Caspase-1 targets the TLR adaptor Mal at a crucial TIR-domain interaction site

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Summary

Toll-like receptors (TLRs) are crucial components of innate immunity, ensuring efficient responses against invading pathogens. After ligand binding, TLR signaling is initiated by recruitment of adaptor molecules, a step mediated by homotypic Toll-IL-1 receptor (TIR) domain interactions. Four TIR-containing TLR adaptor molecules are described, all of which are susceptible to modification and strict regulation. For example, caspase-1 is reported to cleave the TLR adaptor Mal at position D198, an event that is indispensable for Mal function. In this report, we use the mammalian two-hybrid technique MAPPIT to study the implications of Mal cleavage. We show that a Mal mutant, which mimics caspase-1 cleavage and a caspase-1-uncleavable MalD198A mutant, are abrogated in their bridging function and lose the ability to activate NF-κB. A MalD198E mutant is still fully functional, suggesting that caspase-1 cleavage of Mal is not necessary for Mal-mediated signaling. D198 of Mal is conserved in MyD88 and TLR4 TIR domains and the negatively charged amino acid at this position is crucial for the interactions and function of Mal, MyD88 and TLR4 TIR. Our data suggest an inhibitory, rather than an activating role for caspase-1 in Mal regulation, and show that the caspase-1 cleavage site in Mal is part of a TIR-domain interaction site.

Key words: Caspase-1, Mal, TLR, TIR domain

Introduction

Toll-like receptors (TLRs) are on the frontline of innate immunity, ensuring a fast and efficient response against invading pathogens. At present, ten human TLRs are described, all defined by extracellular leucine-rich repeats (LRRs) and an intracellular Toll-IL-1 receptor (TIR) interaction domain. TLRs recognize a whole spectrum of both extracellular and intracellular pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS; TLR4) (Poltorak et al., 1998), bacterial lipoproteins (TLR2) (Yoshimura et al., 1999) or double-stranded RNA (TLR3) (Alexopoulou et al., 2001). Upon ligand binding, various adaptor proteins are recruited to the activated TLRs. This event is mediated by TIR-TIR domain interactions. Four of these TIR-containing adaptor molecules have been identified so far. Myeloid differentiation factor 88 (MyD88) is considered to be the universal adaptor and is recruited by every TLR, except TLR3 (Wesche et al., 1997). MyD88 adaptor-like (Mal) is used by TLR2 and TLR4, and acts as a bridging adaptor, linking MyD88 to the activated receptor (Fitzgerald et al., 2001; Horng et al., 2001; Horng et al., 2002; Kagan and Medzhitov, 2006; Ulrichts et al., 2007; Yamamoto et al., 2002). TIR-domain-containing adaptor-inducing IFN-β (Trif) is involved in TLR3 and TLR4 signaling (Oshiumi et al., 2003a). Finally, Trif-related adaptor molecule (Tram) is solely recruited to TLR4 where it couples Trif to the receptor (Fitzgerald et al., 2003; Kagan et al., 2008; Oshiumi et al., 2003b). TLR signaling is further propagated by a cascade of phosphorylation and ubiquitinylation events, ultimately resulting in the activation of transcription factors such as NF-κB, AP-1 and various interferon-regulatory factors (IRFs) (Kawai and Akira, 2006). This leads to the induction of pro-inflammatory cytokines and type 1 interferons, which initiate a potent immune reaction. However, uncontrolled TLR signaling and the concomitant excessive inflammation are extremely harmful for the host. Mammals have therefore evolved several mechanisms to prevent inordinate TLR-induced pro-inflammatory cytokine production. This attenuation can be achieved at multiple levels, either by interfering with ligand binding, by inhibiting receptor expression or by targeting intracellular signaling components (Lang and Mansell, 2007).

Recently, the family of cysteine-aspartic acid proteases (caspases) was shown to target intracellular components of TLR signaling. Activation of caspases by various pro-apoptotic signals or upon viral infection results in the cleavage and subsequent inactivation of the TLR adaptor Trif; and hence inhibits NF-κB and IRF activation (Rebsamen et al., 2008). However, which caspase is involved in this attenuation and the exact modalities of its activation remain to be clarified. In addition to its cardinal role in inflammasome-mediated cleavage of pro-IL-1β and pro-IL-18β, caspase-1 seems also to be involved in the regulation of TLR signaling, because TLR2 and TLR4 signaling in caspase-1−/− macrophages are abrogated (Miggin et al., 2007). Miggin and co-workers demonstrated a direct interaction of Mal with caspase-1, and Mal is cleaved by caspase-1. Mass spectrometry analysis identified D198 of Mal as the caspase-1 cleavage site. A Mal D198A mutant is no longer cleaved by Mal, shows no NF-κB activation and acts as a dominant-negative inhibitor of LPS- and Pam3Cys-induced signaling. These data suggest that Mal cleavage at D198 by caspase-1 is indispensable for Mal-mediated signaling.

In this report, we further investigate the structural and functional implications of Mal cleavage by caspase-1. We investigate how the interaction of Mal with TLR4 or MyD88 is affected by mutations that inhibit or mimic caspase-1 cleavage. For the study of these interactions, we use the mammalian two-hybrid system MAPPIT
Caspase-1 disrupts Mal interactions (mammalian protein-protein interaction trap) (Eyckerman et al., 2001). Our data show that caspase-1-mediated Mal cleavage is not required for Mal interaction with TLR4 or MyD88, nor for Mal induced NF-κB activation. A mutant that mimics caspase-1-cleaved Mal shows no interaction with TLR4 and MyD88. Our data suggest that the role of caspase-1 in TLR2 and TLR4 signaling is not dependent on Mal cleavage by caspase-1. We show that D198 is close to a very conserved surface area in Mal, which is likely to be involved in Mal function. Mutations of D198 and mutations of the corresponding residue in the TIR domain of TLR4 and MyD88 disrupt their TIR-TIR interactions. The TIR surface area corresponding to D198 in Mal is important for TIR-TIR interactions in Mal, MyD88 and TLR4.

Results

Mutation of the caspase-1 cleavage site in Mal prevents its interaction with MyD88 and TLR4

Caspase-1-dependent cleavage was previously shown to be an essential event for Mal activation and downstream TLR2 and TLR4 signal transduction (Miggin et al., 2007). This processing occurs at the C-terminal portion of its ‘TIR’ domain at the aspartic acid at position 198. Based on a model of Mal (Núñez et al., 2007), the removal of the E helix in the TIR domain of Mal by caspase-1 was argued to be essential for the generation of a steep groove involved in MyD88 recruitment. Mutation of aspartic acid at position 198 of Mal to alanine completely blocks TLR2 and TLR4 signaling. This effect was attributed to a failing caspase-1 cleavage of this mutant form of Mal (Miggin et al., 2007). Using MAPPIT (Fig. 1), we investigated whether this MalD198A mutant indeed fails to recruit MyD88. As expected, a clearly reduced interaction of this mutant form of Mal with MyD88 could be detected (Fig. 2A) and the mutant could not bridge MyD88 to TLR4 (Fig. 2B).

Cleaved Mal does not interact with MyD88 and TLR4

Caspase-1-mediated cleavage of Mal was proposed to be essential for Mal-MyD88 binding (Núñez et al., 2007). We therefore cloned the caspase-cleaved variant of Mal (containing only amino acids 1-198; further referred to as Malcasp), as both MAPPIT bait and prey, and tested its association with MyD88 and TLR4. Interaction of truncated Mal with full-length MyD88 or its TIR domain (MyD88TIR) was completely abrogated (Fig. 3A). In addition, the reciprocal MAPPIT set-up also showed a clear reduction of association (Fig. 3B). These MAPPIT results were confirmed by co-immunoprecipitation (Fig. 3C). FLAG-tagged MyD88 or

Fig. 1. The MAPPIT concept. A ‘bait’ is C-terminally fused with the transmembrane and intracellular part of a leptin receptor that is deficient in STAT3 recruitment. The extracellular domain of either the leptin receptor (LR) or of the erythropoietin (EpoR) can be used. The ‘prey’ protein is linked to a series of six functional STAT3 recruitment sites of the gp130 chain. Association of bait and prey and ligand (L) stimulation leads to STAT3 activation and induction of a STAT3-responsive luciferase reporter (rat PAPI-Luci).
MyD88TIR could be precipitated using wild-type E-tagged Mal, but not by E-tagged Malcasp. Next, we investigated the interaction of Mal with TLR4 (Fig. 3D). As previously described, using the intracellular part of TLR4 as bait and Mal as prey, a clear MAPPIT signal could be monitored. Again, this interaction was clearly negatively affected by Mal truncation. Finally, we also demonstrated that Malcasp loses its ability to bridge MyD88 to TLR4 (Fig. 3E).

**Caspase-1 targets Mal at an acidic amino acid that is crucial for interaction**

To find out whether the altered interaction profile of MalD198A occurs because of a loss of caspase-1 cleavage or to a loss of ‘negative charge’ at position 198, we repeated the same experiments with a Mal mutant in which aspartic acid 198 was mutated to glutamic acid (MalD198E). Since it was shown that substitution of the aspartic acid to glutamic acid in the P1 position of caspase-1 cleavage sites reduces selectivity over 1000-fold, the MalD198E mutant is expected to be resistant to caspase-1 cleavage (Stennicke et al., 2000). To unequivocally demonstrate the impossibility of MalD198E cleavage by caspase-1, both overexpression assays (Fig. 4A), as well as in vitro caspase-cleavage assays with purified Mal(mutant) protein (Fig. 4B) were performed. A modest caspase-1-dependent cleavage could be observed using wild-type Mal. This phenomenon was completely absent when performing the assays with MalD198E, indicating that this mutant indeed is not cleaved by caspase-1.

**Fig. 3. Mal cleavage disrupts its interaction with MyD88 and TLR4.** (A,B) Heterodimerization of Mal or Malcasp and MyD88. HEK293T cells were transiently co-transfected with various combinations of MAPPIT bait vectors pCLL-Mal, pCLL-Malcasp, pCLL-MyD88, together with prey plasmids pMG2-MalTIR, pMG2-MalTIRcasp, pMG2-MyD88, pMG2-MyD88TIR and the rat PAPI-luci reporter. Experimental set-up was as in Fig. 2. (C) Co-immunoprecipitation analysis. HEK293T cells were transfected with combinations of pMet7-MyD88-FLAG, pMET7-MyD88TIR-FLAG, pCAGGSE-Mal and pCAGGSE-Malcasp. Cell lysates were immunoprecipitated with an anti-E-tag antibody and subsequently immunoblotted (IB) with anti-FLAG or anti-E-tag. (D) TLR4 interaction profile. HEK293T cells were transiently co-transfected with pCLL-TLR4ic, the rat PAPI-luci promoter and either Mal or Malcasp prey. (E) Cleavage of Mal results in a loss of its bridging function. HEK293T cells were transiently co-transfected with pCLL or pCLL-TLR4ic, the MyD88TIR prey, a Mal expression vector (pCAGGSE-Mal or pCAGGSE-Malcasp) and the rat PAPI-luci reporter. Experimental set-up was as in Fig. 2.
Caspase-1 disrupts Mal interactions

Strikingly, and in contrast to the MalD198A mutant, the MalD198E mutant still interacted with MyD88 and exerted a bridging function for the MyD88-TLR4 interaction in a MAPPIT context (Fig. 5A,B). These data indicate that an acidic amino acid at position 198, rather than caspase-1-mediated cleavage at this site, is indispensable for Mal-TIR interactions.

Mal cleavage is not essential for NF-κB activation

Next, we investigated the potency of Mal, Malcasp, MalD198A and MalD198E to activate NF-κB signaling. We therefore cloned these Mal forms into a doxycyclin-inducible expression vector (pTre-tight, Clontech). In accordance with previous reports (Fitzgerald et al., 2001), we saw clear NF-κB activation upon Mal expression (Fig. 6A). This effect seemed to be dependent on a full-length form of Mal, because Malcasp had no effect. The MalD198A mutant also failed to induce NF-κB signaling, a feature that has already been described (Miggin et al., 2007). Strikingly, mutating the aspartic acid at position 198 into a glutamic acid only partially affected NF-κB induction. This reduction can partially be attributed to a reduced expression of the MalD198E mutant, as shown by western blot analysis (Fig. 6A). In addition, pre-incubation with the specific caspase-1 inhibitor z-WEHD-fmk (Fig. 6B) or the broad-range caspase inhibitor z-VAD-fmk (data not shown), did not drastically affect Mal-induced NF-κB activation.

Additionally, we tested the role of caspase-1 cleavage of Mal on TLR-induced NF-κB activation in a Mal-deficient background. In analogy with a recent report (Nagpal et al., 2009), immortalized Mal-deficient macrophages were transduced with retroviruses encoding Mal, Malcasp or MalD198E and an internal ribosomal entry site (IRES)-encoded green fluorescence protein (GFP). GFP-positive cells were sorted in bulk, and Mal(mutant) expression was assayed (Fig. 7A). Mal-deficient macrophages were severely impaired in TNF-α production in response to lipoteichoic acid (LTA) (Fig. 7B) and LPS (Fig. 7C), as measured by ELISA. Transduction of these Mal-deficient cells with wild-type Mal led to restored LTA- and LPS-induced TNF-α production. This functional complementation was not observed when transducing the Malcasp knockouts with the Malcasp mutant. MalD198E-transduced cells, on the other hand, displayed a comparable response to LTA and LPS as seen in wild-type Mal-transduced cells. Together with the interaction experiments (see above), these data contradict the hypothesis that Mal cleavage is crucial for Mal function.

Mutations in TLR4 and MyD88 corresponding to D198A disrupt TIR-TIR interactions

The D198A mutation in Mal clearly disrupts its interaction with MyD88 and TLR4, and our data indicate that this might result from
HEK293T cells were transiently co-transfected with a NF-κB-inducible luciferase reporter (pNF-conluc), the expression vector for the transactivator (rtTA-advanced) and doxycyclin-inducible expression vectors pTRE-tight-Mal, pTRE-tight-Malcap, pTRE-tight-MalD198A, pTRE-tight-MalD198E or empty vector. 24 hours after transfection, cells were stimulated with doxycyclin (1 μg/ml) for another 24 hours or were left untreated. Luciferase measurements were performed in triplicate and mean ± s.d. was plotted.

**Discussion**

The recognition of pathogens by TLRs results in the recruitment of one or more adaptor molecules to the activated receptor complex, which in part accounts for receptor-specific responses. This TLR-adaptor association is mediated by homotypic TIR-TIR domain interactions. TIR domain crystal structures reveal a central five-stranded parallel β-sheet, enclosed by five α-helices (Khan et al., 2004; Nyman et al., 2008; Xu et al., 2000). Various regions in the TIR domain seem to be involved in TIR-TIR association. The importance of the BB loop, linking the second β-strand with the second α-helix, is demonstrated in several studies (Jiang et al., 2006; Poltorak et al., 1998; Ulrichts et al., 2007). The TLR adaptor Mal is cleaved by caspase-1, an event that is hypothesized to be essential for TLR2 and TLR4 signaling (Miggin et al., 2007). Núñez and colleagues presented a model of Mal, where the E-helix of Mal is removed after caspase-1 cleavage (Núñez et al., 2007). Removal of the E-helix leads to formation of a deep groove, which is proposed to be involved in MyD88 recruitment. In this model, MalS180, is buried in this cleft and exposed after removal of the E-helix. A common Mal S180L polymorphism in humans clearly attenuates TLR2 and TLR4 signaling, indicating that this residue is important for signaling (Khor et al., 2007). In analogy with a previous study (Ulrichts et al., 2007), we tried to gain better insight in the effects of caspase-1 cleavage of Mal for its TIR domain interactions using the MAPPIT two-hybrid technique. A D198A mutation severely affects the interaction of Mal with the TLR4 and MyD88 TIR domains. The D198A mutant also loses the importance of the BB loop, linking the second β-strand with the second α-helix, as demonstrated in several studies (Jiang et al., 2006; Poltorak et al., 1998; Ulrichts et al., 2007). The TLR adaptor Mal is cleaved by caspase-1, an event that is hypothesized to be essential for TLR2 and TLR4 signaling (Miggin et al., 2007). Núñez and colleagues presented a model of Mal, where the E-helix of Mal is removed after caspase-1 cleavage (Núñez et al., 2007). Removal of the E-helix leads to formation of a deep groove, which is proposed to be involved in MyD88 recruitment. In this model, MalS180, is buried in this cleft and exposed after removal of the E-helix. A common Mal S180L polymorphism in humans clearly attenuates TLR2 and TLR4 signaling, indicating that this residue is important for signaling (Khor et al., 2007). In analogy with a previous study (Ulrichts et al., 2007), we tried to gain better insight in the effects of caspase-1 cleavage of Mal for its TIR domain interactions using the MAPPIT two-hybrid technique. A D198A mutation severely affects the interaction of Mal with the TLR4 and MyD88 TIR domains. The D198A mutant also loses the bridging function of Mal in our MAPPIT assay. We therefore investigated the behavior of a Mal mutant corresponding to residues 1-198 of Mal. This truncated Mal mutant mimics the caspase-1-cleaved Mal. A clear reduction in the MAPPIT signal compared with wild-type Mal was observed when assaying heterodimerization properties of Malcap and MyD88 (Fig. 3A,B).
Caspase-1 disrupts Mal interactions

These MAPPIT data were confirmed by co-immunoprecipitation (Fig. 3C). We also found that the recruitment of Mal to TLR4 was affected in the Malcasp mutant (Fig. 3D). In line with above-mentioned observations, we also demonstrated that the bridging function of Mal was abolished when using the Malcasp mutant (Fig. 3E). The effects of the Malcasp mutation suggest that caspase-1 cleavage would inhibit, rather than promote protein interactions of Mal. This hypothesis is further supported by the observation that reconstitution of a Mal-deficient cell line with the Malcasp mutant does not lead to functional complementation (Fig. 7B,C).

In an attempt to further assess the importance of caspase-1 cleavage for the interactions of Mal, we tested the effects of a Mal D198E mutation. The Mal D198E mutant conserves the negative charge at position 198, but is not cleaved by caspase-1 (Fig. 4). In contrast to the MalD198A mutant, the MalD198E mutation had minimal effects on MAPPIT interaction with MyD88 (Fig. 5A) and bridging of MyD88 to TLR4 in MAPPIT (Fig. 5B). Overexpression of the D198E mutant strongly induces NF-κB activation, indicating that this mutant is fully functional (Fig. 6A).

Our data demonstrate that a conserved acidic amino acid at position 198, rather than cleavage by caspase-1, is crucial for TIR-TIR domain interactions and NF-κB signaling induced by Mal. This is supported by the effects of a caspase-1 inhibitor on NF-κB activation, which is induced by Mal overexpression. We did not observe a strong effect of a specific caspase-1 inhibitor on NF-κB activation induced by overexpression of Mal (Fig. 6B). Moreover, TLR2 and TLR4 responses in MalD198E-transduced Mal-deficient macrophages were comparable with that in cells transduced with wild-type Mal (Fig. 7B,C). These data indicate that Mal-induced NF-κB activation does not require a catalytically active caspase-1 protein.
D198 in Mal corresponds with negatively charged residues in the sequence of TLR1, TLR2, TLR4 and MyD88. E796 in TLR4 and D275 in MyD88 align with the caspase-1 cleavage site D198 of Mal (Fig. 9A). In an alanine scan of TLR4, mutation of residues 796-798 leads to loss of all downstream signaling (Ronni et al., 2003). We performed a mutagenesis study on the TIR domain of TLR4. Using TLR4ic as a bait, we could clearly demonstrate homodimerization of TLR4ic and association of Tram and TLR4ic (Fig. 8A). These interactions were abrogated when two glutamic acid residues E796 and E798 in the βE strand were mutated to alanine, indicating that these residues are crucial for TLR4 interactions. Mutation of the corresponding D275 in MyD88 to alanine was reported to disrupt MyD88 homo-oligomerization, whereas a MyD88 TIR domain with the D275A mutation loses its dominant-negative effect on IL-1 receptor signaling (Li et al., 2005). In our MAPPIT assays, the MyD88 D275A mutant lost its interaction with Mal and MyD88 (Fig. 8B). Together, the data suggest that the conserved negative charge is important for TIR-TIR interaction and possibly part of a binding site in different TIR domains.

In a homology model of TLR4, E796 is part of a strongly conserved surface patch, which is likely to be important for protein interactions of TLR4 (Fig. 9B). The conserved surface patch consists mainly of residues of the DE loop in Mal. In an alanine scan of TLR4, mutations in this surface patch lead to loss of downstream activation (Ronni et al., 2003). In our homology model for Mal (Fig. 9C), D198 was found next to a highly conserved surface patch, which consists mainly of residues of the DE loop at a similar location as in TLR4. Mal phosphorylation by Bruton’s tyrosine kinase is required for TLR2 and TLR4 signal transduction. The conserved surface patch contains the possible Bruton’s tyrosine kinase target Y187. Mutation of Y187 in Mal to phenylalanine reduces Mal phosphorylation and LPS signaling, whereas NF-κB activation by overexpression of this Mal mutant is reduced (Gray et al., 2006). WHISCY analysis of the model confirms that the surface patch is a good candidate for a protein-protein interaction site (Fig. 9E). The D198A and Malcasp mutations and caspase-1 cleavage might disrupt this interaction.
site, whereas phosphorylation of Y187 by Bruton’s tyrosine kinase might be required for interactions via this site. Alternatively, mutation of Y187 in the conserved surface patch might directly inhibit the protein interactions of Mal, leading to decreased tyrosine phosphorylation of Mal. Interestingly, the position of the conserved surface patch partially superposes with a strongly conserved patch in TLR4, whereas residues R192, F193 and D198 are identical in MyD88. It is therefore possible that the conserved surface patch has an important role in protein interactions of Mal, MyD88 and TLR4. The effect of the D198A mutation in Mal might be a consequence of disruption of interactions via this surface patch. Interestingly, R192 and F193 are part of a proposed TRAF6-binding-site motif 188PEPFRL193 of Mal (Mansell et al., 2004). Overexpression of a Mal E190A mutant does not induce any of the signal transduction pathways normally induced by Mal overexpression. However, the E190A mutant retains wild-type levels of TRAF6 binding, making it questionable that the motif is an actual TRAF6 binding site. The role of the conserved surface patch in Mal, MyD88 and TLR4 is currently under investigation.

Our data and homology model for Mal do not agree with the model of Núñez and colleagues (Núñez et al., 2007). We found that removal of the E-helix by caspase-1 is not necessary for the interactions of Mal with MyD88 or TLR4, whereas a mutant where the E-helix is removed loses this interaction. In our homology model, S180 was found at the C-terminal end of beta strand D, where it is surface exposed (Fig. 8D). D198 is found at the C-terminal end of beta strand E, next to S180, but the side chains of S180 and D198 protrude towards opposite sides of the central beta-sheet.

In summary, our data show that caspase-1 cleavage is not required for interaction of Mal with MyD88 or TLR4, or for Mal-induced NF-kB activation. Mutations of D198 might affect a nearby binding site. Two mechanisms have been proposed for the requirement of Mal cleavage by caspase-1. In one model, the C-terminal fragment is proposed to be inhibitory, and its release allows interactions of Mal. In the second model, the released C-terminal fragment itself has a signal transduction function. Our data exclude a role for either mechanism in NF-kB activation induced by Mal, and suggest that caspase-1 cleavage of Mal is inhibitory, rather than required. This would parallel the recent finding that the TLR adaptor Trif and the antiviral adaptor Cardif are cleaved and inactivated by caspases (Rebsamen et al., 2008). Our data clearly indicate that the inhibitory effect of the Mal D198A mutation is insufficient evidence for a requirement of caspase-1 cleavage of Mal in TLR2 and TLR4 signaling. However, we cannot exclude a role for caspase-1 in TLR2 and TLR4 pathways downstream of Mal. Caspase-1 itself can mediate NF-kB activation, and this property does not rely on its enzymatic activity (Lamkanfi et al., 2004; Sarkar et al., 2006). Sarkar and co-workers demonstrated that overexpression of a catalytically inactive mutant of caspase-1 in caspase-1-/- macrophages corrected the decreased NF-kB activation upon LPS stimulation (Sarkar et al., 2006). It was shown that caspase-1 associates with receptor interacting protein 2 (RIP2), which led to the proposition that this interaction is crucial for RIP2-IKK association and hence for NF-kB activation. Moreover, RIP2-deficient cells are severely impaired in TLR2-, TLR4- or TLR3-induced cytokine production (Kobayashi et al., 2002), further underscoring this hypothesis. Our data are in accordance with these reports, because no apparent negative effect of a specific caspase-1 inhibitor on Mal induced NF-kB activation could be demonstrated.
deoxycholic acid, Complete Protease Inhibitor Cocktail (Roche) were incubated with protein G-Sepharose (GE Healthcare). After immunoprecipitation, SDS-PAGE and western blotting, interactions were detected using anti-FLAG antibody (Sigma) and anti-E antibody (GE Healthcare).

Caspase-cleavage assays
We transfected 4×10^6 HEK293T cells with different combinations of pTRE-tight-Mal or pTRE-tight-MalD198E, pCAGGS-p10, pCAGGS-p20 together with the pTET-ON advanced vector. 24 hours after transfection, cells were stimulated with 20 μM of the proteasomal inhibitor MG132 (Callebaut) for 6 hours before cell lysis. Cleared lysates (modified RIPA lysis buffer) were analysed after SDS-PAGE and western blotting, with an monoclonal anti-E antibody. The pET30His-Mal and pET30His-MalΔ198E plasmids were electroporated into E. coli BL21(DE3) cells. This allows the expression of His-tagged human Mal in the cytoplasm of E. coli BL21(DE3) cells. The cytoplasmic fraction was prepared by French pressing using a lysis buffer containing 20 mM Tris-HCl, pH 8, 2 mM MgCl₂ later, cells were incubated with 20 μM of the proteasomal inhibitor MG132 (Callebaut) for 6 hours before cell lysis. Cleared lysates (modified RIPA lysis buffer) were analysed after SDS-PAGE and western blotting, with an monoclonal anti-E antibody.

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Transduction of immortalized Mal knockout macrophages
The immortalized Mal-knockout macrophage cell line (a gift from Jonathan Kagan, Harvard Medical School, Boston, MA) was transfected with retrovirus, produced using the pMX-Mal(mutant)-IRE3-GFP vectors. GFP-positive cells were sorted using a DAKO/Cytomation Mo-Flo fluorescence-activated cell sorter. Expression of Mal or its mutants was assayed using an anti-Mal antibody (FL-235; Santa Cruz Biotechnology).

TNF-α ELISA
Reconstituted macrophages were seeded at a density of 4×10⁴ cells per well and stimulated for 6 hours with various TLKR ligands. Supernatants were collected and assayed for TNF-α using the DuoSET ELISA kit from R&D Systems, according to the manufacturer’s instructions.

Sequence analysis and homology modeling of Mal and TLR4
The crystal structures of the TIR domains of TLR1, TLR2, TLR10 and IL-1RAFP and an NMR structure of MyD88 were structurally superposed via the molecular operating environment (moe) program (chemical computing group). In moe, this structural superposition was aligned with the TIR domains of orthologs of Mal, MyD88 and TLR4 collected from the nr and Ensemble databases and with the TIR domain sequences of all human TLRs. This alignment was used to build homology models for the TIR domain of human Mal and TLR4 in moe. The structure of the TIR domain of TLR1 (pdb code 1FVY) (Xu et al., 2000) was used as template for the TLR4 model. The model of Mal is based on the NMR structure of MyD88 (Protein Data Bank code 225V) (Ohnishi et al., 2009).

Using the alignment of the orthologs and the render by conservation function of the Multialign Viewer of the UCSF Chimera package (Petterson et al., 2004), we visualized the amino acid conservation of Mal or TLR4 orthlogs onto the corresponding model structures. The conservation is colored from red (highly conserved) to blue (low conservation) on a linear scale. The program WHISCY (de Vries et al., 2006) was used to predict possible protein-protein interaction sites on the surface of our Mal homology model. The alignment of the TIR domains of the Mal orthlogs and the Mal TIR model structure were submitted to the WHISCY server (http://nmr.chem.uu.nl/ Software/whisky/index.html). The server returns a pdb file where the B-factor is replaced by the WHISCY prediction scores. The WHISCY prediction scores were visualized in UCSF Chimera by coloring residues according to their B-factor using the ‘render by attribute’ function. The residues are colored on a linear scale according to their WHISCY score (red, high WHISCY score; blue, low WHISCY score).

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