Signalling of Toll-Like Receptors

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Abstract Since Toll-like receptor (TLR) signaling was found crucial for the activation of innate and adaptive immunity, it has been the focus of immunological research. There are at least 13 identified mammalian TLRs, to date, that share similarities in their extracellular and intracellular domains. A vast number of ligands have been identified that are specifically recognized by different TLRs. As a response the TLRs dimerize and their signaling is initiated. The molecular basis of that signaling depends on the conserved part of their intracellular domain; namely the Toll/IL-1 receptor (TIR) domain. Upon TLR dimerization a TIR-TIR structure is formed that can recruit TIR-containing intracellular proteins that mediate their signaling. For this reason these proteins are named adapters. There are five adapters identified so far named myeloid differentiation primary response protein 88 (MyD88), MyD88-adapter like (Mal) or TIR domain-containing adapter (TIRAP), TIR domain-containing adapter inducing interferon-β (IFN-β) (TRIF) or TIR-containing adapter molecule-1 (TICAM-1), TRIF-related adapter molecule (TRAM) or TICAM-2, and sterile α and HEAT-Armadillo motifs (SARM). The first four play a fundamental role in TLR-signaling, defining which pathways will be activated, depending on which of these adapters will be recruited by each TLR. Among these adapter proteins MyD88 and TRIF are now considered as the signaling ones and hence the TLR pathways can be categorized as MyD88-dependent

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and TRIF-dependent. Mal and TRAM have recently been shown to be required for the recruitment of MyD88 and TRIF, respectively, to TLR(s) and are therefore called bridging adapters. The MyD88- and TRIF-dependent pathways activate not only the transcription factor nuclear factor-kappaB (NF-κB), mitogen-activated protein kinases (MAPKs) but also certain interferon-regulated factors (IRFs). Recently, it was discovered that the remaining adapter SARM has an inhibitory role in TRIF-dependent signaling by binding to TRIF. Apart from SARM there are several proteins of the host that target the adapters in order to suppress their signaling. In addition, some viral proteins have been identified that inhibit TLR-signaling via their interaction with the TLRs, preventing in that way the activation of the immune system of the host and acting beneficially for the survival of the virus.

1 Introduction

The discovery of Toll-like receptors (TLRs) altered significantly our understanding on the initiation and activation of the innate immune response. It is now clear that innate immunity is more specific than it was originally thought. When an organism-host is invaded by several types of microbes such as bacteria, yeast, viruses, fungi, and parasites, they are sensed by the TLRs that are expressed in a wide range of different cell types such as those involved in innate and adaptive immunity. Examples are macrophages, dendritic cells (DCs), B- and certain types of T-cells.

TLRs recognize a wide variety of molecules from the invaders and initiate various signal transduction cascades that activate and regulate the host's immune response. All the members of this family of receptors as well as the interleukin-1 receptor type I (IL-1RI) members share homology in their cytoplasmic part. In particular, they contain a domain called the Toll/IL-1 receptor (TIR) domain. TIR also exists in intracellular proteins that are called adapters because they can bind via this domain to the liganded receptors, thereby transmitting signaling inside the cells. One of these adapters, which not only binds to almost all signaling TLRs but also associates with IL-1RI, is MyD88. Thus, TLRs and IL-1RI share some similar pathways that induce the activation of the transcription factor nuclear factor-κB (NF-κB) and the mitogen-activated protein kinases (MAPKs), p38, and the c-Jun N-terminal kinase (JNK). As a result a common set of genes are expressed which produce essential molecules for the activation and the regulation of both innate and adaptive immunity, such as cytokines, chemokines and co-stimulatory molecules.

However, apart from MyD88 there are another four adapter molecules: the MyD88–adapter-like or TIR domain-containing adapter (Mal/TIRAP); the TIR domain-containing adapter-inducing interferon-β (IFN-β) or TIR-containing adapter molecule-1 (TRIF/TICAM-1); the TRIF-related adapter molecule (TRAM), also known as TICAM-2; and the protein that contains sterile α and HEAT-Armadillo motifs (SARM). The existence of adapter molecules explains to some extent the separate signaling cascades that are initiated and regulated by different TLRs. The reason is that not all the members of the TLR family bind the same adapter(s) which

specifically mediate certain pathways. For example, some of these proteins are essential for the activation of various members of the family of transcription factors called (IFN) regulatory factors (IRFs) in response to TLR activation. These transcription factors are responsible for the induction of type I IFNs and IFN-induced genes. This is also a main difference between IL-1RI and some TLRs, with IL-1RI being unable to activate IRFs.

In general, it is not certain if all the signaling pathways activated by the TLRs have been discovered yet. In addition, the exact molecular mechanisms by which the known TLR signal transduction pathways are initiated and regulated have not been completely uncovered. However, a lot has already been understood regarding the TLR signaling cascades and their specificity in terms of which genes they induce to ensure that the host will respond efficiently to a certain invasion.

In this chapter, we discuss the current knowledge on TLRs in terms of their signaling pathways. In particular, we describe TLR-signaling on the basis of which adapter molecules—the first intracellular proteins in the cascade known to specify which cascades are initiated—are used.

2 TLRs and Their Ligands

The first receptor belonging to the family of TLRs to be discovered was the *Drosophila melanogaster* protein Toll (or dToll). Initially, its only identified function was its essential role for the formation of the dorsoventral axis of the fly embryo (Hashimoto et al. 1988). Later, it was found that Toll had a cytosolic portion homologous to that of IL-1RI (Dower et al., 1985; Bird et al., 1988; Urdal, et al., 1988; Gay and Keith, 1991), the active receptor for IL-1, a cytokine that plays a very significant role in innate and adaptive immunity by mediating a variety of local and systemic effects (reviewed in (Dinarello, 1994; 1996)). Subsequently, Toll was found to be important for the antifungal defense of the adult fly (Lemaitre et al., 1996).

Since that discovery, at least 13 mammalian TLRs have been identified, which identification is based on their shared sequence similarities. Most of these TLRs have already been shown to recognize pathogen-associated molecular patterns (PAMPs) from a wide range of invading agents.

In general, TLRs can be categorized into two main groups, according to their ligands. One group consists of TLR1, 2, 4, and 6 that recognize PAMPS from lipids, and the other group consists of TLR3, 7, 8, and 9 that recognize PAMPS from nucleic acids.

Very briefly, as the TLR ligands have already been discussed in more detail in Chapter of this volume, TLR2 binds lipopeptides from the cell wall of Gram-positive bacteria, lipomannans from mycobacteria, phospholipomannan from fungi, and glycosylphosphatidylinositolmucin from protozoan parasites. TLR2 is also activated by proteins. Such examples are porins from the cell wall of Gram-negative bacteria and other proteins derived from viruses. TLR2 can actually heterodimerize with TLR1 or TLR6. The TLR1/TLR2 complex is responsible for the

recognition of bacterial and mycobacterial diacyl lipopeptides and bacterial triacylated lipoproteins; such as the synthetic compound Pam3Cys. The TLR2/TLR6 complex recognizes diacylated lipoproteins from mycoplasma, such as the mycoplasma lipoprotein-2, and the bacterial glycolipid lipotechoic acid.

TLR4 is activated by endotoxin also known as lipopolysaccharide (LPS), which is derived from the cell wall of Gram-negative bacteria (Poltorak et al., 1998; Hoshino et al., 1999; Qureshi et al., 1999; Arbour et al., 2000; Agnese et al., 2002; Lorenz et al., 2002), mannan from fungi, glycoinositolphospholipids from protozoan parasites, and several viral proteins, such as the fusion protein of the respiratory syncytial virus.

TLR3, TLR7, and TLR8 are key receptors for antiviral responses. TLR3 initiates signaling for double-stranded RNA. TLR7 and TLR8 are induced by single-stranded RNA (Alexopoulou et al., 2001). Furthermore, TLR3 recognizes polyriboinosinic: polyribocytidylic acid (poly(I:C)) (a synthetic analogue of dsRNA) and TLR7, several imidazoquinolines (e.g., imiquimod and R-848). Other ligands for TLR7 include guanosine analogues (e.g., Loxoribin). R-848 also activates the human TLR8 (Hemmi et al., 2002; Lee et al., 2003; Heil et al., 2004; Lund et al., 2004). The mouse TLR8 has been shown to be unable to initiate signal transduction. Last, TLR9 recognizes unmethylated CpG-DNA motifs from bacteria and mycobacteria. It also senses genomic DNA from parasites (Hemmi et al., 2000; Bauer et al., 2001). It has to be mentioned that, as in the case of TLR2 that forms a complex with either TLR1 or TLR6, TLR8 can interact with TLR7 or TLR9 (Wang et al., 2006). Furthermore, TLR9 can also interact with TLR7. However, in contrast to the formation of the TLR1/2 and TLR2/6 complexes, which initiate signaling, the TLR9-TLR7 interaction antagonizes TLR7 signaling. In addition, the TLR8 ligands act as antagonists for the signaling cascades induced by TLR7 and 9.

Apart from these two main groups of TLRs there are also the TLR5 and the murine TLR11 (its human homologue is inactive) that recognize the protein flagellin from bacteria (Hayashi et al., 2001) and a profilin-like molecule (Yarovinsky et al., 2005), respectively. Ligands for the remaining TLRs—TLR10, 12, and 13—have not been identified yet. TLR10 has been found to be expressed in humans but not in mice, and the other two in mice instead of humans.

Except from the mentioned exogenous ligands, TLRs are also activated by several endogenous molecules. For example, TLR2 and TLR4 have been reported to be activated by heat shock proteins (Ohashi et al., 2000; Vabulas et al., 2001; Vabulas et al., 2002) and fragments of the polysaccharide hyaluronan (Termeer et al., 2002; Jiang et al., 2005; Scheibner et al., 2006). Last, TLR3 and TLR9 have been shown to recognize host mRNA (Kariko et al., 2004) and DNA, respectively (Leadbetter, 2002).

The structural basis that explains the wide variety of ligands recognized by the TLRs and how exactly their signaling is initiated has not been completely elucidated yet. The main reason is that no liganded TLR or TLR-adapter structures have been solved so far. However, a small number of TLR structures are available and several studies on the conserved regions among the TLRs (e.g., identification of mutations that make them inactive) enable us to have a good understanding of the first events in TLR signaling.

3 Structure and Signaling of TLRs

3.1 Structural Characteristics of the TLRs and Initiation of Signaling

The structure of TLRs and the recognition of their ligands will be described concisely as they are discussed in more detail in other chapters of this volume. Briefly, TLRs are type 1 transmembrane receptors that contain an extracellular domain consisting of leucine-rich repeat (LRR) motifs that recognize the ligands. The crystal structure of the LRR domain of TLR3 has been solved at 2.3 Å (Bell et al., 2005; Choe et al., 2005). It contains 23 LRR motifs that build a horseshoe-shaped solenoid structure that is highly glycosylated. Binding of the monomeric TLR3 ligand dsRNA is believed to occur at a surface of the LRR domain that is free of glycosylation. Liganding of TLR3 causes symmetrical dimerization of its ectodomains (Bell et al., 2006). As a result, conformational changes occur that bring the intracellular part of two TLR3 molecules that contain the highly conserved TIR domain into close proximity, thereby forming a TIR-TIR structure. It is believed that in general, liganded TLRs form dimers in order to signal (Ozinsky et al., 2000). These dimers are either homodimers, as in the case of TLR3 and the *Drosophila* Toll (Weber et al., 2003; Weber et al., 2005); or heterodimers, like those of TLR2 with TLR1 or TLR6; or that of IL-1RI with its accessory protein (IL-1RAcP), which also contains a TIR domain.

The TIR domains are between 135 and 160 amino acids in length. The molecular structures of the TIR domains of TLR1 and TLR2 have been resolved. They contain a central, five-stranded, parallel β-sheet which is surrounded by a total of 5 α-helices on both sides (Xu et al., 2000). In terms of its sequence, each TIR domain contains three conserved regions named Box 1, Box 2, and Box 3. Box 1 is the signature sequence of all TLRs; Box 2 contains a loop termed the "BB loop," which is important for signaling, and Box 3 contains certain amino acids that have been identified as important for signaling, at least in the case of IL-1RI. The BB loop contains a conserved proline. When a missense mutation in the *tlr4* gene changed this proline to histidine, the expressed TLR4 protein was unable to signal (Poltorak et al., 1998; Qureshi et al., 1999). There is also another important loop that has been identified as essential for signaling, named the "DD loop." It has been shown that the DD loop of TLR2 interacts with the BB loop of TLR1 (Gautam et al., 2006). Thus, a possible model for the formation of a TIR-TIR structure could be based on an interaction between the DD loop of one TIR domain, with the BB loop of the other. Such a model is also supported by the TIR-TIR interactions among certain TLRs and their adapter molecules. In general, it is now believed that the formation of a TIR-TIR structure at the receptor level provides the place of association with the TIR domain-containing adapters that mediate signaling by linking the receptors to downstream intracellular proteins.

3.2 MyD88

3.2.1 Recruitment of MyD88 by the TLRs

The first adapter to be found essential for the TLR signaling cascades was MyD88. MyD88 had already been identified as a protein expressed in myeloid tissues, and its mRNA levels were used as markers for differentiation (Lord et al., 1990a; Lord et al., 1990b). In particular, MYD88 was induced when M1D+ myeloid precursors were differentiated in response to IL-6. This is why it was named MyD88: "MyD" stands for myeloid differentiation and "88" is the number of the genes induced expressing the MyD88 protein. Four years later, MyD88 was identified as a member of the Toll/IL-1 receptor family (Hultmark, 1994; Yamagata et al., 1994) and consequently, its function was found to be crucial for signaling induced by IL-1, as well as several TLRs (Bonnert et al., 1997; Muzio et al., 1997; Wesche et al., 1997; Burns, 1998; Medzhitov et al., 1998; Muzio et al., 1998). The development of MyD88 knock out mice was normal but their cells were completely unresponsive to IL-1, IL-18 (Adachi et al., 1998), and endotoxin (Kawai et al., 1999; Takeuchi et al., 2000). In general, MyD88 mediates the signaling of all TLRs, apart from TLR3.

The basic characteristics of its structure are a death domain "DD" and a TIR domain at positioned at its N- and C-terminals, respectively. These two domains are linked by an intermediate domain "ID."

The recruitment of MyD88 to TLR2 is based on the DD loop of its TIR domain. In particular, both the BB loop of the TLR2 and the DD loop of the MyD88 are required for the interaction of these two proteins (Xu et al., 2000; Dunne et al., 2003). In addition, site-directed mutagenesis of the amino acids 195–197 in the Box 2 of MyD88 impaired its recruitment to IL-1RI (Li et al., 2005), whereas the conserved proline in its the BB loop (see Section 3.1) is not required for its interaction with TLR2 or TLR4 (Dunne et al., 2003).

In 2006 germ-line mutagenesis offered further insight into the interactions between MyD88 and the TLRs (Jiang et al., 2006). The TLR phenovariant produced in that way was named Pococurante (Poc) and resulted in the MyD88 I179N mutation. Abolished was the ability of the mice of that phenotype to respond to TLR ligands that in turn require MyD88 in order to signal. Only the TLR2/6 signaling heterodimer was active in these mice as they could respond to diacyl lipopeptides. Thus, it was suggested that I179 in MyD88 (Poc site) is required for its interaction with the BB loop of all TLRs apart from those of the TLR2/6 complex. Moreover, the mice having the Poc mutation were different than the MyD88-deficient ones in terms of their susceptibility to infection from *Streptococcus pyogenes*. In particular, the mice that were not expressing MyD88 were highly susceptible to such an infection, in contrast to the mice carrying the Poc mutation. Thus, it can be concluded that the TLR2/6 complex is essential in the host's defence against *Streptococcus pyogenes*.

3.2.2 MyD88-Dependent Pathways

MyD88-Dependent Activation of NF-κB, p38, and JNK

All TLRs that recruit MyD88 signal in a MyD88-dependent manner, which is very similar to the IL-1RI pathway in its activation of NF-κB and the MAPKs. Employment of MyD88 to liganded IL-1RI or TLRs results in the association of the interleukin-1 receptor-associated kinases (IRAKs) to the receptor signaling complexes (Figure 1).

It was first observed that although IL-1RI, IL-1RacP, and MyD88 were not protein kinases an IL-1RI-associated kinase activity could be co-precipitated with the receptor from cells stimulated by IL-1 (Martin et al., 1994). The associated kinase could phosphorylate the exogenous substrate myelin basic protein *in vitro* and an endogenous substrate of approximately 60 kDa (referred to as p60). This kinase activity seemed necessary for IL-1 responsiveness (Croston et al., 1995). Subsequently, a serine/threonine kinase associated with the receptor was purified from a human embryonic kidney (HEK) 293-cell line, which had been stably transfected to overexpress IL-1RI. This enzyme was named IRAK (Cao et al., 1996a). Its murine homologue was also identified (Trofimova et al., 1996) and named mouse Pelle-like protein kinase, based on its similarity to the kinase Pelle of the Toll pathway in the *Drosophila melanogaster*.

IRAK-1 seemed to possess a very important role in IL-1RI signaling. Its overexpression resulted in activation of downstream signaling events (Knop et al., 1998) and mutant HEK 293 cells not expressing IRAK-1 did not respond to IL-1 (Li et al., 1999c). In addition, fibroblasts from IRAK-1 knock out mice showed impaired IL-1 response *in vivo* (Thomas et al., 1999). Upon IL-1 stimulation, IRAK-1 associates with the receptor complex and becomes multiply phosphorylated. Initially, it was thought that this phosphorylation was very likely an autophosphorylation because a kinase-defective IRAK-1 (IRAK-1-Asp^{340}Asn) was not phosphorylated *in vitro* (Maschera et al., 1999). However, when the ATP binding site of IRAK-1 was mutated (K239A) to prevent its autophosphorylation, and transfected into IRAK-1$^{-/-}$ cells, the mutant became phosphorylated. It was thus proposed that IRAK-1 was phosphorylated by another kinase (Li et al., 1999c). Furthermore, the kinase activity of IRAK-1 was not necessary for IL-1RI signaling because kinase-dead IRAK-1 restored IL-1 responsiveness to IRAK-1$^{-/-}$ cells (Knop and Martin 1999; Li et al., 1999c; Maschera et al., 1999; Vig et al., 1999). Later, another kinase was identified based on its similarity to IRAK-1 and named IRAK-4 (Li et al., 2002). The discovery of this kinase could explain the observations regarding the phosphorylation and function of IRAK-1. It was shown that recombinant IRAK-4 could be autophosphorylated and also phosphorylate recombinant IRAK-1 *in vitro*. In contrast, recombinant IRAK-1 was able to become autophosphorylated, but could not phosphorylate IRAK-4 *in vitro*. IRAK-4 knock out mice presented a stronger phenotype than the IRAK-1 ones. IL-1 and TLR downstream signaling was severely impaired (Suzuki et al., 2002). Furthermore, IRAK-4-deficient humans were identified and they were susceptible to pyogenic bacterial infections (Picard et al., 2003). Thus the

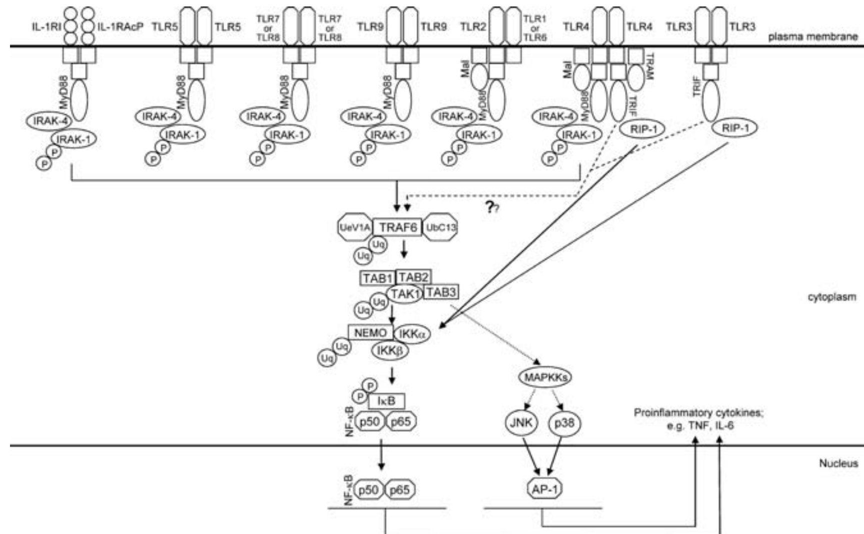

Fig. 1 TLR signaling pathways leading to the activation of NF-κB, p38, and JNK. IL-1RI and all the functional TLRs apart from TLR3, employ the adapter MyD88 in order to signal via intracellular proteins and activate NF-κB and MAPKs. MyD88 recruits IRAK-4 and IRAK-1 in the complex. IRAK-4 is the crucial serine/threonine kinase of the complex. It phosphorylates IRAK-1, which also becomes autophosphorylated to become completely activated. The IRAKs link the receptor complexes with TRAF6, which interacts with the ubiquitin-conjugating enzymes Uev1A and Ubc13 and becomes polyubiquitinated in order to become activated. This TRAF6 complex interacts with the TAK1/TAB1/TAB2/TAB3 complex. TAK1 seems to be the main kinase that phosphorylates and activates the NEMO/IKKα/IKKβ complex. The activation of the IKK complex also requires NEMO to become polyubiquitinated by the TRAF6/Uev1A/Ubc13 complex. In its turn, the IKKs phosphorylates IκB, which is pre-associated with NF-κB consisting of the subunits p50 and p65. This phosphorylation leads to proteosomal degradation of IκB, which enables NF-κB to translocate to the nucleus and bind to genes that contain NF-κB binding motifs; this leads to their transcription and expression. In this way, proinflammatory cytokines such TNFα and IL-6 are produced. TAK1 is also linked to the MAPK cascades. Two main proteins of these cascades that become activated are p38 and JNK. In their turn, they activate the transcription factor AP-1, that, like NF-κB, leads to production of proinflammatory cytokines. In the case of TLR4 signaling, MyD88 is not able to directly associate with the receptor. It requires Mal, which acts as a bridging adapter, bringing MyD88 to the membrane and allowing its downstream signaling. TLR4 utilizes all four signaling adapters. Apart from MyD88 and Mal, it can associate with TRIF and TRAM. TRAM is the bridging adapter that enables the association of TRIF with the receptor. TLR4 also activates the IKK complex through TRIF that can interact with RIP1. The same proteins are used by TLR3 in order to activate NF-κB. TLR3 only utilizes TRIF from the four signaling adapters. Some major inhibiting proteins at the adapter level are MyD88s, , IRAK-M, and RIP3, which are not shown in the figure, for simplicity sake (see text for details)

role of IRAK-4 protein in innate immunity was proved to be essential. However, the role of the kinase activity of IRAK-4 in IL-1RI/TLR signaling had not been completely understood until very recently. Initially, one study showed by reconstituting IRAK-4-deficient cells with kinase inactive IRAK-4 that its kinase activity was not necessary for IL-1RI-induced signaling and for the activation of IRAK-1

in vivo (Qin et al., 2004). On the contrary, another study showed that such a reconstitution partially restored the activation of IRAK-1 and signaling induced by IL-1 (Lye et al., 2004). Two very recent studies finally clarified the contradiction between the previous observations (Kawagoe et al., 2007; Koziczak-Holbro et al., 2007). In both studies “knock in” mice were generated; these carry a kinase-dead variant of IRAK-4 instead of the wild type. Comparison of these knock in mice with wild-type IRAK-4 mice and IRAK-4-deficient ones showed that the kinase activity of IRAK-4 is required for IL-1RI/TLR-induced responses and the phosphorylation of IRAK-1.

Thus, in the MyD88-dependent IL-1RI-TLR signaling (Figure 1), recruited IRAK-4 was the missing kinase required to phosphorylate IRAK-1. The protein IRAK-1 is subsequently autophosphorylated to become fully activated (Kollewe et al., 2004), leaves the receptor(s), and eventually is degraded by proteosomes (Yamin and Miller, 1997). Very recently, using a proteomic approach, it was shown that the originally phosphorylated *in vitro* endogenous substrate p60 observed in the IL-1RI signaling complex was actually autophosphorylated IRAK-4 (Brikos et al., 2007). Thus, the initially discovered IL-1RI-associated kinase activity (Martin et al., 1994) was partially or totally due to IRAK-4. This finding clarified the contradiction between the studies showing that the IL-1RI-associated kinase activity was essential for NF-κB activation (Croston et al., 1995) and those claiming that the kinase activity of IRAK-1 was not necessary for signaling (Knop and Martin 1999; Li et al., 1999c; Maschera et al., 1999; Vig et al., 1999).

Apart from IRAK-1 and IRAK-4, two additional members in the family of IRAKs—IRAK-2 and IRAK-M—have been identified (Muzio et al., 1997; Wesche et al., 1999). They are both inactive kinases because a critical aspartate residue in the catalytic site of IRAK-1 (D340) is replaced by a serine or asparagine, respectively. Overexpression of human IRAK-2 restored IL-1 and LPS responsiveness to cells that lack IRAK-1 (Li et al., 1999c; Wesche et al., 1999) and dominant negative forms of IRAK-2 inhibited IL-1RI activity. A murine homologue of IRAK-2 was also identified (Rosati and Martin, 2002) and later it was discovered that there are four murine alternative spliced isoforms of IRAK-2, two of which induce and two that inhibit signaling that leads to activation of NF-κB (Table 1). No IRAK-2 knock out mice have been reported, so the exact role of IRAK-2 in signaling remains to be resolved. IRAK-M (Wesche et al., 1999), when overexpressed in IRAK-1-deficient HEK 293 cells, could also reconstitute their responsiveness to IL-1. However, the IRAK-M knock out mice showed that this inactive kinase is a negative regulator of IL-1RI and certain TLRs (Kobayashi et al., 2002). It acts by inhibiting the dissociation of IRAK-1 from MyD88 and the formation of the complex of IRAK-1 with the next protein downstream: tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (Table 1).

The exact ways by which the IRAKs are recruited to the receptor complexes via MyD88 are not yet completely clear. All the complexes contain an N-terminal DD like MyD88. It was initially thought that this domain of IRAK-1 was necessary for interaction with the DD of MyD88 (Muzio et al., 1997; Wesche et al., 1997). However, in the case of IL-1RI signaling, mutated IL-1RI that could not recruit MyD88 still associated with IRAK-1. In addition, an alternatively spliced variant

Table 1 TLR signaling-inhibitory proteins at/near the adapter level

Protein	Type of inhibitor/host	Mechanism of inhibition
A20	Endogenous	Removes ubiquitin molecules from TRAF6, inhibiting MyD88- but also TRIF-dependent signaling
A46R	Exogenous/viral	Binds to MyD88, Mal, TRIF and TRAM but to SARM, inhibiting activation of NF-κB, the MAPKs and IRF3
A52R	Exogenous/viral	Interacts with IRAK-2 and TRAF6 inhibiting NF-κB activation
IRAK-2	Endogenous/2 out of the 4 spliced isoforms that exist in mice but not in human	Unknown-possibly they prevent the recruitment of the active IRAKs to MyD88 and IL-1RI or TLR signaling complexes
IRAK-M	Endogenous/shown inhibitory in mice	Prevents IRAK-1/IRAK-4 dissociation from MyD88
IRF4	Endogenous	Associates with MyD88 preventing its interaction with IRF5
MyD88s	Endogenous	Prevents IRAK-4 recruitment
NS3/4A	Exogenous/viral	Serine protease that cleaves TRIF inducing its degradation, thereby inhibiting TLR3-induced IRF3 and NF-κB activation
PIASy	Endogenous	Interacts with TRIF, IRF3 and IRF7 preventing their signaling
RIP3	Endogenous	Associates with RIP1 preventing its interaction with TRIF
SARM	Endogenous	Interacts with TRIF preventing directly or indirectly its signaling
SHP-2	Endogenous	Binds to TBK1 and inhibits TRIF-dependent signalling
SIKE	Endogenous	Prevents interactions of TBK1 and IKKε to TRIF and IRF3, inhibiting TRIF-dependent signalling through IRF3
SOCS1	Endogenous	Important for Mal degradation
ST2	Endogenous	Sequesters MyD88 and Mal competing their interaction with the TLRs
TGF-β	Endogenous	Causes ubiquitination and proteosomal degradation of MyD88
TRAF1	Endogenous	TRIF causes cleavage of TRAF1 (via a caspase) releasing a fragment with inhibitory function for TRIF-dependent signalling
TRAF4	Endogenous	Sequesters TRIF and TRAF6

Abbreviations

DCs, dendritic cells; DD, death domain; ID, intermediate domain; HCV, hepatitis C virus; HEK, human embryonic kidney; IκB, inhibitor of NF-κB; IKK, IκB kinase; IL-1, interleukin-1; IL-1RAcP, IL-1 receptor accessory protein; IL-1RI, interleukin-1 receptor type I; IRAK, IL-1 receptor-associated kinase; IRF, interferon-regulated factor; ISRE, IFN-stimulated response element; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; LRR, leucine-rich repeat; MAPK, mitogen-activated protein kinase; Mal, MyD88-adapter like; MyD88, myeloid differentiation primary response protein 88; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-kappaB; PAMPs, pathogen-associated molecular patterns; Poc, Pococurante; RIP, receptor interacting protein; SARM, sterile α and HEAT-Armadillo motifs; SH2, Src homology 2; SHP-2, SH2-containing tyrosine phosphatase 2; SIKE, suppressor of IKKε; SOCS1, suppressor of cytokine signaling 1; TAB, TAK1-binding protein; TAK-1, TGF-β-activated kinase 1; TBK1, (TANK)-binding kinase 1; TANK, TRAF-family-member-associated NF-κB activator; TGF, transforming growth factor; TICAM, TIR-containing adapter molecule; TIR, Toll/IL-1 receptor; TIRAP, TIR domain-containing adapter; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor; TRAM, TRIF-related adapter molecule; TRIF, TIR domain-containing adapter inducing IFN-β.

of MyD88 (MyD88s) which still contains the DD, inhibits IL-1RI/TLR signaling (Burns et al., 2003) (Table 1). MyD88s is shorter than MyD88 because it lacks the ID of MyD88. This domain enables MyD88 to associate with IRAK-4 but not with IRAK-1. The association of IRAK-4 to MyD88 is most likely happening via binding of the IRAK-4 DD to the MyD88 ID. Thus, MyD88s inhibits signaling because it interferes with this interaction, and as a result IRAK-1 cannot bind to IRAK-4 and become phosphorylated and degraded (Burns et al., 2003). In more detail, by using *in vitro* pull down assays it was shown that the parts of MyD88 which are responsible for its association with the IRAK-4 DD are a C-terminal portion of the MyD88 ID and its TIR domain (Lasker and Nair, 2006).

As mentioned, when IRAK-1 becomes multiply phosphorylated, it dissociates from the receptor(s) (in contrast to IRAK-4 (Brikos et al., 2007)) and binds to TRAF6. In IL-1RI signaling, however, TRAF6 has been shown to be recruited to the signaling complex via IRAK-1, which is acting as an adapter (Jiang et al., 2002). Consequently, both IRAK-1 and TRAF6 dissociate from the receptor complex together. TRAF6 (Cao et al., 1996b) is the protein considered to link the IL-1RI/TLR complexes with the activation of the NF-κB and the MAPK cascades (Figure 1). It interacts with the ubiquitin-conjugating enzyme E2 variant 1 (Uev1A) and the ubiquitin-conjugating enzyme 13 (Ubc13), becomes polyubiquitinated, and oligomerizes (Deng et al., 2000; Chen, 2005). In that way TRAF6 becomes activated and associates with the downstream proteins, transforming growth factor (TGF)-β-activated kinase 1 (TAK1) and the TAK1-binding proteins, TAB1, TAB2, and TAB3 (Wang et al., 2001). The series of events continues with TAK1 being ubiquitinated as well. TAK1 phosphorylates the inhibitor of NF-κB (IκB) kinases (IKK) complex, which is also polyubiquinated by Uev1A/Ubc13/TRAF6. The activated IKKs phosphorylate the IκB protein(s) that are pre-associated with NF-κB dimers in the cytoplasm of resting cells. This phosphorylation event leads to the release of NF-κB. The predominating dimer of NF-κB consists of two proteins, p50 and the p65, (also known as RelA). The protein p50 is responsible for the assembly of this dimer with IκBα, whereas p65 is needed for transactivation of gene expression. In MyD88-dependent signaling, IκBα becomes phosphorylated, polyubiquitinated, and then degraded (Brockman et al., 1995; Brown et al., 1995; DiDonato et al., 1995; Traenckner et al., 1995; Whiteside et al., 1995; DiDonato et al., 1996; Karin and Ben-Neriah, 2000). Consequently, NF-κB is able to translocate to the nucleus, where it binds and induces its target genes, which are responsible for inflammatory responses.

In more detail, the IKK complex consists of three subunits: one regulatory subunit IKKγ (Cohen et al., 1998; Rothwarf et al., 1998), also named as NF-κB essential modulator (NEMO) (Yamaoka et al., 1998) and two catalytic ones: IKKα and IKKβ (Verma et al., 1995; Rothwarf et al., 1998; Rottenberg et al., 2002). NEMO is a scaffold protein essential for the assembly of the IKKs (Li et al., 2001). Moreover, it also links the IKKs to the activated downstream molecule IκB (Yamamoto et al., 2001). NEMO is thus essential for the pathway. In cells which do not express it, NF-κB activation did not occur (Yamaoka et al., 1998). In addition, either disrupting the association of NEMO with IKKs or overexpressing a deletion-mutant of NEMO

blocks the activation of NF-κB (Le Page et al., 2001; May et al., 2002). The kinases of the complex, IKKα and IKKβ, can both phosphorylate IκBα on serines 32 and 36 (Lee et al., 1998). IKKβ is crucial for NF-κB activation and cannot be substituted by IKKα (Li et al., 1999b). It has been shown that phosphorylation of IKKβ at two sites in its activation loop is important for the activation of IKK (Delhase et al., 1999). In contrast, it has not been clarified whether IKKα, which was found to regulate IKKβ (O'Mahony et al., 2000), is a target for IL-1 (Delhase et al., 1999; Hu et al., 1999; Takeda et al., 1999). Two independent studies of IKKα knock out mice showed that it was not essential for activation of IKK complex and NF-κB by IL-1 (Hu et al., 1999; Takeda et al., 1999), but in another IKKα knock out mice report, a high reduction of activation of NF-κB was observed (Li et al., 1999a).

The TAK1 complex is not only essential for IKK activation. It is also linked to the MAPK kinase (MAPKK) 6 (or MKK6) and other MAPKKs such as MKK3 and MKK7 (Figure 1). Phosphorylation of MKK6 leads to the activation of JNK, whereas MKK3 and MKK7 are responsible for the activation of p38. JNK and p38 culminate in the activation of the transcription factor, activator protein-1 (AP-1), which plays a crucial role in the induction of inflammatory-response genes.

MyD88-Dependent Activation of the IRFs

Among the extremely significant recent discoveries on TLR signaling is that the MyD88-dependent pathway also activates some IRFs. One of them is IRF7, which was shown to participate in TLR7 and TLR9 signaling in a cell-specific manner (Figure 2). Specifically, IRF7 activation in these TLR pathways occurred in plasmacytoid, but not in conventional DCs. MyD88, IRAK-1, IRAK-4, and TRAF6 have been shown to directly associate with IRF7 (Hochrein et al., 2004; Honda et al., 2004; Kawai et al., 2004; Honda et al., 2005; Uematsu et al., 2005). Consequently, IRF7 translocates into the nucleus where it binds IFN-stimulated response element (ISRE) motifs. As a result, type I IFNs are produced. The role of IRAK-1 in these pathways is essential, as shown by the IRAK-1-deficient mice, which do not produce IFN-α following activation of either the TLR7 or TLR9 signaling pathways (Uematsu et al., 2005). It is therefore likely that IRAK-1 needs to phosphorylate IRF7 to induce its activation. In TLR9 signaling, it has also been shown that MyD88 needs to stably associate with the TIR domain of that receptor in order for the activation of IRF7 to occur (Honda et al., 2005).

Another transcription factor from the same family that is activated via MyD88 is IRF5 (Figure 2). In TLR4 and TLR9 signaling, IRF5 has also been found in a complex with MyD88 and TRAF6 (Takaoka et al., 2005). Consequently, it moves into the nucleus and binds ISRE motifs in the promoter regions of cytokine genes, inducing the production of IL-6, IL-12, and TNFα.

Last, in myeloid DCs, MyD88 has been shown to associate with IRF1 (Figure 2) (Negishi et al., 2006). Following that interaction, IRF1 translocates to the nucleus and activates several TLR-dependent genes. IRF1 is induced by the IFN-γ receptor 1 (IFNγR1), affecting in that way the TLR signaling cascades indirectly. Another

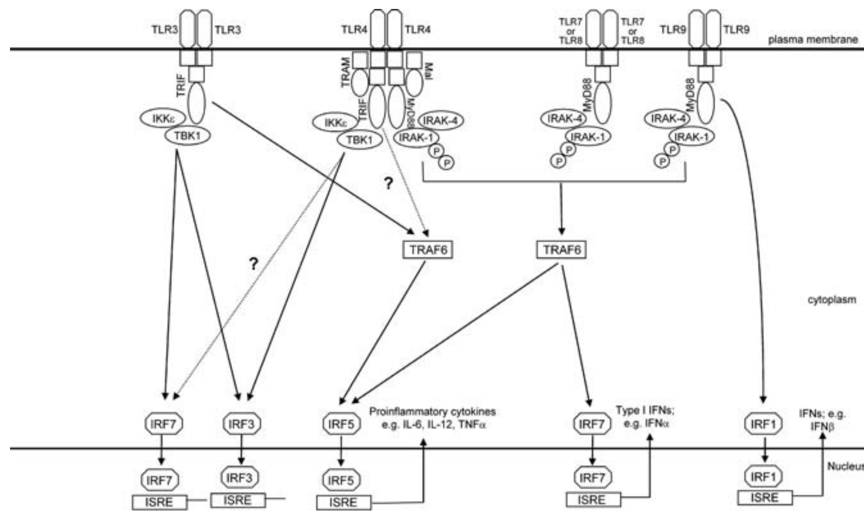

Fig. 2 TLR activation of IRFs. The TLRs that have been found to activate IRFs are TLR3, TLR4, TLR7, TLR8 and TLR9. Apart from the proteins responsible for the NF-κB activation, TRIF that can interact with IKKε and TBK1, which have been shown to induce the activation of IRF3 and IRF7 in the TLR3 and TLR4 signaling cascades (see text for details). IRF3 and IRF7, when activated, translocate into the nucleus, where they bind genes to their ISRE motifs and cause their subscription, leading to production of IFNs type I. TLR3 interacts via TRIF with TRAF6 and activates IRF5, which also translocates into the nucleus and binds to ISRE motifs of genes. However, in that case, instead of IFNs, proinflammatory cytokines are produced. Apart from these pathways, all the other TLR cascades that lead to activation of IRFs are MyD88-dependent. MyD88 and the IRAKs can interact with TRAF6 and IRF5 or IRF7, causing their activation and translocation to the nucleus. MyD88 also acts in a cell-specific way. In myeloid DCs, MyD88 binds to IRF1 in response to TLR7, 8, and 9. (In the figure, for simplicity sake, only the TLR9-recruited MyD88 is linked to IRF1 by an arrow.) MyD88 and IRF1 translocate together into the nucleus, where IRF1 binds to ISRE motifs, inducing the production of IFNs

discovery linking the TLR pathways with that of IFNγR1 is that, although this receptor does not contain a TIR domain, it can recruit MyD88 and thus activate p38 (Sun and Ding, 2006).

Apart from TRAF6, another member of the same family, TRAF3, was also found to participate in MyD88-dependent signaling of some TLRs. In particular, an interaction was observed between TRAF3 and MyD88. Furthermore, TRAF3-deficient mice presented an impaired production of type I IFNs and IL-10 in their TLR4 and TLR9 pathways (Hacker et al., 2006).

Mal—A Bridging Adapter in MyD88-Dependent TLR Signaling

MyD88 is not the only adapter protein required for TLR signal transduction. This notion became evident by the fact that in MyD88-deficient mice, the activation of NF-κB and the MAPKs, was only eliminated in response to IL-1 and the ligands

for TLR5, TLR7, TLR8 and TLR9. In response to TLR4 agonists, such activation was only delayed. Eventually, four more adapter molecules homologous to MyD88 were identified that could explain this phenomenon.

The first of these four adapters was Mal/TIRAP (Fitzgerald et al., 2001; Horng et al., 2001). It was discovered based on the similarity of its sequence to that of MyD88. Mal was initially shown to have an important role in TLR4 but not in IL-1RI signaling (Fitzgerald et al., 2001) because when a proline in its Box 2 was mutated to histidine, it blocked the response to endotoxin, but not to IL-1. Similarly, a peptide based on the BB loop of Mal could inhibit TLR4 but not TLR9 signaling. Later, Mal-deficient mice agreed with these original observations (Horng et al., 2002; Yamamoto et al., 2002a). The Mal knock out mice were very similar to the MyD88 ones in terms of TLR4 signaling. In both of these types of knock out mice, the activation of NF-κB and the MAPKs was delayed instead of abolished in response to TLR4. This activation was still induced by IL-1 and various TLR ligands, with an exception of those for TLR2. Interestingly, TLR2 signaling was even more affected than that of TLR4 in the Mal-deficient mice. In particular, NF-κB and p38 activation was completely impaired. Thus, it became clear that Mal is a member of the TLR2 and TLR4 MyD88-dependent pathways. The importance of Mal in the activation of innate immunity was also demonstrated by humans having a single nucleotide polymorphism in the gene encoding the adapter (Khor et al., 2007). This polymorphism results in the S180L Mal phenotype, which attenuates TLR2-induced signaling. It was discovered that the S180L heterozygosity protects its carriers against invasive pneumococcal disease, bacteremia, malaria, and tuberculosis.

The role of Mal in TLR4 signaling was uncovered recently. In contrast to MyD88, Mal has a binding domain for one of the main structural components of the plasma membrane; the phosphatidylinositol-4, 5-bisphosphate (PIP2), (Fitzgerald and Chen, 2006; Kagan and Medzhitov, 2006). Via this domain Mal can interact with PIP2, thereby facilitating the recruitment of MyD88 to the membrane. In that way, Mal and MyD88 can be in close proximity to TLR4 and thus able to associate with it. Thus, Mal is the bridging adapter, enabling MyD88 to be a component of the TLR4 signaling complex, and MyD88 is the signaling adapter, leading to the downstream events. Apart from the PIP2 binding domain, there are several other differences between Mal and MyD88. The first difference that became obvious since the discovery of Mal's sequence is that it does not contain a DD. In addition, in contrast to MyD88, Mal can associate with TRAF6. So it is likely that Mal is responsible for the recruitment of TRAF6 in the TLR2 and TLR4 signaling complexes (Mansell et al., 2004). Last, Mal is able to interact with Bruton's tyrosine kinase (Btk). This protein phosphorylates Mal and makes possible its downstream signaling (Gray et al., 2006). Btk has been shown to participate in TLR2 and TLR4 signaling (Jefferies et al., 2003; Liljeroos et al., 2007) by being involved in the phosphorylation of NF-κB on its p65 subunit (Doyle et al., 2005). However, the tyrosine phosphorylation of Mal is also required for its degradation (Mansell et al., 2006), providing a means of termination of its signaling. An essential protein for the occurrence of this degradation is the suppressor of cytokine signaling 1 (SOCS1)

and cells that cannot express SOCS1 respond to endotoxin at a higher extent than SOCS1 wild-type cells (Kinjyo et al., 2002; Nakagawa et al., 2002) (Table 1).

The discovery of Mal was the first evidence that different TLRs recruit different adapter molecules. Furthermore, the generation of Mal-MyD88 double knock out mice showed a delayed instead of abolished response in terms of TLR4 stimulation, indicating the existence of another protein that could lead to downstream signaling (Yamamoto et al., 2002a). Last, the MyD88 knock out mice presented signal transduction that was independent of MyD88 when not only TLR3, but also when TLR4 were liganded (Fitzgerald et al., 2003a; Fitzgerald et al., 2003b). In particular, that pathway culminates in the activation of the transcription factors IRF3 and IRF7, leading to the induction of IFN-α/β and IFN-inducible genes.

3.3 TRIF—The Signaling Adapter for the MyD88-Independent Pathways

3.3.1 TRIF-Dependent Pathways

The adapter that was eventually found to be essential for the MyD88-independent pathway is TRIF (Yamamoto et al., 2002b; Hoebe et al., 2003; Oshiumi et al., 2003a). It was discovered in two independent ways. One was by searching the databases for TIR-containing proteins (Yamamoto et al., 2002b) and the other was by using the yeast two-hybrid system using a part of TLR3 as a bait (Oshiumi et al., 2003a). The TRIF-deficient mice clearly identified the specificity and the role of the adapter. The inflammatory cytokine production was impaired in the TLR4 signaling, but unaffected following activation of TLR2, 7, and 9 (Figure 1). The activation of IRF3 and the following induction of IFN-β was impaired in both the TLR3 and the TLR4 pathways (Yamamoto et al., 2003a) (Figure 2). Thus, TRIF was the missing adapter that was utilized by TLR3 and was also required for TLR4-induced signal transduction. The TRIF/MyD88 double knock out mice shed further light on the function of TRIF in the TLR4 signaling cascades. These mice were unable to activate NF-κB (Hirotani et al., 2005). Thus, TRIF was responsible for the delayed activation of NF-κB that was observed in the MyD88 and Mal knock out mice (discussed in "Mal-A bridging adapter in MyD88-Dependent TLR signaling" under Sect. 2.3.2.2).

In order to signal downstream in both the TLR3 and TLR4 pathways, TRIF forms a complex with an IKK-like kinase named the TRAF-family-member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) (Fitzgerald et al., 2003a), the IKK homolog IKKε and IRF3. These interactions result in the phosphorylation of IRF3 by TBK1 and IKKε, leading to its activation (Figure 2). Consequently, IRF3 binds to the ISRE on target genes and induces the production of IFN-α/β (Sato et al., 2003). Similar to IRF3, IRF7 becomes activated via its phosphorylation by TBK1 and IKKε, and induces the expression of genes, by binding to their ISREs (Kawai et al., 2004; Honda et al., 2005; Uematsu et al., 2005) (Figure 2).

Furthermore, IRF5-deficient mice showed that IRF5 is also activated via TRIF in TLR3 signaling inducing IL-6, IL-12, and TNFα production (Takaoka et al., 2005) (Figure 2).

In TLR3 signaling, TRIF has also been shown to activate NF-κB (Figure 1). TRIF can bind to the TRAF6-TAK1-TAB2 complex that activates IKK (see Section 2.3.2.3.1) (Jiang et al., 2004). Very likely, this interaction occurs because TRIF contains consensus TRAF6-binding motifs. However, the exact role of TRAF6 in NF-κB activation via TRIF remains unclear, as two studies using TRAF6-deficient mice disagree with each other. In one of them TLR3-induced NF-κB activation from TRAF6-deficient murine embryonic fibroblasts was completely abolished (Jiang et al., 2004), whereas in the other study such signaling was unaffected (Gohda et al., 2004). An explanation for this contradiction could be that there is cell specificity in the usage of TRAF6 in order to activate NF-κB via TRIF.

TRIF also contains a receptor interacting protein (RIP) homotypic interaction motif, and thus it can associate with RIP1 and RIP3 (Meylan et al., 2004). RIP1 is important for TLR3-induced NF-κB activation and RIP3 has an inhibiting effect, preventing the interaction of RIP1 with TRIF (Table 1). In the case of TLR4 signaling, the role of RIP1 is unclear. According to Meylan et al., (2004), in RIP1-deficient murine embryonic fibroblasts the NF-κB activation was completely abolished in response to poly(I:C), but not to endotoxin. However, another study (Cusson-Hermance et al., 2005) showed that the activation was affected in both of the TLR3 and TLR4 pathways.

A difference among the TLR3 and TLR4 pathways that seems clear concerns the activation of NF-κB. In contrast to TLR3 signaling, TLR4 also leads indirectly to NF-κB activation. This is happening via TRIF, which, as previously mentioned, induces TNFα production via the activation of IRF3 (Figure 1). The produced TNFα is then secreted from the stimulated cells and binds to its receptor (TNFR) (Covert et al., 2005). Signaling induced by TNFR also culminates in NF-κB activation. In this way endotoxin can cause a prolonged activation that is important for the defense of the host.

Another difference between TLR3 and TLR4 is evident in the TRIF-dependent pathway that leads to binding of IRF3 to ISRE motifs (Figure 2). In the case of the TLR3 pathway, IRF3 forms homodimers that bind ISRE. In contrast, following activation of TLR4, IRF3 forms a heterocomplex with the p65 subunit of NF-κB which then binds ISRE (Wietek et al., 2003).

Apart from the pathways mentioned above, TRIF in contrast to the other adapter proteins is shown to be important for the induction of apoptosis in response to the activation of TLR3 and TLR4 (Han et al., 2004; Ruckdeschel et al., 2004; De Trez et al., 2005; Kaiser and Offermann, 2005). Along with TRIF this pathway requires involvement of the proteins RIP1, FADD, and caspase-8.

Finally, very recently TRIF was shown to play an important role in the induction of MHCII expression in DCs, which is essential for CD4 T cell activation (Kamon et al., 2006). This process occurs in response to endotoxin and involves the proteins RhoB and the guanine nucleotide exchange factor (GEF)-H1.

3.3.2 TRAM—A Bridging Adapter in TRIF-Dependent TLR Signaling

From the previous section it is clear that TRIF seems to interact with a wider range of proteins in comparison to the other adapters. It is thought that TLR3 associates with TRIF *directly*. On the other hand, TLR4 requires TRAM to *engage* TRIF (Fitzgerald, et al., 2003b; Oshiumi et al., 2003b; Yamamoto et al., 2003b; McGettrick et al., 2006; Rowe et al., 2006). Thus, TRAM is also a bridging adapter like Mal and is different than all the other adapters in the fact that it is strictly utilized by TLR4 (Figure 2). As in the case of Mal, TRAM also associates with the plasma membrane. However, unlike Mal, this depends on myristioylation of the N-terminus of TRAM. Mutation of this myristioylation motif makes TRAM inactive. Furthermore, the activation of TRAM is dependent on the phosphorylation of its serine 16 by protein kinase Cε. Mutant TRAM that cannot be phosphorylated at this site does not function, and inhibition of this phosphorylation impairs TRAM signaling. Thus, this modification is required in order for TRAM to signal, but its exact role is unknown.

3.4 Inhibition of TLR Signaling at the Adapter Level

3.4.1 SARM and Its Role in Suppressing TLR Signaling

The fifth and last adapter is SARM. It was discovered as a human gene that encodes a protein of unknown function with a sterile α-motif (SAM), and it was found to be structurally similar to Armadillo/β-catenin (it contains HEAT/Armadillo repeats) (Mink et al., 2001). It was also shown that SARM was conserved in *Caenorhabditis elegans, Drosophila melanogaster*, and *Mus musculus*. The existence of its TIR domain was observed later (Couillault et al., 2004; Liberati et al., 2004). Initially, the significance of SARM in TLR signaling had not been recognized, as it could not activate NF-κB when overexpressed. In that aspect it is dissimilar to all the other TIR-containing adapters. It was only recently that its role started to be revealed. In contrast to the other adapters, SARM acts as an inhibitor of NF-κB and IRF activation through its interaction with TRIF (Carty et al., 2006) (Table 1). However, exactly how SARM prevents the function of TRIF remains to be identified. It is not clear if SARM forms a complex with TRIF, disabling it to interact with other proteins downstream, or if SARM facilitates the interaction of TRIF with another inhibitory protein. The knockdown of SARM expression using small interfering RNA (siRNA) leads to an increased production of chemokines and cytokines in response to TLR3 and TLR4 activation. Very interestingly, the expression of SARM is increased in response to LPS. It is therefore very likely that this is a negative feedback mechanism used to inhibit prolonged TLR3 and TLR4 activation that could have dangerous effects for the host.

3.4.2 Additional Negative Regulators of TLR Adapter Signaling

Endogenous Inhibitory Proteins at the Adapter Level

Apart from SARM, MyD88s, IRAK-M, SOCS1, and RIP3 mentioned in the previous sections, there are several other proteins of the host that inhibit TLR signaling at the adapter level (Table 1).

One of these proteins is the antiinflammatory cytokine-transforming growth factor-β1 (TGF-β1) (Table 1) (Naiki et al., 2005). It has been shown that TGF-β1 inhibits NF-κB activation via the MyD88-dependent pathway in response to ligands for TLR2, 4, and 5. In particular, it induces ubiquitination of MyD88 and its subsequent proteosomal degradation. Thus, it decreases the levels of MyD88 in the cells of the host without affecting its mRNA levels. TGF-β1 does not affect the TRIF-dependent pathway of TLR4.

Another inhibitory protein is ST2 (Table 1) (Brint et al., 2004). This transmembrane protein belongs to the IL-1RI/TLR family of receptors because it also contains a TIR domain. When overexpressed, ST2 has been found to inhibit MyD88-dependent signaling induced by IL-1RI and TLR4, but not by TLR3, as it does not utilize that adapter. A recombinant ST2 protein construct is able to interact with Mal and MyD88, but not with TRIF. Thus, the molecular basis of the inhibitory role of ST2 could be its sequestration of the adapters MyD88 and Mal.

Additional adapter-mediated signaling inhibitory proteins belong to the family of TRAFs. In contrast to TRAF6 and TRAF3, which are essential for TLR signaling (see previous sections), TRAF1 and TRAF4 play an inhibitory role (Table 1) (Takeshita et al., 2005; Su et al., 2006). TRAF1 can associate with the adapter TRIF, and its overexpression prevents TLR3 signaling, thereby inhibiting NF-κB, ISRE, and IFN-β promoter activation. In addition, TRIF induces cleavage of TRAF1 (via a caspase), which is essential for its inhibitory function. TRAF4 can interact also not only with TRIF but also with TRAF6. Thus, TRAF4 is able to prevent NF-κB activation induced by TLR2, 3, 4, and 9, and IFN-β promoter activation mediated by both TLR3 and TLR4.

Another inhibitory protein of TLR signal transduction is IRF4 (Table 1) (Negishi et al., 2005). As mentioned in the MyD88-dependent signaling pathways section (2.3.2.2.2), IRF5 and 7 associate with MyD88, which leads to production of type I IFNs and proinflammatory cytokines. IRF4 can bind MyD88, thereby not allowing formation of the MyD88-IRF5 complex. Conversely, the association of IRF4 with MyD88 does not prevent the formation of the MyD88-IRF7 complex. Consequently, IRF4 only inhibits MyD88-IRF5-mediated signaling. In particular, IRF4 suppresses the translocation of IRF5 into the nucleus in response to TLR9. The fact that IRF4 can be a negative feedback regulator is supported by the observation that its mRNA levels are increased following treatment of murine peritoneal macrophages with ligands for TLR4, 7, and 9, all of which activate the MyD88-IRF5 pathway.

At the adapter-IRF level, TLR signaling can be further controlled, by a member of the PIAS family, called PIASy (Table 1) (Zhang et al., 2004). PIAS are protein

inhibitors of the activated signal transducer and activator of transcription (STAT) family. PIASy interacts with TRIF, IRF3, and IRF7 and inhibits their activation of ISRE. It also suppresses NF-κB activation and IFN-β production induced by TRIF. It does not, however, affect the apoptotic effects of TRIF (mentioned in the previous section 3.4.1).

Activation of the TRIF-dependent pathway, can also be regulated by the suppressor of IKKε (SIKE) (Table 1) (Huang et al., 2005). Apart from IKKε, SIKE also interacts with TBK1, but not with RIP1 and TRAF6. In fact SIKE is pre-associated with TBK1 in nonstimulated cells, and dissociates from it when the cells are stimulated with TLR3 ligands. As a result, SIKE was shown to inhibit the association of TBK1 and IKKε with TRIF and IRF3, disrupting in that way ISRE and IFN-β promoter activation. On the other hand, as SIKE does not affect the interactions of TRIF with TRAF6 and RIP1, it does not act as an inhibitor of the TLR3-induced NF-κB activation.

Src homology 2 (SH2)-containing tyrosine phosphatase 2 (SHP-2) has been identified as an additional endogenous inhibitor of TRIF-dependent signaling (Table 1) (An et al., 2006). It also, like SIKE, associates with TBK1 and blocks TBK1-induced IFN-β expression. In general, SHP-2 was shown to downregulate IFN-β production in TLR3 and TLR4 signaling. SHP-2 also negatively affected the production of IL-6, TNFα, and the activation of the MAPKs in response to TLR3. This inhibitory function of SHP-2 does not depend on its tyrosine phosphatase activity.

Last, another cytoplasmic protein of the host which is important for terminating TLR signaling is A20 (Table 1) (Boone et al., 2004). This is an enzyme that can remove ubiquitin moieties from TRAF6. As mentioned in a previous section, the ubiquitination of TRAF6 is essential for its signaling resulting in NF-κB activation. In particular, it was shown that A20 is crucial in the termination of NF-κB activation induced by TLR4. However, A20 does not only suppress TLR4 signaling; A20-deficient macrophages express enhanced levels of TNF, IL-6, and nitric oxide in response to ligands for TLR2, TLR3, and TLR9, suggesting that A20 negatively regulates these TLRs as well.

Exogenous Inhibitory Proteins at the Adapter Level

Apart from the previously mentioned endogenous proteins, there are several exogenous proteins of invading agents that are used by them in order to prevent the activation of TLR signaling. Two of these inhibitors, A46R and A52R (Table 1), are expressed by the vaccinia virus. A46R contains a TIR domain and is able to interact with MyD88. In particular, it inhibits NF-κB and MAPKs activation in response to IL-1. A46R also blocks NF-κB activation induced by ligands for TLR2/1, TLR2/6, TLR4, TLR5, TLR7, and TLR9. However, apart from MyD88, A46R can also interact with TLR4, Mal, TRAM, and TRIF, but not with SARM. In that way, the viral protein can completely abolish TLR4 signaling that leads to NF-κB activation, and it can inhibit IRF3 activation mediated by TLR4 and TLR3. The other viral protein, A52R (Harte et al., 2003), can also disrupt MyD88-dependent signaling although it does not contain a TIR domain. A52R can interact with IRAK-2 and TRAF6, and

blocks NF-κB activation by TLR1/2, TLR2/6, TLR3, TLR4, and TLR5. In another study, a peptide that was designed based on the sequence of A52R, could suppress TLR signaling and reduce bacterial middle ear inflammation (McCoy et al., 2005).

Last, an additional suppressor of TLR signaling is the serine protease NS3/4A of hepatitis C virus (HCV) (Table 1). NS3/4A cleaves TRIF, causing its degradation and disrupting TLR3-induced IRF3 and NF-κB activation.

4 Final Perspectives

Since the discovery of the essential role that TLRs play for the innate and adaptive immunity, a lot of discoveries have been made regarding their signaling cascades. Continuous research widely increases the number of proteins that participate in the TLR pathways and the interactions that take place during their course. Currently, there is a quite clear understanding on the molecular basis of the initiation of signaling by the TLRs and those sets of genes that they activate in order to activate an immune response.

Several studies have shown that TLRs are activated by a wide spectrum of ligands. As a consequence of this liganding the first step in the initiation of signaling by the TLRs is their homo- or heterodimerization. This pairing provides the structural platform in the cytoplasm of the cells, where the adapter molecules bind and transmit signaling intracellularly.

The existence of these adapters explains, to some extent, the differences in the TLR pathways. The reason is that not all TLRs recruit the same adapter molecule(s). Thus, depending on which adapter or adapters associate with a certain TLR, several different biochemical pathways are activated that culminate in the production of proinflammatory cytokines or IFNs and the genes induced by them. In addition, the adapters link the TLRs to other biochemical pathways by interacting with some of their components. One of the recent discoveries is that TLR signaling leads through TRIF to apoptosis.

However, the adapter molecules are not only responsible for determining which pathways are going to be activated in response to the variety of TLR ligands. The adapters are also essential for the regulation of the TLR cascades.

In fact, one of the very recent discoveries is that one of the adapters—SARM—plays an inhibiting role in TLR signaling. Apart from this adapter, several endogenous proteins have been identified that target the TLR signaling pathways at the adapter level in order to inhibit them and avoid their overactivation that could be extremely dangerous for the host.

This method of inhibiting TLR signaling is also used by certain viruses in order to block the activation of the immune system and survive. In particular, these invaders have evolved to express proteins that are inhibiting the interactions of the adapters with other proteins of the TLR signaling pathways. Furthermore, one of the adapters (TRIF) can also be cleaved by an HCV protease, preventing in that way its signaling.

It is now believed that the adapter molecules provide potential targets for therapeutic intervention. It is hoped that soon new drugs will be designed as adapter-mimetics that could have antimicrobial action or suppress chronic inflammation.

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