Minireview

Signalling adaptors used by Toll-like receptors: An update


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ABSTRACT

Research into the five Toll/IL1 receptor (TIR) adaptor proteins involved in innate immunity continues to advance. Here we outline some of the more recent findings. MyD88 has a key role in signalling by the IL1 receptor complex and TLRs. However, a MyD88-independent pathway of IL1β signalling in neurons has been described which involves the protein kinase Akt, and which has an anti-apoptotic effect. This pathway may also be important for the mechanism whereby Alum exerts its adjuvant effect since this depends on IL1β but is MyD88-independent. MyD88 is also involved in tumourigenesis in models of hepatocarcinoma and familial associated polyposis (FAP); negative regulation of TLR3 signalling and in PKCε activation. The adaptor Mal is regulated by phosphorylation and caspase-1 cleavage. A variant form of Mal in humans termed S180L confers protection in multiple infectious diseases. TRAM is controlled by myristoylation and phosphorylation and the localisation of TRAM with TLR4 to endosomes is required for activation of IRF3 and induction of IFNβ. Finally SARM has been shown to regulate TRIF and also appears to be involved in neuronal injury mediated by oxidative stress in mouse neurons. These advances confirm the importance for the TIR domain-containing adapters in host defence and inflammation.

1. Introduction

The study of Toll-like receptor (TLR) signalling pathways has proven to be a very fruitful area for investigators interested in signal transduction during innate immunity and inflammation [1]. Newly discovered classes of signalling adaptors (the Toll/IL1 receptor (TIR) domain-containing adaptors) [2], serine/threonine kinases (the IL1 receptor associated kinases (IRAKs)) and transcription factors such as NFκB, IRF3 and IRF7 [3] were discovered culminating in recent attempts to provide a system-wide description of how TLRs such as TLR4 (the sensor for LPS) elicit the enhanced expression of immune and inflammatory genes that lie at the heart of host defence [4].

Signalling is likely to be initiated by the TIR adaptors when they are recruited to a TIR–TIR interface formed by TLR dimerisation. Five TIR adaptors have been discovered—myeloid differentiation factor-88 (MyD88), MyD88 adapter-like protein (Mal), TIR domain-containing adaptor protein inducing IFNβ (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α- and armadillo-motif-containing protein (SARM). Their roles in TLR signalling have been extensively reviewed elsewhere, and we have learned much about their regulation [5,6]. Here, we discuss newer insights into their function and importance, which provides us with ongoing details on the molecular basis for the initiation of innate immunity.

2. MyD88

MyD88 is the universal adaptor for TLRs and also members of the IL1 receptor subfamily, which similar to the TLRs signal via the TIR domain [7]. Four new insights into MyD88 have been made, which further our knowledge of its important role in inflammation and host defence.

2.1. MyD88-independent IL1R signalling

The first finding, curiously, concerns MyD88-independent signalling by IL1β [8]. The current consensus is that IL1β signalling has an absolute requirement for MyD88 as MyD88-deficient cells are apparently unresponsive to IL1β [9]. However, it has recently been shown that the IL1R1/IL1RαC complex is capable of signalling in the absence of MyD88 in anterior hypothalamic neurons (Fig. 1A). A synthetic low molecular weight peptide prevented MyD88 binding to the IL1R1/IL1RαC complex but did not prevent activation of the serine/threonine kinase Akt as anticipated. It was revealed that the p85 subunit of PI3-kinase binds directly to IL1R when the receptor is phosphorylated leading to the activation of Akt in the absence of MyD88. Conversely the tyrosine kinase Src required the presence of MyD88 to signal in these cells. Src activation leads to induction of cyclooxygenase (COX) and interleukin 6 (IL6) among other molecules [10] whereas Akt activation has neuroprotective effects in response to IL1β.

These results were confirmed with cells from MyD88-deficient mice. Upon IL1β stimulation Src was not activated in these cells...
but Akt activation was normal. The interaction of the p85 subunit of PI3-kinase with IL1RI also occurred in a MyD88-independent manner. This study postulates that the low molecular weight peptide used or a peptide mimetic could be used to treat neurodegenerative diseases by blocking the inflammatory response due to IL1 but allowing its neuroprotective effect to remain intact.

A MyD88-independent role for IL1β has also been indicated from the adjuvant effect of Alum [11]. This has recently been shown to depend on Nalp3, the key regulator of caspase-1, which is required to process the pro-form of IL1β. However, the effect of Alum is MyD88-independent which by extension indicates that if IL1β is involved, its adjuvant effect is MyD88-independent. These studies may therefore herald a new phase for IL1/TLR signalling if IL1 is required to process the pro-form of IL1β and prevented PKCε activation, 14-3-3 binding and for the ability of PKCε to interact with TLR4.

There is however, an inconsistency which needs to be resolved. If PKCε is required for TRAM to signal how can MyD88 be involved, since the TRAM pathway can function in the absence of MyD88 [16]. It is possible that additional mechanisms exist for PKCε activation in TLR signalling.

2.3. MyD88 as a negative regulator of TLR3 signalling

TLR3 uses the adaptor TRIF to signal in response to double-stranded RNA. MyD88 was thought not to be involved in TLR3 signalling until a recent study demonstrated that MyD88-deficient corneal epithelial cells show an enhanced response to poly I:C [17] (Fig. 1C). Treatment of corneas from MyD88-deficient mice with poly I:C led to an exacerbation of neutrophil infiltration into the region and an increase in corneal haze in comparison to wild type mates. Macrophages were ruled out as being important here since stimulation of MyD88-deficient macrophages with poly I:C resulted in normal cytokine production relative to wild type macrophages. The use of human corneal epithelial cells (HCECs) with MyD88 knocked down by siRNA demonstrated that a lack of MyD88 led to enhanced RANTES production. Further investigation using the HCECs showed that MyD88 acts as a negative inhibitor of JNK but not p-38 phosphorylation on the TLR3 pathway. The mechanism is not clear however.

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**Fig. 1.** Recent advances in MyD88 signalling. (A) Stimulation of the IL1RI/II1RAcP complex by IL1β leads to the activation of two signalling pathways in neurons. The first involves MyD88-dependent induction of Src resulting in an inflammatory response. The second involves MyD88-independent activation of Akt by PI3-kinase which binds directly to the IL1RI when the receptor is phosphorylated. This pathway has a neuroprotective effect. (B) MyD88 is required for the activation of PKCε. The interaction between MyD88 and PKCε leads its phosphorylation allowing it to interact with TLR4 and 14-3-3. (C) MyD88 acts downstream of the TRIF pathway in TLR3 signalling as a negative inhibitor. It inhibits the activation of JNK but not p-38.
This study suggests that negative regulation of TRIF by MyD88 occurs in certain cell types as a control mechanism to prevent overproduction of RANTES (among other chemokines) in areas where an inflammatory response is more detrimental (such as in the eye).

2.4. MyD88 and cancer

Three recent studies implicate MyD88 in tumourigenesis. MyD88 is thought to have a role in cancer due to the activation of the inflammatory response during carcinogenesis. It has been suggested that MyD88 is involved in colon cancer development. Mice with a mutation in their adenomatous polyposis coli (APC) gene develop familial associated polyposis (FAP), a colon cancer. However, when these mice are crossed with MyD88-deficient mice there is decreased mortality, tumour size and tumour number [18]. This suggests that MyD88 is involved in the induction of tumour-specific modifier genes linked to intestinal tumourigenesis.

A separate study also found a role for MyD88 in cancer progression. A link between MyD88-dependent IL6 production and the onset of hepatocellular carcinoma (HCC; the most common form of liver cancer) was shown using MyD88- and IL6-deficient mice [19]. HCC represents an inflammation-linked cancer hence the requirement of IL6. Male mice treated with the carcinogen diet-dimethylnitrosamine had higher IL6 production than female mice. This IL6 production in males is dependent on IKKβ and is produced mainly by Kupffer cells in the liver. Estrogens act as inhibitors of IL6 production in female Kupffer cells hence less female mice developed the cancer. In MyD88-deficient male mice treated with diethylnitrosamine there was a marked decrease in tumour size and number. This study therefore provides a connection between MyD88-dependent IL6 production and the progression of HCC. A key question regarding both of these studies is what is activating MyD88?

However, a somewhat different role for MyD88 in colon cancer has also been presented. In a recent study nude mice lacking either MyD88 or TLR5 were injected with the human colon cancer cell line DLD-1. The lack of MyD88 in these mice led to enhanced tumour growth, inhibited necrosis and reduced neutrophil invasion into tumours [20]. The administration of the TLR5 ligand flagellin to wild type mice injected with DLD-1 cells resulted in diminished tumour growth and induced necrosis. These results suggest that TLR5 activation is protective in colonic cancer cells and this protection requires MyD88 activity.

3. Mal

Mal acts a bridging adaptor for MyD88, but only in the context of TLR2 and TLR4 stimulation [21]. MyD88, TLR2 and TLR4 are largely electropositive on their surfaces hence MyD88 is unable to bind these TLRs. Mal on the other hand is mainly electronegative on its surface allowing it to bind TLR2 and 4 and bring MyD88 into the signalling complex [22]. Three recent important findings have been made in relation to Mal.

3.1. Mal is cleaved by caspase-1

A region in the C-terminus of Mal contains a caspase-1 cleavage site [23] (Fig. 2A). A yeast two-hybrid screen using full length human Mal as bait and a cDNA spleen library as prey first revealed...
the potential of caspase-1 to interact with this C-terminal region. The caspase-1 inhibitor YVAD-Cmk prevented NFκB activation in response to LPS and phosphorylation of p-38 in response to Pam3Cys linking caspase-1 activity to both these TLRs. Bioinformatics hinted at a putative caspase-1 cleavage site in Mal at amino acid 198 (an aspartic acid). Treatment of Mal with caspase-1 led to the cleavage of Mal at this position and this cleavage could be blocked with the caspase-1 inhibitor. The cleavage of Mal is required for its activation in the signalling pathways of TLR2 and 4. This finding therefore points to an important synergy between TLRs and NLRs that activate caspase-1. Activation of NLRs such as Nalp3 will therefore potentiate signalling by TLR2 and TLR4 via cleavage of Mal.

3.2. Mal S180L is an immunoprotective polymorphism

A single nucleotide polymorphism (SNP) in the gene encoding Mal leads to a change in amino acid at position 180 converting a serine to a leucine. This SNP is protective against several diseases (Fig. 2B). In the first study published a link between the Mal S180L variant and protection against malaria, TB, bacteremia and invasive pneumonococcal disease (IPD) was reported, with heterozygotes showing a near halving of disease risk. The study also showed that the Leu 180 variant of Mal was unable to activate NFκB when reconstituted into Mal-deficient murine embryonic fibroblasts. It behaved as a dominant negative when introduced into cells containing Ser 180 Mal. Modelling studies concluded that the Leu 180 variant was unable to bind TLR2 due to the close proximity of position 180 to the DD loop (thought to be involved in TLR2 binding). The leucine is more exposed in this region than the serine resulting in disruption of the interaction. This was confirmed with protein interaction studies showing that Mal-Leu 180 could not bind TLR2.

Other investigations into the Mal variant and disease have led to its implication in other diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (the TLR4 SNP G299D was also determined not to be protective against rheumatoid arthritis (Fig. 2B)). The data showed that the absence of IFNα and IL10 production indicating that activation of the TRIF pathway via TLR3 was sufficient to allow tolerance on the TLR4 pathway. Finally the role of TRIF in tolerance was linked to the production of IFNβ through the use of a neutralising antibody against IFNβ. Treatment of cells with this antibody and lipid A for 24 h followed by restimulation with lipid A resulted in a restoration of TNFα production. This demonstrates that tolerance has a requirement for IFNβ production.

These data suggest that lipid A-induced tolerance involves the TRIF-dependent pathway and is MyD88-independent. TLR activation is required to allow tolerance to develop which is vital for prevention of over activation of the host. However, it must be pointed out that the absence of IFNβ does not abolish tolerance completely therefore other pathways must also be involved.

4.2. Adjuvant effects of poly(I:C) require TRIF

The adjuvant effect of poly(I:C) has been recently been shown to be due to TRIF and IFNβ promoter stimulator 1 (IPSI) activity [33]. IPS1 is the downstream adaptor used by the receptors Retinoic acid-inducible gene 1 (RIG I) and Melanoma differentiation-associated gene 5 (Mda5). These receptors are found in the cytoplasm and are crucial for binding dsRNA. IPS1 is then recruited to activate the transcription factors IRF3, NFκB and API [6]. In a recent study bone marrow derived dendritic cells (BMDCs) from TRIF- and IPS1-deficient mice or mice lacking both were analysed for their ability to respond to poly(I:C). The IPS1-deficient mice showed low levels of IFNα and β in their serum after injection with poly(I:C) whereas the TRIF-deficient mice had levels of both comparable to wild type
mice. At the mRNA level the IPS1-deficient mice showed a marked reduction in the expression of many cytokines including IFNα and β, RANTES and IL6. mRNA from the TRIF-deficient mice also showed a decrease in expression of these cytokines but not to the same extent as IPS1.

To examine the adjuvant effects of polyI:C mice were immunised with OVA in Alum with or without polyI:C stimulation and antibody titres were measured. This revealed a requirement for IPS1 and TRIF to allow a robust response as titre levels dropped in the absence of either. Both were also required for the increase in Ag-specific CD8⁺ T cell activation and hence IFNγ production. In all experiments the responses to polyI:C were completely diminished in the doubly deficient mice therefore indicating that both TRIF and IPS1 are required to produce an adaptive immune response to double-stranded RNA. Both the endosomal membrane bound TLR3 and the cytoplasmic RIGI or Mda5 pathways are necessary to provide a robust response to dsRNA and neither can function sufficiently in the absence of the other.

5. TRAM

TRAM acts as a bridging adaptor allowing activation of the TRIF-dependent pathway in response to LPS. The downstream effect of TRAM is the activation of IRF3 and late activation of NFκB (as outlined in Section 4). It is regulated by myristoylation, which is required to localise it to the plasma membrane, and as stated in Section 2.2 it is also regulated by PKCε.

TLR4 signalling therefore involves all four adaptor proteins with MyD88/Mal believed to be involved in the early LPS responses and TRIF/TRAM pathway initiated at a later stage. It is yet to be clarified if Mal and TRAM bind to TLR4 in a mutually exclusive manner. Two recent studies into TRAM localisation have clarified this to some degree.

One study published that stimulation with LPS led to TLR4 and TRAM co-localisation at the plasma membrane and also recruited TRIF. This LPS stimulation then caused co-translocation of TRAM and TLR4 to endosomes where TRAF3 was recruited to TRIF allowing the activation of IFNβ.

The second study identified the method for translocation of TRAM and TLR4 (Fig. 3). Through the use of the dynamin inhibitor dynasore it was shown that endocytosis of TLR4 was controlled by dynamin. The ability of TRIF/TRAM to lead to the phosphorylation of IRF3 with subsequent induction of IFNβ and RANTES was blocked in the presence of dynasore indicating TLR4 must be endocytosed to activate the TRIF-dependent pathway. However, dynasore did not affect the localisation of TRAM to the membrane and early endosomes suggesting that TLR4 and TRAM traffic to endosomes via different routes.

The study also identified a bipartite localisation motif in TRAM consisting of the previously mentioned N-terminus myristoylation site followed by a polybasic domain. Other proteins that contain this bipartite motif are involved in signalling at both the plasma membrane and in endosomes. Examination of this motif through mutation studies revealed that both regions are required for plasma membrane localisation but the myristoylation site is solely needed for endosomal localisation. Cells from TRAM-deficient mice were reconstituted with various forms of TRAM to clarify these roles. TRAM mutants lacking the myristoylation site were unable to produce IL6 or RANTES in response to LPS whereas mutations in the polybasic region did not affect these responses. The replacement of the localisation motif of TRAM with a sequence from the plasma membrane-targeting domain of Fyn did not reconstitute...
the TLR4/TRAM signal indicating that endosomal localisation is required for TRAM to signal.

Finally this study revealed how the results above fit with the current model of TLR signalling. The TLRs found on the plasma membrane are unable to induce IFNs, with the exception of TLR4, whereas all the TLRs found in the endosomal membrane can induce this response. Therefore if TLR4 must be translocated to the endosome to activate the TRIF-dependent pathway (which induces IFNs) this segregation between membranes stands true. The reasoning behind this segregation is the absence of TRAF3 in the plasma membrane. TRAF3 is required for IFN production and interacts with both TRIF (in the case of TLR3 and TLR4 signalling) and MyD88 (in the case of TLR7, 8 and 9) on the endosomes. Mutations in TRAF3 allowing it to bind the plasma membrane resulted in Pam3Cys-induced IFNβ production by TLR1/2 thus demonstrating the ability of plasma membrane TLRs to produce IFNs but only if TRAF3 is available to them in this artificial way.

The conclusions drawn from these studies are that TRAM functions as a bridging adaptor like Mal but signals from endosomal compartments.

6. SARM

SARM is the fifth TIR domain-containing adaptor that also contains sterile α-(SAM) and HEAT/armadillo (ARM) motifs. There are conflicting reports as to its function.

6.1. SARM negatively regulates TRIF

The first studies into SARM demonstrated that it could not induce NFκB activation when overexpressed [38]. A subsequent study in 2006 confirmed that SARM could not activate NFκB or IRF3 but expanded on this with the recognition that SARM in fact downregulated the TRIF but not the MyD88-dependent pathway [39] (Fig. 4A). It found that SARM protein levels but not mRNA levels were greatly upregulated after LPS stimulation. The examination of components of the TRIF pathway showed that SARM acted directly on TRIF. Truncated forms of SARM were used to identify the domains in SARM necessary to cause this inhibitory effect. Both the TIR and the ARM domains were proven to be vital. Deletion of the N-terminus of SARM prevented its upregulation after LPS stimulation hinting at a domain present in that region involved in the post-transcriptional regulation of SARM.

RNA interference of TRAM expression led to enhanced ifnb promoter, TNF and RANTES expression upon polyI:C and LPS stimulation.

6.2. SARM mediates stress-induced toxicity in the brain

SARM-deficient mice were generated to test its role in apoptosis. (A) In humans SARM has been identified as an inhibitor of TRIF signalling. As it contains a TIR domain it binds directly to the TIR domain of TRIF thus blocking the interaction of TRIF with TLR3 or TRAM in the case of TLR4 signalling. This interaction prevents the activation of IRF3, IRF7, NFκB and apoptosis by TRIF. (B) SARM has been shown to interact with the outer membrane of mitochondria in mouse neurons. It binds the membrane via its ARM domain and recruits JNK3 to mitochondria when the cell is stressed. This leads to the activation of cell death.

The functions of SARM may be species-dependent. (A) In humans SARM has been identified as an inhibitor of TRIF signalling. As it contains a TIR domain it binds directly to the TIR domain of TRIF thus blocking the interaction of TRIF with TLR3 or TRAM in the case of TLR4 signalling. This interaction prevents the activation of IRF3, IRF7, NFκB and apoptosis by TRIF. (B) SARM has been shown to interact with the outer membrane of mitochondria in mouse neurons. It binds the membrane via its ARM domain and recruits JNK3 to mitochondria when the cell is stressed. This leads to the activation of cell death.

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this cell death. Neurons from SARM-deficient mice were more resistant to apoptosis induced by oxygen and glucose deprivation (OGD) than neurons from wild type mice demonstrating the role of SARM in inducing stress-related cell death in neurons. Macrophages from these mice responded normally to TLR2, 3, 4 and 9 ligands indicating no role for SARM in these TLR pathways.

The role of SARM differs greatly in these two studies so a clear picture has yet to emerge. Carty et al. revealed an inhibitory role for SARM in TLR3 and TLR4 signalling due to its ability to block TRIF activity. However, the generation of SARM-deficient mice by Kim et al. has revealed no apparent role for SARM in TLR signalling. Its expression appears to be limited to neurons where it activates cell death if the neurons become stressed. Since the study of Carty et al. was in human cells there may be an important species difference in relation to the function of SARM, which will require testing.

7. Final perspectives

A large body of knowledge strongly indicates that the TIR adaptors are central to the innate immune response, triggering the production of inflammatory cytokines, chemokines, IFNs and ultimately the activation of the adaptive immune system allowing the host to clear infection.

Discoveries regarding the biological roles of the adaptors continue to be made, with MyD88 in particular showing key involvement in tumourigenesis and as a negative regulator of TRIF. The involvement of TRIF in tolerance is a novel discovery with much potential for future work. Negative regulation and tolerance are crucial factors to a successful immune response and prevention of inflammatory disease development. The hunt for negative regulators of the innate immune system has been extensive but little work has been carried out on the role the adaptors may play. These insights could lead to in-depth investigations into the role of all the TIR adaptors in dampening down our innate immune responses.

Complex biochemical regulation of Mal and TRAM has been revealed with TRAM signalling NFκB at the plasma membrane and IRF3 on endosomes. A variant of Mal which confers protection against multiple infectious diseases has been described. The accumulating evidence on the control of the bridging adaptors may lead to the development of mechanisms to block their signalling without affecting other pathways. Blocking peptides or small molecules that target these adaptors would be of great interest to test in models of infectious and inflammatory diseases.

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A negative role for SARM in the regulation of TRIF in humans has been discovered whereas in mice it appears to have a role in preventing neuronal apoptosis. The functions of SARM require further investigation.

We can anticipate further findings into the function and role of this family of proteins, and possibly novel therapies aimed at limiting them in disease.

References


