3 dependent, respectively. The TAK1-dependent pathway leads to IKK α / β phosphorylation and IKK γ activation, resulting in classical NF κ B activation through I κ B α phosphorylation and degradation (Yao *et al*, 2007). The TAK1-independent MEKK3-dependent pathway involves IKK γ phosphorylation and IKK α activation, which leads to NF κ B activation through I κ B α phosphorylation and subsequent dissociation from NF κ B but without I κ B α degradation. While TLR/IL-1R regulates gene transcription, they also induce gene expression by stabilizing otherwise unstable mRNAs of pro-inflammatory genes. Many cytokine and chemokine mRNA exhibit very short half-lives due to the presence of AU-rich sequence elements (AREs) located within their 3' untranslated regions (Anderson, 2008). Therefore, the regulation of mRNA stability is an important control of inflammatory gene expression. We have previously reported that the kinase activity of IRAK-4 is required for TAK1-dependent NF κ B activation and mRNA stabilization of cytokines and chemokines, but not for MEKK3-dependent NF κ B activation (Kim *et al*, 2007; Fraczek *et al*, 2008). Based on these findings, we propose that IRAK-4 mediates IL-1R-TLR-induced receptor-proximal signalling events through its kinase activity to coordinately regulate TAK1-dependent NF κ B activation and mRNA stabilization pathways to ensure robust production of cytokines and chemokines during inflammatory response. In addition to mRNA stabilization, TLR signalling is also necessary for efficient and sustained translation of cytokine and chemokine mRNAs

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(Fukunaga and Hunter, 1997; Ueda *et al*, 2004). The ratios of LPS-induced cytokine and chemokine mRNAs in translation-active versus translation-inactive pools were lower in IRAK-2-deficient macrophages compared with wild-type macrophages, indicating the requirement of IRAK-2 for sustained translation of these mRNAs (Wan *et al*, 2009).

In this study, we investigated the functional relationships among IRAK family members and their roles in TLR signalling by analysing mice deficient in IRAK-1, IRAK-2 and/or IRAK-M. We found that IRAK-1 and IRAK-2, substrates of IRAK-4, are required for TAK1-dependent NF κ B activation and mRNA stabilization of cytokines and chemokines, but not for MEKK3-dependent NF κ B activation. In contrast to the direct inhibitory role of IRAK-M in TLR signalling, IRAK-M was able to interact with MyD88-IRAK-4 to form IRAK-M Myddosome to mediate TAK1-independent MEKK3-dependent NF κ B activation in the absence of IRAK-1 and IRAK-2. This IRAK-M-dependent pathway is required for the second wave of TLR7-induced NF κ B activation in the presence of IRAK-1/IRAK-2, which is uncoupled from post-transcriptional regulation. Thus, the IRAK-M-dependent pathway only induced expression of genes that are not regulated at the

post-transcriptional levels (including inhibitory molecules SOCS1, SHIP1, A20 and I κ B α), exerting an overall inhibitory effect on inflammatory response. Taken together, this study for the first time demonstrates unique positive engagement of IRAK-M in transmitting signalling upon TLR activation, providing important insight into the mechanistic roles of IRAK-M in modulating TLR signalling.

Results

IRAK-M mediates TLR-induced MEKK3-dependent NF κ B activation in the absence of IRAK-1 and IRAK-2

Although IRAK-M has been implicated as a negative regulator in TLR signalling, the precise molecular mechanism for how IRAK-M participates and modulates TLR signalling remains unclear. We recently found that substantial TLR7-induced NF κ B activation was retained in IRAK-1/2-double deficient (DKO) bone marrow-derived macrophages (BMDMs), whereas completely abolished in MyD88- or IRAK-4-deficient BMDMs (Figure 1A and data not shown). These results suggest that MyD88-IRAK-4 either themselves or in conjunction with IRAK-M can mediate NF κ B activation in IRAK-1/

Figure 1 IRAK-M mediates TLR7-induced NF κ B activation in the absence of IRAK-1 and IRAK-2. (A) Nuclear extracts prepared from wild-type (WT), IRAK-1/2-double deficient (IRAK-1/2 DKO), IRAK-1/2/M-triple deficient (IRAK-1/2/M TKO), IRAK-4-deficient (IRAK-4 KO) bone marrow-derived macrophages (BMDMs) untreated or treated with TLR7 ligand R848 (1 μ g/ml) for the indicated times were analysed by electrophoresis mobility shift assay using an NF κ B-specific probe. (B) Cell lysates from wild-type (WT), IRAK-1/2-double deficient (IRAK-1/2 DKO), IRAK-1/2/M-triple deficient (IRAK-1/2/M TKO) BMDMs untreated or treated with TLR7 ligand R848 (1 μ g/ml) for the indicated times were analysed by western blot analysis with antibodies against IRAK-1, IRAK-2, p-IKK α/β , TAK1, MEKK3, p-I κ B α , I κ B α , p-JNK, p-P38, p-ERK and actin. The experiments were repeated for five times with similar results. Source data for this figure is available on the online supplementary information page.

2-DKO-BMDMs. To determine the role of IRAK-M in this process, we compared TLR7-mediated NF κ B activation in IRAK-1/2-DKO with that in IRAK-1/2/M triple deficient (TKO) BMDMs. TLR7-mediated NF κ B activation was completely abolished in IRAK-1/2/M-TKO-BMDMs, indicating the importance of IRAK-M in mediating TLR7-dependent NF κ B activation in the absence of IRAK-1 and IRAK-2 (Figure 1A).

We previously uncovered two parallel TLR-mediated MyD88/IRAK-4-dependent signalling pathways for NF κ B activation, TAK1 dependent and independent, respectively (Yao *et al*, 2007). The TAK1-dependent pathway leads to IKK α /IKK β phosphorylation and IKK β activation, resulting in classical NF κ B activation through I κ B α phosphorylation and degradation. The TAK1-independent pathway involves activation of MEKK3 and IKK α , resulting in NF κ B activation through I κ B α phosphorylation and subsequent dissociation from NF κ B but without I κ B α degradation. TLR7-induced TAK1 activation (shown as slower mobility shift bands) was greatly reduced in IRAK-1/2-DKO-BMDMs, while IRAK-1/2-double deficiency also substantially decreased TLR7-induced IKK α /IKK β phosphorylation, indicating the important role of IRAK-1/IRAK-2 in mediating TAK1-dependent NF κ B activation. TLR7-induced TAK1-mediated IKK activation leads to the phosphorylation and degradation of I κ B α . Indeed, TLR7-induced I κ B α degradation was attenuated in the IRAK-1/2-DKO-BMDMs. However, consistent with the NF κ B gel-shift assay, TLR7-induced I κ B α phosphorylation was still retained in IRAK-1/2-DKO-BMDMs, which was completely abolished in IRAK-1/2/M-TKO-BMDMs, indicating the importance of IRAK-M in mediating TLR7-induced TAK1-independent NF κ B activation in IRAK-1/2-DKO-BMDMs (Figure 1B). Importantly, through co-immunoprecipitation we found that IRAK-M formed a complex with MEKK3, but not with TAK1 (Figure 2A and B). Furthermore, TLR7-induced MEKK3 modification was still retained in IRAK-1/2-DKO-BMDMs, whereas abolished in IRAK-1/2/M-TKO-BMDMs (Figure 1A). Taken together, these results suggest that IRAK-M probably mediates TLR7-induced NF κ B activation through the TAK1-independent MEKK3-dependent pathway in the absence of IRAK-1 and IRAK-2. In addition to NF κ B activation, TLR7-mediated JNK and ERK phosphorylation was completely abolished in IRAK-1/2/M-TKO-BMDMs, although some levels of phosphorylation of p38 were still retained. Importantly, this positive signalling role of IRAK-M was not restricted to TLR7. Similarly, TLR2- and TLR9-induced phosphorylation of I κ B α was still retained in IRAK-1/2-DKO-BMDMs, which was completely abolished in IRAK-1/2/M-TKO-BMDMs, implicating the participation of IRAK-M in TLR2- and TLR9-mediated signalling. However, it is important to point out, while TLR9-induced I κ B α phosphorylation in IRAK-1/2-DKO-BMDMs was comparable to that in wild-type cells, TLR2-induced I κ B α phosphorylation in IRAK-1/2-DKO-BMDMs was substantially reduced compared to that in wild-type cells, suggesting differential utilization of IRAK-M by different TLRs (Supplementary Figure 1).

IRAK-M mediates IL-1R-induced NF κ B activation through formation of Myddosome with MyD88-IRAK-4

Lin *et al* (2010) recently reported the crystal structure of the MyD88-IRAK-4-IRAK-2 DD complex, which revealed a left-handed helical oligomer that consists of six MyD88, four

IRAK-4 and four IRAK-2 DDs (Lin *et al*, 2010). This helical signalling tower is referred as Myddosome complex and its assembly is sequential, in which MyD88 recruits IRAK-4 and the MyD88-IRAK-4 complex recruits the IRAK-4 substrates IRAK-2 or the related IRAK-1. This study has provided great insight into how IL-1R and also TLRs utilize adaptor molecule MyD88 to recruit IRAKs to mediate its signalling. The question here was how TLR/IL-1R utilizes IRAK-M to mediate signalling in the absence of IRAK-1 and IRAK-2. Since MyD88- or IRAK-4-deficient BMDMs had completely abolished TLR/IL-1R-mediated NF κ B activation, indicating that TLR/IL-1R-induced IRAK-M-mediated NF κ B activation in the IRAK-1/2-DKO-BMDMs must be MyD88-IRAK-4 dependent. In the light of the crystal structure of MyD88-IRAK-4-IRAK-2 DD Myddosome, we hypothesized that IRAK-M might also be capable of forming a complex with MyD88-IRAK-4 to mediate signalling. We indeed detected IL-1-induced interaction of IRAK-M with MyD88, IRAK-4, TRAF6 and MEKK3, but not with TAK1 when we restored IRAK-M-deficient MEFs with tagged-IRAK-M (Figure 2A). Furthermore, TLR-induced interaction of IRAK-M with MyD88, IRAK-4, TRAF6 and MEKK3 (but not with TAK1) can take place in the absence of IRAK-1 and IRAK-2 when we introduced tagged IRAK-M into the IRAK-1/2/M-TKO-BMDMs (Figure 2B). These data indicate the formation of IRAK-M Myddosome (MyD88-IRAK-4-IRAK-M), which is probably responsible for TLR-induced IRAK-M-mediated NF κ B activation in the absence of IRAK-1 and IRAK-2 through the MEKK3-dependent, but not the TAK1-dependent pathway.

The N-terminal DD of IRAK-M shares a considerable sequence identity to those of other members of the IRAK family. The similarity of topologies within this region (DD) of IRAK-M to the region of IRAK-2 suggested that this region of IRAK-M may adopt a similar protein fold. To test this prediction, we built a three-dimensional model of the region of IRAK-M DD (residues 14–105). The model has secondary structure elements similar to those of other IRAK DDs with a hexahelical bundle (Weber and Vincenz, 2001). The model places a cluster of hydrophilic residues on the surface that is reminiscent to that of IRAK-2 DD when bound to IRAK-4 DD (Lin *et al*, 2010). IRAK-4-binding residues with IRAK-2 are highly conserved in IRAK-M DD, suggesting that IRAK-M is assembled with IRAK-4 DD in a similar manner to IRAK-2 (Figure 2C). In particular, the conserved W74 (W62 in IRAK-2) in helix H4 of the IRAK-M DD is likely a key residue for interacting with IRAK-4 DD. The model also a conserved salt bridge between E71 (E59 in IRAK-2) in helix H4 of the IRAK-M and R54 in IRAK-4, suggesting a critical role of the helix H4 segment in IRAK-M for recognizing IRAK-4. Interestingly, Q78 in IRAK-M is a polar residue and distinct from the equivalent residue in IRAK-2 (M66) but the hydrophobic side chain portion of Q78 seems to make the hydrophobic interaction with IRAK-4, whereas the hydrophilic part of Q78 in IRAK-M may interact with the backbone oxygen atoms of F25, E92 and F93 in IRAK-4.

To test the proposed binding interface, we made individual and combined point mutants of IRAK-M in retroviral vector. We have previously shown that IRAK-M was able to restore IL-1-induced NF κ B activation in 293-derived IRAK-1-deficient cells (293-I1A cells), in which IRAK-M and IRAK-2 expression was not detectable (absent or very low) (Muzio *et al*, 1997). Therefore, this cell line was used to test the ability of IRAK-M

to form Myddosome in response to IL-1 stimulation. Figure 2C shows that E71A and Q78G each showed partial defect, whereas the combination of these two mutations (E71A/Q78G) drastically decreased the binding of IRAK-M

to IRAK-4. Furthermore, the W74A IRAK-M mutant completely lost the interaction with IRAK-4 (Figure 2D). We next used this cell line to examine the ability of IRAK-M mutants in mediating IL-1-dependent NF κ B activation.

IRAK-M wild-type and mutants were co-transfected with NF κ B-dependent luciferase reporter construct (E-selectin-Luc) into 293-I1A cells, followed by IL-1 treatment and luciferase assay. The W74A IRAK-M mutant completely lost the ability to activate NF κ B in 293-I1A cells upon IL-1 stimulation (Figure 2E). On the other hand, while E71A and Q78G exhibited partial defect, the combination of these two mutations (E71A/Q78G) greatly decreased the IL-1-induced NF κ B-dependent luciferase activity in 293-I1A cells (Figure 2E). The same results were obtained from the analysis of the IRAK-M mutants in NF κ B electrophoretic mobility shift assay (Figure 2F). Consistent with its ability in mediating IL-1-induced NF κ B activation, IRAK-M was also able to restore IL-1-induced IL-8 and TNF α gene expression in 293-I1A cells (Figure 2G). Importantly, W74A and E71A/Q78G IRAK-M mutants failed to restore the IL-8 and TNF α gene expression in 293-I1A cells in response to IL-1 stimulation (Figure 2G). Moreover, since we predict the hydrophilic part of Q78 in IRAK-M may interact with the backbone oxygen atoms of F25 in IRAK-4, we generated the IRAK-4 F25D mutant. When tested in IRAK-4-deficient cells, this F25D mutant showed decreased interaction with IRAK-M compared with wild-type IRAK-4 upon IL-1 stimulation (Supplementary Figure 2), confirming the critical contact between Q78 in IRAK-M and F25 in IRAK-4.

We then confirmed the binding interface of IRAK-M with IRAK-4 in response to TLR7 stimulation. IRAK-M wild-type

and mutants were expressed in IRAK-1/2/M-TKO-BMDMs by adenovirus infection. Consistent with the results for the IL-1 response, wild-type IRAK-M was able to interact with IRAK-4 and restored TLR7-induced NF κ B activation in IRAK-1/2/M-TKO-BMDMs, whereas W74A mutant lost its ability to interact with IRAK-4 and failed to mediate NF κ B activation. While E71 and Q78G showed partial reduction, the combination mutant E71A/Q78G was unable to interact with IRAK-4 with abolished TLR7-induced NF κ B activation (Figure 2H and I). Furthermore, W74A and E71A/Q78G mutants also failed to restore CXCL1, TNF α and IL-6 gene expression in IRAK-1/2/M-TKO-BMDMs upon TLR7 stimulation (Figure 2J). Taken together, these results provide strong supporting evidence for the interaction between DDs of IRAK-M and IRAK-4 and demonstrate that this interaction is critical for IRAK-M to mediate IL-1R/TLR-induced NF κ B activation.

IRAK-M deficiency resulted in decreased second wave TLR7-induced NF κ B activation in the presence of IRAK-1 and IRAK-2

Previous studies suggested that IRAK-M functions as a negative regulator that prevents the dissociation of IRAKs from MyD88, thereby inhibits all downstream signalling events including NF κ B activation (Kobayashi *et al*, 2002). Considering how IRAK-M can form Myddosome and is capable of mediating TLR/IL-1R signalling in the absence of IRAK-1/IRAK-2, we decided to re-investigate the impact

Figure 2 IRAK-M forms Myddosome with MyD88–IRAK-4 to mediate signalling through MEKK3 and TRAF6. (A) IRAK-M-deficient mouse embryonic fibroblast (MEF) infected with retroviruses containing empty vector construct (Vector) and FLAG-IRAK-M were treated with IL-1 β (1 ng/ml) for the indicated times, followed by immunoprecipitation (IP) with anti-FLAG antibody and analysed by western blot analyses using antibodies against MyD88, IRAK-4, TRAF6, MEKK3, TAK1 and FLAG. The whole cell lysates (WCLs) were subjected to the same western analyses, including actin as a control. The experiments were repeated for five times with similar results. (B) IRAK-1/2/M-triple deficient (IRAK-1/2/M TKO) BMDMs infected with adenovirus containing empty vector construct (Vector) and HA-tagged IRAK-M (HA-IRAK-M) were treated with R848 (1 μ g/ml) for the indicated times, followed by IP with anti-HA antibody and analysed by western blot analysis with antibody against MyD88, IRAK-4, TRAF6, MEKK3, TAK1 and FLAG. The WCLs were subjected to the same western analyses, including actin as a control. The experiments were repeated for five times with similar results. (C) Conserved binding interface in the interaction of IRAK-M with IRAK-4 (left). A three-dimensional model of the MyD88–IRAK-4–IRAK-M Myddosome complex (middle). A putative binding interface in the death domain (DD) between IRAK-M (blue) and IRAK-4 (yellow), as guided by molecular modelling study. The helix H4 of IRAK-M DD seems to be a critical element for the interaction with IRAK-4 DD. Putative binding residues in the helix H4 in the IRAK-M in the interaction with IRAK-4 are highlighted. Specifically, the surface-exposed E71 on the helix H4 of IRAK-M makes a salt bridge with R54 from one IRAK-4 DD molecule, and this salt bridge formation is conserved in the IRAK-2 DD/IRAK-4 DD interface. The E71-mediated IRAK-M DD/IRAK-4 DD interaction is further stabilized by a hydrophilic interaction with Q29 from another neighbouring IRAK-4 DD molecule upon the Myddosome formation. W74 of IRAK-M is situated in the centre of the helix H4 and packs against a hydrophobic environment that is created by two IRAK-4 DD monomers upon Myddosome formation. Q78 in IRAK-M may play an important role both in the hydrophilic and hydrophobic interactions with IRAK-4 (see text). The significance of these side chains of IRAK-M in the interaction with IRAK-4 was verified by our site-directed mutagenesis study (right). The interaction interface in the DD between IRAK-2 (magenta) and IRAK-4 (yellow), as evidenced by crystallographic study. (D) IRAK-1-deficient 293-IL1R (I1A) cells transfected with empty vector, FLAG-tagged wild-type IRAK-M (WT), FLAG-tagged IRAK-M mutants (F18A, E71A, Q78A, W74A and E71A/Q78G) were treated with IL-1 β (1 ng/ml) for the indicated times, followed by IP with anti-IRAK-4 antibody and analysed by western blot analysis with antibody against FLAG to detect IRAK-M. The experiments were repeated for five times with similar results. (E) IRAK-M wild-type and mutants (F18A, E71A, Q78A, W74A and E71A/Q78G) were transiently co-transfected with NF κ B-dependent luciferase reporter construct (E-selectin-Luc) into IRAK-1-deficient 293-IL1R (293-I1A) cells, followed by IL-1 β (1 ng/ml) treatment for 6 h and luciferase assay. The experiment was repeated three times. Data represent mean $\pm$ s.e.m.; * P < 0.05, versus WT treated group (two tailed t -test). (F) Nuclear extracts prepared from 293-I1A cells transiently co-transfected with wild-type (WT), W74A and E71A/Q78A untreated or treated with IL-1 β (1 ng/ml) for the indicated times were analysed by electrophoretic mobility shift assay using an NF κ B-specific probe. The experiments were repeated for five times with similar results. (G) IRAK-1-deficient 293-IL1R (I1A) cells transiently transfected with empty expression vector, wild-type IRAK-M and IRAK-M mutants (W74A and E71A/Q78G) were treated with IL-1 β (1 ng/ml) for the indicated times. Total RNAs from these cells were subjected to real-time PCR analysis for the levels of human IL-8 and TNF α mRNAs. The experiments were repeated three times. Data represent mean $\pm$ s.e.m.; * P < 0.05 (two tailed t -test). (H) IRAK-1/2/M-triple deficient (IRAK-1/2/M TKO) BMDMs infected with adenovirus expressing HA-tagged wild-type IRAK-M (WT) and HA-tagged IRAK-M mutants (F18A, E71A, Q78A, W74A and E71A/Q78G) were treated with R848 (1 μ g/ml) for the indicated times, followed by IP with anti-IRAK-4 antibody and analysed by western blot analysis with antibody against HA to detect IRAK-M. The experiments were repeated for five times with similar results. (I) IRAK-1/2/M-triple deficient (IRAK-1/2/M TKO) BMDMs infected with adenovirus expressing HA-tagged wild-type IRAK-M (WT) and HA-tagged IRAK-M mutants (F18A, E71A, Q78A, W74A and E71A/Q78G) were treated with R848 (1 μ g/ml) for the indicated times, followed by western blot analyses with antibodies against p-p65 p-I κ B α , I κ B α and actin. The experiments were repeated for five times with similar results. (J) IRAK-1/2/M-triple deficient (IRAK-1/2/M TKO) BMDMs infected with adenovirus expressing HA-tagged wild-type IRAK-M (WT) and HA-tagged IRAK-M mutants (F18A, E71A, Q78A, W74A and E71A/Q78G) were treated with R848 (1 μ g/ml) for the indicated times. Total RNAs from these cells were subjected to real-time PCR analysis for the levels of mouse CXCL1, TNF α and IL-6 mRNAs. The experiment was repeated three times. Data represent mean $\pm$ s.e.m.; * P < 0.05 (two tailed t -test). Source data for this figure is available on the online supplementary information page.

of IRAK-M single deficiency on TLR7-mediated signalling. To our surprise, IRAK-M deficiency decreased TLR7-induced late phase NF κ B activation (after 0.5 h) in a gel-shift assay (Figure 3A). The TAK1-dependent downstream signalling events (phosphorylation of TAK1 and IKK α / β and I κ B α degradation) were similar or slightly enhanced in IRAK-

MKO-BMDMs compared to that in control cells. However, whereas TLR7-induced I κ B α and p65 phosphorylation was comparable at early times (within 10 min), they were substantially reduced at later times (after 30 min) (Figure 3B). These results suggest that IRAK-M Myddosome contributes to the second wave of TLR7-induced NF κ B activation in the pre-

sence of IRAK-1 and IRAK-2, probably through the TAK1-independent pathway.

Our previous studies showed that MG132, which inhibits proteasome-dependent protein degradation, can block TLR-mediated TAK1-dependent pathway (phosphorylation of TAK1 and IKK α / β and I κ B α degradation), since IRAK-1 ubiquitination and degradation is critical for TAK1-dependent downstream signalling (Xiao *et al*, 2008; Figure 3C). On the other hand, MG132 does not block the TLR-induced MEKK3-dependent pathway, as evident by intact TLR-induced phosphorylation of MEKK3 and I κ B α ; and substantial residual JNK, p38 and ERK activation after MG132 treatment. Therefore, to confirm the role of IRAK-M in TLR7-induced TAK1-independent MEKK3-dependent signalling, we examined the impact of MG132 on TLR7-induced signalling in IRAK-MKO BMDMs. We indeed found that pretreatment of MG132 greatly reduced TLR7-induced phosphorylation of MEKK3, I κ B α , JNK, p38 and ERK in IRAK-M-deficient BMDMs compared to its impact on wild-type control cells. These results showed that IRAK-M-mediated signalling becomes the dominant pathway when the TAK1 pathway is blocked by MG132, supporting that IRAK-M Myddosome might mediate the second wave of TLR7-induced NF κ B activation through the TAK1-independent MEKK3-dependent pathway (Figure 3D). To confirm the role of MEKK3 in IRAK-M-mediated NF κ B activation, IRAK-M was co-transfected with NF κ B-dependent luciferase reporter construct (E-selectin-Luc) into scramble shRNA-transfected or MEKK3 knockdown 293-I1A cells, followed by IL-1 treatment and luciferase assay. IL-1-induced IRAK-M-mediated NF κ B activation was greatly reduced in MEKK3 knockdown cells compared to that in the control cells (Figure 3E and F). Taken together, these data support that IRAK-M Myddosome-mediated NF κ B activation is through the MEKK3-dependent pathway.

IRAK-M is required for TLR7-induced expression of inhibitory molecules SHIP1, SOCS1, A20 and I κ B α

One important question is then whether TLR-induced IRAK-M-mediated signalling has any impact on gene expression. IRAK-1/IRAK-2-double deficiency results in substantial impairment of TLR-mediated production of pro-inflammatory cytokines and chemokines (Kawagoe *et al*, 2008). It is important to note that the TLR-induced IRAK-M-mediated signalling events in IRAK-1/2-DKO-BMDMs allowed the induction of cytokines and chemokines mRNAs at early times (peak at 30 min), such as TNF α and CXCL1 (KC), which was completely abolished in IRAK-1/2-M-TKO-BMDMs (Figure 4A). However, since IRAK-2 is required for

post-transcriptional control of the TNF α and CXCL1 (KC) mRNAs, we failed to detect TNF α and CXCL1 (KC) mRNAs at later times (after 1 h) and thus little protein production of TNF α and CXCL1 (KC) in IRAK-1/2-DKO-BMDMs (Figure 4B). Therefore, the IRAK-1/IRAK-2-mediated coupling of the TAK1-dependent NF κ B activation and post-transcriptional regulation plays an essential role in the production of these pro-inflammatory cytokines and chemokines. Although TLR7-induced IRAK-M-mediated signalling in IRAK-1/2-DKO-BMDMs can mediate the induction of cytokines and chemokines mRNAs at early times, this IRAK-M-mediated pathway alone in the absence of IRAK-1 and IRAK-2 is insufficient to induce the production of cytokines and chemokines that are under the post-transcriptional control. Similar results were obtained for TLR2 and TLR9 signalling pathways (Supplementary Figure 3).

We have previously also identified a group of TLR-induced genes that are not regulated at post-transcriptional levels (Fraczek *et al*, 2008). The TLR7-induced IRAK-M-mediated signalling events in IRAK-1/2-DKO-BMDMs allowed sustained induction of these genes, which was completely abolished in IRAK-1/2-M-TKO-BMDMs (Figure 5A). Furthermore, the expression of these genes was also substantially reduced in IRAK-M-KO-BMDMs compared to that in wild-type cells (Figure 5B). Several of the genes are important for the activation and homeostasis of the macrophages (including ETS2, SOD2, Bcl-2A1D and GPR84) (Figure 5A and B). Another group of IRAK-M-dependent genes is inhibitory molecules, including A20, SOCS1, SHIP1 and I κ B α (Figure 5A and B; Supplementary Figure 4). The gene expression data were consistent with the role of IRAK-M in TLR7-induced second wave NF κ B activation, since most of the IRAK-M-dependent genes were late genes (induced after 1 h). Although A20 was readily induced at 30 min, its expression peaked at 4 h of treatment. Taken together, these results suggest that at least one potential importance of TLR7-induced IRAK-M-mediated signalling is to induce the expression of several inhibitory molecules (SHIP1, SOCS1, A20 and I κ B α), which might serve as a negative feedback control, implicating a novel inhibitory mechanism by which IRAK-M modulates TLR signalling.

IRAK-M inhibits translational control of pro-inflammatory cytokines via its interaction with IRAK-2

Although it is clear that IRAK-M contributes to TLR7-induced gene transcription through the activation of second wave of NF κ B activation, the production of some pro-inflammatory cytokines and chemokines (including CXCL1, TNF α and IL-6)

Figure 3 The role of IRAK-M in second wave of TLR-mediated NF κ B activation. (A) Nuclear extracts prepared from wild-type (WT), and IRAK-M-deficient (MKO) BMDMs untreated or treated with R848 (1 μ g/ml) for the indicated times were analysed by electrophoretic mobility shift assay using an NF κ B-specific probe. The experiments were repeated for five times with similar results. (B) Cell lysates from WT, and IRAK-M-deficient (MKO) BMDMs untreated or treated with R848 (1 μ g/ml) for the indicated times were analysed by western blot analyses with antibodies against TAK1, MEKK3, p-IKK α / β , p-p65, p-I κ B α , I κ B α , p-JNK, p-ERK, p-p38 and actin. The experiments were repeated for five times with similar results. (C) WT BMDMs pre-treated with MG132 (20 μ M) or DMSO for 2 h, were treated with R848 (1 μ g/ml) for the indicated time, followed by western blot analyses with antibodies against TAK1, MEKK3, p-IKK α / β , p-I κ B α , I κ B α , p-JNK, p-ERK, p-p38 and actin. The experiments were repeated for five times with similar results. (D) BMDMs from WT and IRAK-M-deficient (MKO) mice were pre-treated with MG132 (20 μ M) for 2 h followed by R848 (1 μ g/ml) treatment for the indicated time. Western blot analysis was performed by antibodies against TAK1, MEKK3, p-IKK α / β , p-I κ B α , I κ B α , p-JNK, p-ERK, p-p38 and actin. The experiments were repeated for five times with similar results. (E) MEKK3 knockdown (MEKK3 KD) cells and control (Scrambled) cells were transiently co-transfected with IRAK-M and NF κ B-dependent luciferase reporter construct (E-selectin-Luc) followed by IL-1 (1 ng/ml) treatment for 6 h and luciferase assay. The experiments were repeated three times. Data represent mean $\pm$ s.e.m.; * P < 0.05 (two tailed t -test). (F) FLAG-tagged IRAK-M-transfected MEKK3 knockdown (MEKK3 KD) cells and control (Scrambled) cells were treated with IL-1 (1 ng/ml) for indicated time, followed by western blot analyses with antibodies against MEKK3, FLAG, p-I κ B α , I κ B α and actin. The experiments were repeated for five times with similar results. Source data for this figure is available on the online supplementary information page.

Figure 4 IRAK-1 and IRAK-2 are required for TLR7-induced pro-inflammatory gene expression. (A) Total mRNAs from BMDMs of wild-type (WT), IRAK-1/2-double deficient (DKO) and IRAK-1/2/M-triple deficient (TKO) mice treated with R848 (1 μ g/ml) for the indicated times, were subjected to RT-PCR analyses for the levels of CXCL1, IL-6 and TNF α expression. (B) BMDMs from WT, IRAK-1/2-double deficient (DKO) and IRAK-1/2/M-triple deficient (TKO) mice were treated with R848 (1 μ g/ml) for the indicated time. CXCL1, IL-6 and TNF α concentrations in the supernatant were measured by ELISA. The experiments were repeated three times. Data represent mean $\pm$ s.e.m.; ** P < 0.01; * P < 0.05 (two tailed t -test).

was actually enhanced in the IRAK-MKO-BMDMs compared to that in the control cells (Kobayashi *et al*, 2002; Figure 6B). Since the mRNAs of these pro-inflammatory genes were induced at similar or reduced levels in IRAK-MKO-BMDMs as compared to wild-type cells (Figure 6A), we investigated the possible role of IRAK-M in regulation of cytokine and chemokine translation. BMDMs from IRAK-M-deficient mice and wild-type mice were treated with TLR7 ligand R848 for 1.5 h and a sucrose gradient was used to separate the mRNA translation-inactive pool (free ribosome) and translation-active pool (poly-ribosomes) (Figure 7A). Compared with wild-type BMDMs, IRAK-M KO BMDMs had more cytokine and chemokine mRNAs (IL-6, KC and TNF α) in the translation-active pool. IRAK-M deficiency thus resulted in the increased ratios of TLR-induced cytokine and chemokine mRNAs in translation-active pool versus translation-inactive pool (Figure 7B; Supplementary Figure 5). On the other hand, IRAK-M deficiency had no impact on the translation of mRNAs that are not regulated at the post-transcriptional levels, including the inhibitory molecules (ETS2, SOD2, Bcl-2A1D, GPR84, SHIP1, SOCS1, A20 and I κ B α) (Figure 7C and D; Supplementary Figure 5).

We previously reported that IRAK-2 plays an important role in the regulation of TLR-mediated protein translation of cytokines and chemokines, including IL-6, CXCL1 (KC) and TNF α . Through co-immunoprecipitation experiment, we found that IRAK-M specifically bound to IRAK-2, not to IRAK-1 (Figure 7E). Furthermore, we have previously reported that IRAK-2 is required for TLR-induced phosphorylation of MNK1, MK2 and eIF4e, which are involved in protein translational control. Interestingly, IRAK-M deficiency resulted in increased TLR7-induced phosphorylation of MNK1, MK2 and eIF4e (Figure 7F), supporting the role of

IRAK-M in inhibition of TLR7-induced pro-inflammatory cytokine and chemokine translation through its interaction with IRAK-2.

Discussion

Although previous studies suggested that IRAK-M prevents the dissociation of IRAKs from MyD88, suppressing all downstream signalling, there have not been sufficient experimental evidences to fully support this hypothesis. In this manuscript, we provided two novel mechanisms for the regulatory role of IRAK-M in TLR signalling (Figure 8). First, IRAK-M is able to function as an intermediate signalling component to transmit signalling upon TLR activation. IRAK-M interacts with MyD88-IRAK-4 to form IRAK-M Myddosome to mediate TLR7-induced MEKK3-dependent second wave NF κ B activation. However, this IRAK-M-dependent pathway only induces expression of genes that are not regulated at the post-transcriptional levels (including inhibitory molecules SOCS-1, SHIP-1, A20 and I κ B α), exerting an overall inhibitory effect on inflammatory response. The second novel mechanism is that IRAK-M specifically interacts with IRAK-2, but not with IRAK-1, and suppresses TLR7-induced IRAK-2-mediated translation of cytokines and chemokines. These findings present a new outlook for how IRAK-M modulates TLR signalling and TLR-mediated inflammatory responses.

The role of IRAK-M in MEKK3-dependent NF κ B activation was best demonstrated in IRAK-1/2-DKO-BMDMs versus IRAK-1/2/M-TKO-BMDMs. While IRAK-1/2-double deficiency abolished TAK1-, but not MEKK3-dependent pathway, TLR7-induced NF κ B activation was no longer detectable in IRAK-1/2/M-TKO-BMDMs. It is intriguing that IRAK-M single deficiency resulted in loss of TLR7-induced late NF κ B

Figure 5 IRAK-M is required for TLR7-induced expression of inhibitory molecules SHIP-1, SOCS1, A20 and I κ B α . (A) Total mRNAs from BMDMs of wild-type (WT), IRAK-1/2-double deficient (DKO) and IRAK-1/2/M-triple deficient (TKO) mice treated with R848 (1 μ g/ml) for the indicated times were subjected to RT-PCR analyses for the levels of ETS2, SOD2, Bcl-2A1D, GPR84, SHIP-1, SOCS1, A20 and I κ B α expression. (B) Total mRNAs from BMDMs of WT and IRAK-M-deficient (MKO) mice treated with R848 (1 μ g/ml) for the indicated times were subjected to RT-PCR analyses for the levels of ETS2, SOD2, Bcl-2A1D, GPR84, SHIP-1, SOCS1, A20 and I κ B α expression. The experiments were repeated three times. Data represent mean $\pm$ s.e.m.; ** P < 0.01; * P < 0.05 (two tailed t -test).

activation (after 30 min), implicating the role of the IRAK-M-MEKK3 pathway in second wave of NF κ B activation in the presence of IRAK-1/2. The importance of IRAK-M in NF κ B activation was further demonstrated in the MG132 treatment experiment, in which IRAK-M deficiency greatly diminished TLR7-induced NF κ B activation, since MG132 treatment was shown to block the TAK1-dependent pathway, mimicking IRAK-1/2 deficiency (Xiao *et al*, 2008). These mouse genetic experiments on the novel role of IRAK-M were nicely supported by biochemical studies. IRAK-M-mediated NF κ B activation was almost abolished in MEKK3 knockdown cells and IRAK-M specifically interacted with MEKK3, but not TAK1, providing direct biochemical evidence for the role of IRAK-M in MEKK3-, but not in TAK1-dependent pathway. IRAK-1 and IRAK-2 are known to form Myddosomes with MyD88-IRAK-4 to mediate TLR7-induced TAK1-dependent NF κ B activation. Since IRAK-M was able to mediate NF κ B activation in the absence of IRAK-1 and IRAK-2, and interacted with MyD88 and IRAK-4, we proposed the

formation of IRAK-M Myddosome. Through protein modelling, we predicted the critical residues for interaction interface between DDs of IRAK-M and IRAK-4. Mutations in the conserved W74, E71 and Q78 in helix H4 of the IRAK-M DD greatly reduced the interaction of IRAK-M with IRAK-4 and also diminished the ability of IRAK-M to mediate NF κ B activation. These results strongly suggest that IRAK-M probably indeed has the ability to directly interact with MyD88-IRAK-4, like IRAK-1/2, acting as an intermediate signalling component to transmit signal from the receptor to downstream signalling cascade (interaction with TRAF6-MEKK3) to mediate NF κ B activation.

One important question is what is the biological significance of the IRAK-1/2-mediated TAK1- versus IRAK-M-mediated MEKK3-dependent pathway? We have previously shown that the kinase activity of IRAK-4 is required TAK1-, but not MEKK3-dependent NF κ B activation (Kim *et al*, 2007; Fraczek *et al*, 2008). Furthermore, IRAK-4 coordinately regulates TAK1-dependent NF κ B activation and

Figure 6 IRAK-M inhibits TLR7-induced pro-inflammatory gene expression. (A) Total mRNAs from BMDMs of wild-type (WT) and IRAK-M-deficient (MKO) mice treated with R848 (1 μ g/ml) for the indicated times were subjected to RT-PCR analyses for the levels of CXCL1, IL-6 and TNF α expression. (B) BMDMs from WT and IRAK-M-deficient (MKO) mice were treated with R848 (1 μ g/ml) for the indicated time. CXCL1, IL-6 and TNF α concentrations in the supernatant were measured by ELISA. The experiments were repeated three times. Data represent mean $\pm$ s.e.m.; ** P < 0.01; * P < 0.05 (two tailed t -test).

mRNA stabilization pathways to ensure robust production of cytokines and chemokines during inflammatory response (Lye *et al*, 2004). In addition to mRNA stabilization, TLR signalling is also necessary for efficient and sustained translation of cytokine and chemokine mRNAs. We have previously reported that IRAK-2 is essential for sustained translation of TLR-induced cytokine and chemokine mRNAs (Wan *et al*, 2009). Therefore, it is logical to find that IRAK-1/2 (substrates of IRAK-4) are required for TAK1- (not MEKK3-) dependent NF κ B activation, which is coupled with post-transcriptional control to promote the production of pro-inflammatory cytokines and chemokines. On the other hand, the IRAK-M-induced MEKK3-dependent pathway is uncoupled from post-transcriptional regulation. Therefore, the IRAK-M-dependent pathway only induced expression of IRAK-4-kinase-independent genes that are not regulated at the post-transcriptional levels, including inhibitory molecules SOCS1, SHIP1, A20 and I κ B α . The important concept is that IRAK-M is wired to engage an alternative NF κ B activation pathway to produce inhibitory molecules, which can in turn exert an overall inhibitory effect on inflammatory response. The advantage for such inhibitory mechanism could be that through the production of these inhibitory molecules, IRAK-M could exert broader inhibitory effects not just on TLR-IL-1R but also on other cytokine signalling cascades, so that the inflammatory response can be effectively controlled.

It is important to point out that IRAK-M also has the ability to directly impact on TLR-induced production of cytokines and chemokines. Although IRAK-M deficiency did not have much impact on TLR-induced mRNA levels of cytokines and chemokines, their protein production was enhanced in IRAK-M-KO-BMDMs as early as 1 h after stimulation. Through

polysomal fractionation, we found that the mRNAs of cytokines and chemokines were more actively translated in the absence of IRAK-M, indicating the critical role of IRAK-M in translational control. Consistent with the previous finding about the essential role of IRAK-2 in TLR-mediated translational control, we found IRAK-M specially interacts with IRAK-2, but not with IRAK-1. These results suggest that in addition to the formation of IRAK-M Myddosome, IRAK-M probably has the ability to dock onto IRAK-2 Myddosome, thereby interfering IRAK-2-dependent downstream signalling.

While this study has provided important insight into the mechanistic roles of IRAK-M in TLR signalling, many questions still remain regarding the functional relationship of the IRAK family members. Future studies are needed to determine how IRAK-M specifically interacts with IRAK-2, but not with IRAK-1 and what determines the distribution and dynamics of IRAK-M in IRAK-M Myddosome versus IRAK-2 Myddosome. It will be important to determine how the newly found functions of IRAK-M will impact on the interpretation of the role of IRAK-M in inflammatory responses *in vivo* and the drug development of inhibitors for different IRAKs.

Materials and methods

Biological reagents and cell culture

Recombinant human IL-1 was purchased from R&D system. CpG B oligodeoxynucleotide (ODN) and Pam3CSK4 were purchased from Invivogen. LPS (*Escherichia coli* 055:B5) was purchased from Sigma-Aldrich and R848 was obtained from GLS synthesis company. Antibodies against phosphorylated I κ B α (Ser32/S36), JNK, IKK α / β (Ser176/180), Mnk1 (Thr179/202), MK2 (Thr334), eIF4E (Thr70), MKK3 (Ser189)/MKK6 (Ser207), total I κ B α and SOCS1 were purchased from Cell Signaling. Antibody to IRAK-2 was purchased from Abcam. Antibody to IRAK-4 was purchased from Enzo. Antibody to FLAG (anti-FLAG) and haemagglutinin (anti-HA) were purchased

Figure 7 IRAK-M inhibits the translation of TLR7-induced pro-inflammatory genes through its interaction with IRAK-2. (A) Translation-active and -inactive mRNAs from R848-treated BMDMs were isolated by sucrose gradient fraction. (B–D) BMDMs from wild-type (WT) and IRAK-M-deficient (MKO) mice were treated with R848 (1 μ g/ml) for 90 min. (B) IL-6, KC and TNF α ; (C) ETS2, SOD2, Bcl2A1D and GPR84; (D) A20, SOCS1, SHIP1 and I κ B α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs from unfractionated cell lysates, translation-active pools, and translation-inactive pools were analysed by quantitative RT-PCR and normalized to β -actin (see Supplementary Figure 4). The ratios of mRNAs from R848-treated translation-active and -inactive pools are shown in (B–D). The experiments were repeated three times. Data represent mean $\pm$ s.e.m.; **P < 0.01; *P < 0.05 (two tailed *t*-test). (E) IRAK-M-deficient mouse embryonic fibroblast (MEF) infected with retroviruses containing empty vector construct (Vector) and FLAG-IRAK-M were treated with IL-1 β (1 ng/ml) for the indicated times, followed by immunoprecipitation (IP) with anti-FLAG antibody and analysed by western blot analyses using antibodies against IRAK-1, IRAK-2 and FLAG. WCLs, whole cell lysates. The experiments were repeated for five times with similar results. (F) Cell lysates from WT, and IRAK-M-deficient (MKO) BMDMs untreated or treated with R848 (1 μ g/ml) for the indicated times were analysed by western blot analyses with antibodies against p-MKK3/6, p-MNK1, p-MK2, p-eIF4E and actin. The levels of the phosphorylated proteins were analysed by ImageJ 1.43 μ and normalized to actin. *Indicates non-specific band. The experiments were repeated for five times with similar results. Source data for this figure is available on the online supplementary information page.

from Sigma. Antibody to MEKK3 was purchased from BD Biosciences Pharmingen. Antibodies against IRAK, TAK1, SHIP1 and Actin were from Santa Cruz Biotechnologies. MG132 was purchased from Calbiochem. 293-derived IRAK-1-deficient cells (293-I1A cells) and mouse embryonic fibroblast (MEF) were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS), penicillin G (100 μ g/ml), and streptomycin (100 μ g/ml). Bone marrow-derived macrophages were obtained from the bone marrow of tibia and femur by flushing with DMEM. The cells were cultured in DMEM supplemented with 20% FBS, and 30% L929 supernatant for 5 days.

Knockdown of MEKK3

Oligonucleotides encoding either scrambled or MEKK3-specific small hairpin RNAs were cloned into pSUPER to generate

pSUPER-scrambled or pSUPER-MEKK3 respectively. In all, 1 μ g of pSUPER-scrambled or pSUPER-MEKK3 was transfected into 293-I1A cells along with 0.1 μ g of pBabe-puromycin by FuGENE 6 (Roche Applied Science). Two days after transfection, puromycin (1 μ g/ml) containing DMEM was added to the cells to select for puromycin-resistant clones. After 10 days of puromycin selection, single clones were picked and subjected to western analysis to determine the levels of MEKK3. Clones with over 85% knockdown of MEKK3 were pooled as MEKK3 knockdown cells and used for experiments.

Transfection and luciferase assay

Transfection of the 293-I1A cells was performed using the FuGENE 6 transfection reagent as recommended by the manufacturer (Roche Diagnostics). After 24 h, the cells were stimulated with IL-1 β or left untreated for 6 h before harvest. Luciferase activity was determined

Figure 8 Model for the regulatory role of IRAK-M in TLR-IL-1R signalling. IRAK-M is able to function as an intermediate signalling component to transmit signalling upon TLR-IL-1R activation. IRAK-M interacts with MyD88-IRAK-4 to form IRAK-M Myddosome to mediate TLR-IL-1R-induced MEKK3-dependent second wave NF κ B activation. However, this IRAK-M-dependent pathway only induces expression of genes that are not regulated at the post-transcriptional levels (including inhibitory molecules SOCS-1, SHIP-1, A20 and I κ B α), exerting an overall inhibitory effect on inflammatory response. On the other hand, IRAK-M specifically interacts with IRAK-2, but not with IRAK-1, and suppresses TLR-IL-1R-induced IRAK-2-mediated translation of cytokines and chemokines.

by using the luciferase assay system and chemiluminescent reagents from Promega.

Plasmids and retroviruses

Mouse IRAK-M and IRAK-4 cDNA were purchased from Open Biosystems. The IRAK-M mutants and IRAK-4 mutant were generated by site-directed mutagenesis PCR. The wild type and mutants of IRAK-M and IRAK-4 were cloned into pMX retroviral expression vector and transfected into phoenix cell for viral packaging. Cells were infected by the packaged retrovirus for 3 days and selected by puromycin (2 μ g/ml) for 2 days for stable viral integration. For all PCRs high fidelity Pfu Turbo polymerase was used (Stratagene).

Adenovirus infection

IRAK-M wild type and mutants were cloned into pENTR/D-TOPO Vector (Invitrogen). The pENTR clones were packaged into an adenovirus destination vector pAd/CMV/V5-DEST using Gateway Vector Kit according to manufacturer's guidelines (Invitrogen). The adenoviral stocks were prepared using ViraPower Adenoviral Expression System according to manufacturer's guidelines (Invitrogen). The BMDMs were infected at

multiplicity of infection (MOI) of 200. After 16 h of infection, the cells were changed to fresh L929 conditioned medium. Forty-eight hours after infection, the cells were used for experiments.

Immunoblotting

Cell were harvested and lysed in a Triton-containing lysis buffer (0.5% Triton X-100, 20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β -glycerophosphate, 1.5 mM MgCl₂, 10 mM NaF, 2 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail from Roche). Cell lysates were then separated by 10% SDS-PAGE, transferred onto Immobilon-P membranes (Millipore), and subjected to immunoblotting.

Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). In all, 3 μ g of total RNA was then used for reverse transcription reaction using SuperScript-reverse transcriptase (Invitrogen). Q-PCR was performed in AB 7300 RealTime PCR System, and the gene expression of human IL-8, TNF α , actin and mouse CXCL1 (KC), TNF α , IL-6, A20, SHIP1, SOCS1, I κ B α , GPR84, ETS2, SOD2,

Bcl-2AD1 was examined by SYBR GREEN PCR Master Mix (Applied Biosystems). PCR amplification was performed in triplicate, and water was used to replace cDNA in each run as a negative control. The reaction protocol included preincubation at 95°C to activate FastStart DNA polymerase for 10 min, amplification of 40 cycles that was set for 15 s at 95°C, and annealing for 60 s at 60°C. The results were normalized with the housekeeping gene human or mouse β -Actin. Primer sequences were designed using AlleleID 6.0. The following primers were used: mouse TNF α forward, CAAAGG GAGAGTGGTCAGGT; mouse TNF α reverse, ATTGCACCTCAGGGAA GAGT; mouse actin forward, GGTCATCACTATTGGCAACG; mouse actin reverse, ACGGATGTCAACGTCACACT; mouse CXCL1 forward, TAGGGTGAGGACATGTGTGG; mouse CXCL1 reverse, AAATGTCCA AGGGAAGCGT; mouse IL-6 forward, GGACCAAGACCATCCAATTC; mouse IL-6 reverse, ACCACAGTGAGGAATGTCCA; mouse SOCS1 forward, TGAATACCTGAGTTCCTTCC; mouse SOCS1 reverse, ATC TCACCTCCACAACC; mouse SHIP1 forward, GAGGAGACAGGCAA CATC; mouse SHIP1 reverse, TCTTGACACTGAAGGAACC; mouse GPR84 forward, TCAACCTGTGCTCTATGC; mouse GPR84 reverse, GCCTGTCTGCTGAATGG; mouse Bcl2A1D forward, GGAATGGAG GTTGGGAAGATGG; mouse Bcl2A1D reverse, CTGGTCCGTAGTGTT ACTTGAGG; mouse SOD2 forward, ACAACTCAGGTCCGCTCTTCAG; mouse SOD2 reverse, GATAGCCTCCAGCAACTCTCC; mouse ETS2 forward, AGTGTGGTGCTTCCTGTCTTG; mouse ETS2 reverse, TTGCTCTGTCTGTGCTTCTGG; A20 forward, TGAGCAAGTAGGCAA GATAAG; A20 reverse, GTAGACGAGCAGCAATAGC; mouse IkB α forward, TGGAAATCATTTGGTCAGG; mouse IkB α reverse, ACAGGC AAGATGTAGAGG; human IL-8 forward, AGAGACAGCAGAGCACAC; human IL-8 reverse, GTTCTTTAGCACTCCTTGGC; human TNF α forward, TCAGCAAGGACAGCAGAG; human TNF α reverse, GTATG TGAGAGGAAGAGAACC; human actin forward, GTCGGTATGGGTC AGAAAG; human actin reverse, CTCGTGTAGAAAGGTGTGG.

ELISA

Supernatants from cell cultures were collected and measured for the level of mouse cytokines CXCL1, IL-6 and TNF α using DuoSet ELISA kits (R&D system) according to manufacturer's instructions.

Molecular modelling

A theoretical three-dimensional model of the IRAK-M DD region (residues 14–105) was generated via a web-based molecular modelling server (Arnold *et al*, 2006). The crystal structure of the IRAK-2 DD segment (PDB 3MOP) was used as a template because it shares a considerable sequence identity (30%) and topology with IRAK-M DD, as analysed by the structure prediction. The complex of the MyD88 DD–IRAK-4 DD–IRAK-M DD was constructed via two steps. First, four homology models of the IRAK-M DD were superposed onto each IRAK-2 DD segment (chains K to N) in the Myddosome complex (PDB 3MOP) using the secondary structure matching algorithm (Krissinel and Henrick, 2004), as implemented in COOT (Emsley and Cowtan, 2004). Second, these four IRAK-M DD models were assembled into the coordinates of the MyD88 DD–IRAK-4 DD complex in the Myddosome complex after removal of the regions of IRAK-2 DD. Thus, the integrity and physicochemical property of the MyD88 DD–IRAK-4 MM complex were maintained. The hypothetical IRAK-4 DD–IRAK-M DD interface was examined by the server PISA (Krissinel and Henrick, 2007) and inspected by the program PYMOL (www.pymol.org).

Co-immunoprecipitation and immunoblotting

BMDMs and 293-I1A cells were cultured as indicated above. After stimulation with R848 (1 μ g/ml) or IL-1 β (1 ng/ml), the cells were harvested and co-immunoprecipitation and immunoblotting were performed as previously described (Qin *et al*, 2006).

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Electrophoretic mobility shift assay

BMDMs and 293-I1A cells were cultured as indicated above. After stimulation with R848 (1 μ g/ml) or IL-1 β (1 ng/ml), the cells were harvested and nuclear extracts were prepared with the NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo). Labelling of NF κ B-specific oligonucleotides (Santa Cruz) was performed with [γ -³²P]-ATP (Perkin-Elmer) by using T4 polynucleotide kinase (NEB) following manufacturer's guideline. After purification over a Sephadex G-25 column (Amersham), radiolabelled oligonucleotides (around 20 000 c.p.m.) were incubated with nuclear extracts at room temperature for 30 min in binding buffer containing 12 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.8), 60 mM KCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM DTT, 1 mM PMSF, 12% glycerol, 5 μ g bovine serum albumin (BSA) and 2 μ g poly(dI-dC) (Sigma). The same amount of nuclear protein (around 5 μ g) was used in each experiment. The protein concentration was measured using Quick Start Bradford Protein Assay Kit (Bio-Rad) following manufacturer's guideline. DNA–protein complexes were separated using 6% non-denaturing polyacrylamide gel electrophoresis. Signals were visualized by exposing the dried gel to autoradiography film.

Polysome fractionation analysis

Wild-type and IRAK-M-deficient macrophages were stimulated with R848 (1 μ g/ml) for 1.5 h. Cytoplasmic extracts were prepared from BM-derived macrophages stimulated with R848 (1 μ g/ml) as described (Qin *et al*, 2006). Cytoplasmic extracts were carefully layered over 10–50% linear sucrose gradients in polysome buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 50 units of recombinant RNasin (Promega), and 0.1% Igepal-CA630 (Sigma)) and centrifuged at 17 000 r.p.m. in a Beckman SW32.1 Ti rotor for 4 h at 4°C. Gradients were fractionated by using an ISCO gradient fractionation system equipped with a UA-6 detector. Light RNP fractions, 40S, 60S, and 80S and heavy polysome fractions were monitored by the continuous UV absorption profile at A254, and 12 tubes of 750 μ l fractions were collected. The fractions representing light RNP and free ribosomes were used to isolate the translation-inactive pool of mRNAs, and the fractions presenting heavy polysomes were used to isolate the translation-active mRNAs. RNAs were isolated from these fractions by extraction with TRIzol.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: HZ performed all experiments with assistance from MY, JI, PY, WC, KB, WY, TK, WY, VM and JZ; KF conducted structural modelling; JT, JW, JG, JQ, PF and XL interpreted results and provided scientific advice. HZ, MY and XL designed experiments and wrote the manuscript; and XL oversaw the whole study.

Conflict of interest

The authors declare that they have no conflict of interest.

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