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Review

TLR-4, IL-1R and TNF-R signaling to NF-κB: variations on a common theme

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Abstract. Toll-like receptors (TLRs) as well as the receptors for tumor necrosis factor (TNF-R) and interleukin-1 (IL-1R) play an important role in innate immunity by regulating the activity of distinct transcription factors such as nuclear factor-κB (NF-κB). TLR, IL-1R and TNF-R signaling to NF-κB converge on a common IκB kinase complex that phosphorylates the NF-κB inhibitory protein IκBα. However, upstream signaling components are in large part receptor-specific. Nevertheless, the principles of signaling are similar, involving the recruitment of specific adaptor proteins and the activation of kinase cascades in which protein-protein interactions are controlled by poly-ubiquitination. In this review, we will discuss our current knowledge of NF-κB signaling in response to TLR-4, TNF-R and IL-1R stimulation, with a special focus on the similarities and dissimilarities among these pathways.

Keywords. Toll-like receptor 4, interleukin-1, tumor necrosis factor, NF-κB, signal transduction.

NF-κB, does it still need to be introduced?

Nuclear factor κB (NF-κB) is the generic name of a family of transcription factors that regulate the expression of a large number of genes involved in immune and inflammatory responses, as well as in cell survival, cell proliferation and cell differentiation. NF-κB transcription factors are activated in response to various stimuli, including cytokines, infectious agents, injury and other stressful conditions requiring rapid reprogramming of gene expression. Inappropriate activation of the NF-κB signaling pathway is implicated in the pathogenesis of chronic inflammation and autoimmunity, certain hereditary disorders and various cancers. In mammals, the NF-κB family consists of five proteins sharing a highly conserved Rel homology domain: c-Rel, RelB, p65 (= RelA), p105 (= NF-κB1) and p100 (=NF-κB2). The first three contain C-terminal transactivation domains, while the others share a long C-terminal domain with multiple copies of ankyrin repeats, which inhibit their activation.

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Partial proteolysis of p105 and p100 results in the formation of the DNA-binding proteins p50 and p52, respectively. The highly conserved Rel homology domain is responsible for DNA binding, dimerization, nuclear translocation and interaction with the inhibitor of κB (IκB) proteins. All NF-κB family members are able to form hetero- and homodimers, except for RelB, which only forms heterodimers. The prototypical complex corresponds to a heterodimer of p65 and p50 subunits. NF-κB is kept inactive in the cytoplasm by members of the IκB family (IκBα, IκBβ, IκBγ/p105, IκBδ/p100 and IκBε), all containing ankyrin repeats necessary for binding NF-κB and blocking its nuclear import. Upon stimulation with for example tumor necrosis factor (TNF), interleukin-1 (IL-1) or lipopolysaccharide (LPS), different signaling cascades are activated, ultimately resulting in activation of an IκB kinase (IKK) complex, comprising two catalytic kinase subunits (IKKα and IKKβ) and a regulatory protein [IKKγ/NEMO (NF-κB essential modulator)/IKKAP1 (IKK associated protein 1)/FIP3 (type 2 adenovirus E3–14.7-kD interacting protein)]. Each kinase contains in its N-terminus the catalytic domain and in its C-terminus a helix-loop-helix, a leucine zipper structure and the NEMO binding domain [1, 2]. IKKγ contains several structural domains: in its N-terminal half a first coiled-coil motif responsible for interaction with IKK kinases [3, 4], and in its C-terminal half a second coiled-coil motif, a leucine zipper and a zinc finger motif involved in oligomerization and interaction with upstream signaling molecules [5, 6]. In addition, IKKγ contains a ubiquitin binding domain between its second coiled-coil motif and its leucine zipper, shown to bind K63-linked polyubiquitin chains [7, 8]. Activation of the IKK-complex results in phosphorylation of IκBα at two specific serine residues (Ser32 and Ser36) by IKKβ [9,10], followed by poly-ubiquitination and subsequent degradation of IκBα by the 26S proteasome. Thereby, NF-κB is set free and translocates to the nucleus where it can bind the promoters of genes containing specific NF-κB-binding sequences. IκBα poly-ubiquitination on Lys21/Lys22 is mediated by Ubc4/5 ubiquitin conjugating enzyme (E2), together with the E3-ubiquitin-protein ligase SCF-βTrCP [Skp1-Cul1-F-box ligase containing the F-box protein β-transducin repeat-containing protein (βTrCP)]. βTrCP consists of βTrCP1 and βTrCP2, specifically recognizing phosphorylated IκBα, while the SCF complex contains the RING domain protein Roc1/Rbx1 and binds Ubc4/5. Subsequently, Ubc4/5 poly-ubiquitinates IκBα at the two conserved lysine residues [11]. A fourth protein that has been isolated as part of the IKK complex is ELKS, a protein rich in glutamate, leucine, lysine and serine. ELKS has been implicated as an essential scaffolding component in the activation of NF-κB in response to TNF and IL-1, where it would play a role in the recruitment of IκBα to the IKK complex [12]. Nonetheless, the role of ELKS in IKK and NF-κB activation needs to be further confirmed through generation of a knockout mouse strain.

NF-κB activation as described above is referred to as the canonical or classical NF-κB pathway, which is activated by different receptors, including TLRs, TNF-R and IL-1R. Some other receptors such as the lymphotoxin-β receptor can activate an alternative NF-κB signaling pathway that results in the phosphorylation of p100 instead of IκBα [1, 2]. We will only focus on the canonical pathway in this review, as much more data dealing with the roles of post-translational modifications targeting the signaling molecules involved in that pathway have been recently generated. Moreover, the role of the alternative pathway in the cascades triggered upon stimulation by TLR ligands or pro-inflammatory cytokines is not so clearly established.

IL-1R and TLR-4 signaling to NF-κB

The TLR/IL-1R superfamily groups multiple receptors, which all play a crucial role in innate and adaptive immunity [13]. The TLR subfamily consists of 13 members that contain leucine-rich repeat motifs in their extracellular domain, which recognize distinct microbial patterns such as LPS, flagellin, viral double-stranded RNA and unmethylated CpG motifs. Members of the IL-1R subfamily are characterized by Ig(immunoglobulin)-like structures in their extracellular domain that bind specific IL-1 related cytokines, which are involved in multiple immunological and inflammatory processes. In contrast to their distinct extracellular domains, all members of the TLR/IL-1R family are characterized by an intracellular TIR domain. Here we will focus on IL-1RI (the receptor for IL-1) and TLR-4 (which recognizes LPS in cooperation with circulating LPS-binding protein, the co-receptor CD14 [14] and MD-2 [15]) as prototypes for each subfamily.

Upon binding of IL-1, the IL-1RI associates with IL-1 receptor accessory protein (IL-1RAcP), forming a functional signaling receptor complex (Fig. 1) [16, 17]. Secondly, the TIR domain containing adaptor protein MyD88 is recruited to the receptor complex [18]. This leads to the translocation of the serine/threonine kinase IL-1 receptor-associated kinase 1 (IRAK-1), together with the adaptor protein Tollip, into the IL-1RI complex [18–20]. IRAK-1 is a multidomain protein containing an N-terminal death domain (DD) that interacts with the DD of MyD88 [21], followed by

a domain rich in proline, serine and threonine residues (ProST region), a serine/threonine-specific protein kinase domain and a C-terminal domain containing three TNF receptor-associated factor 6 (TRAF6) interaction consensus motifs [22–24]. TRAF6 is a member of a larger TRAF family, containing an N-terminal RING domain, five zinc finger structures and a conserved C-terminal TRAF domain. The TRAF domain is responsible for TRAF6 oligomerization, whereas the RING domain has E3 ubiquitin ligase activity (see also below). Both TRAF6 as well as the IRAK-1 related kinase IRAK-4 are recruited at the activated receptor complex in order to form complex I [21, 25–27]. IRAK-4 becomes activated by intramolecular autophosphorylation of three residues (Thr342, Thr345 and Ser346) within its activation loop, which is required for optimal kinase activity of IRAK-4 [28]. Activation of IRAK-4 leads to phosphorylation of IRAK-1 on Thr209 and Thr387 in its activation loop, leading to full kinase activity [23, 29]. Subsequently, IRAK-1 becomes hyperphosphorylated in its ProST region, probably via autophosphorylation, resulting in its dissociation from MyD88 and Tollip, but not from the downstream signaling molecule TRAF6 [21, 23]. However, the kinase activity of IRAK-1 is dispensable for IL-1 signaling towards NF-κB, as complementation of cells deficient in IRAK-1 with a kinase death mutant can restore IL-1-induced NF-κB activation [29]. In contrast, contradictory data are published about the necessity of the kinase activity of IRAK-4. In human IRAK-4-deficient cells, restoration of IL-1-mediated NF-κB activation can be achieved with a kinase inactive mutant [25], while this was not the case for murine embryonic fibroblasts derived from IRAK-4 knockout mice [26]. Furthermore, Qin et al. demonstrated that only impairment of the kinase activity of both IRAK-1 and IRAK-4 abolishes IL-1 signaling, suggesting redundancy of both kinase activities [25]. These contradictory outcomes might reflect cell type- or species-specific differences.

After dissociation from the receptor complex, the IRAK-1-TRAF6 complex interacts with a pre-existing TAK1 (transforming growth factor β-activated kinase 1)-TAB1 (TAK1-binding protein)-TAB2 (or TAB3) membrane-bound complex, thus forming complex II [27]. The TRAF6-TAK1-TAB1-TAB2/3 complex then translocates to the cytosol, whereas IRAK-1 stays at the membrane and becomes poly-ubiquitinated [27, 29 –31]. The precise function of IRAK-1 poly-ubiquitination is still unclear. Two types of poly-ubiquitination have been described, depending on the specific ubiquitin lysine residue that is used for making the inter-ubiquitin linkage. K48-linked poly-ubiquitination triggers proteasome-dependent degradation, whereas K63-linked poly-ubiquitination does not trigger degradation of the modified protein but forms a recognition signal for the binding of other proteins [11]. IL-1 has been shown to trigger IRAK-1 degradation by the proteasome, which is indicative of K48-poly-ubiquitination [29–31]. However, Cohen and colleagues recently demonstrated that IL-1 triggers K63-poly-ubiquitination of IRAK1 (which helps its binding to NEMO) instead of K48-poly-ubiquitination [32]. Moreover, they showed that the IL-1-mediated degradation of IRAK-1 does not occur through the proteasome, which is in agreement with Ordureau et al. who claimed that the proteasome inhibitor MG-132 had no effect on the IL-1-induced disappearance of IRAK-1 [32, 33]. The identification of the specific ubiquitin ligases for IRAK-1 might further clarify the function of different types of IRAK-1 poly-ubiquitination. In this context, different members of the Pellino family were shown to K63-poly-ubiquitinate IRAK-1 using a novel CHC2CHC2 RING motif, thereby functioning as an E3 ubiquitin ligase for IRAK-1 [33–36]. Furthermore, binding of Pellino proteins with IRAK-1 and IRAK-4 depends on IRAK kinase activity and is associated with phosphorylation of Pellino [33, 34, 37], which has been shown to enhance E3 ligase activity as well as degradative poly-ubiquitination of Pellino [33, 35]. Clearly, the exact function of IRAK-1 and Pellino poly-ubiquitination needs more investigation.

In the cytoplasm, TRAF6 interacts with the E2 ubiquitin-conjugating enzyme complex Ubc13/Uev1A, thus forming complex III. Ubc13/Uev1A cooperates with the RING finger domain of TRAF6, which acts as an E3-ubiquitin ligase, and results in K63-linked poly-ubiquitination of TRAF6, which is necessary for IKK and c-jun N-terminal kinase (JNK) activation [22, 38, 39]. The RING finger domain of TRAF6 is necessary for Ubc13 interaction and self-association [40]. The crucial role of TRAF6 in NF-κB signaling is demonstrated by the fact that cells isolated from TRAF6-deficient mice fail to activate NF-κB in response to IL-1 or LPS [41, 42]. Ubc13 is also required for TRAF6 poly-ubiquitination and IKK activation *in vivo*, as spleen lysates from LPS-treated mice heterozygous for Ubc13 show lower poly-ubiquitination of TRAF6 than isolates from wild-type mice. IκBα degradation in response to LPS is also impaired in macrophages and splenocytes from *Ubc13*$^{+/-}$ mice [43]. In contrast, Yamamoto et al. revealed that Ubc13-deficient murine embryonic fibroblasts show normal activation of NF-κB in response to IL-1, whereas JNK activation is impaired [44]. Furthermore, TRAF6 poly-ubiquitination and TAK1 activation are normal in Ubc13-deficient cells; however, IKKγ poly-ubiquitination upon IL-1 stim-

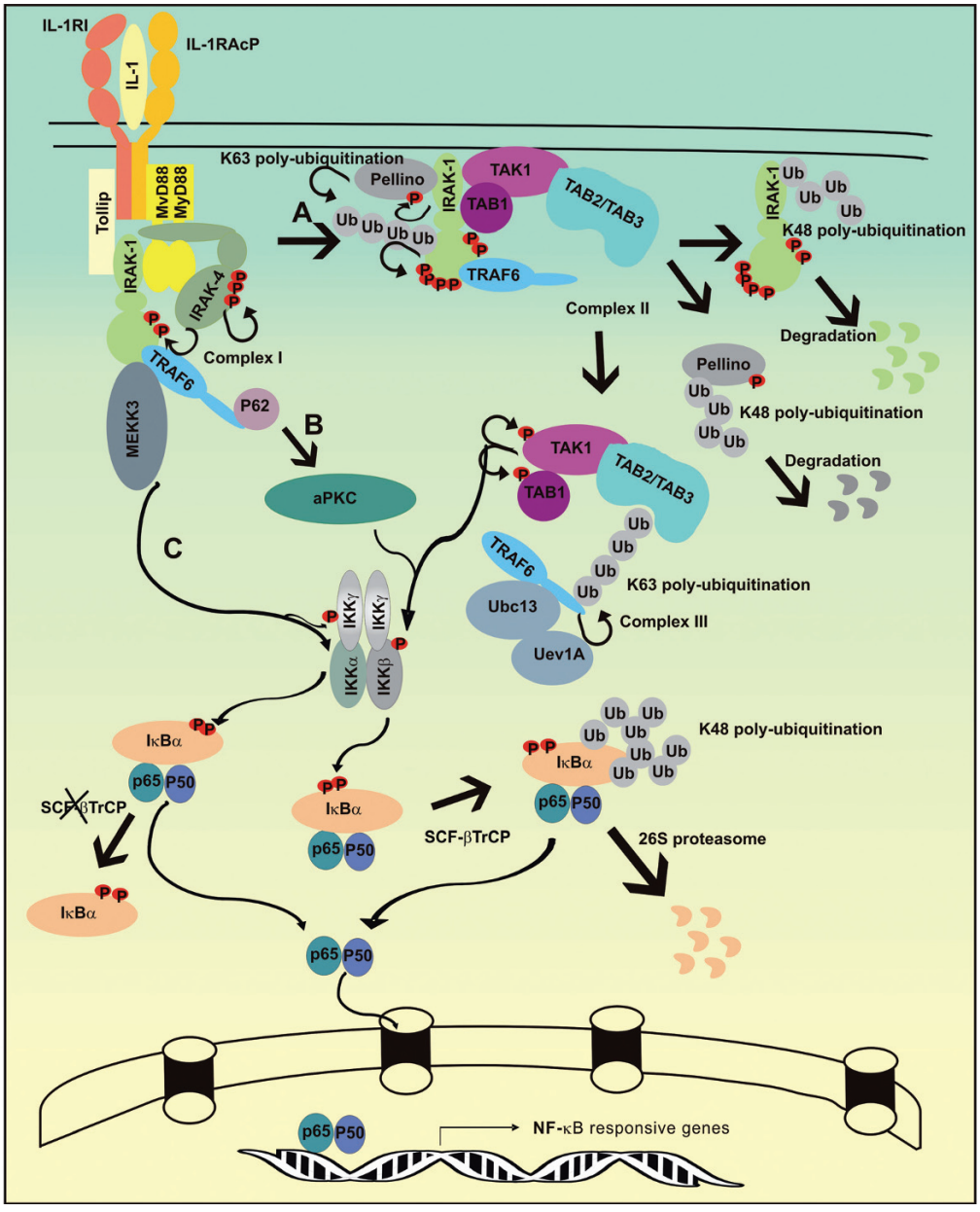

Figure 1. IL-1 signaling pathways to NF-κB. (*A*) TAK1-dependent pathway. Upon receptor triggering with IL-1, IL-1RI forms a complex with IL-R1AcP, leading to recruitment of MyD88, Tollip, IRAK-1, IRAK-4 and TRAF6 to form complex I. Formation of complex I triggers IRAK-4 autophosphorylation and the phosphorylation of IRAK-1. Subsequently, IRAK-1 leaves the receptor complex together with TRAF6 and associates with the pre-formed TAK1-TAB1-TAB2/TAB3 complex at the membrane (complex II). Here IRAK-1 is K63-poly-ubiquitinated by Pellino, which is itself phosphorylated by IRAK-1. Subsequently, IRAK-1 most likely becomes K48-poly-ubiquitinated and degraded. Likewise, phosphorylation of Pellino by IRAK-1 leads to K48-poly-ubiquitination and degradation of Pellino. Then, TRAF6-TAK1-TAB1-TAB2/TAB3 leaves the membrane (complex III). In the cytoplasm, TRAF6 associates with the E2 ubiquitin-conjugating enzyme (Ubc13/Uev1A) and undergoes auto-ubiquitination with K63-linked poly-ubiquitin chains. This triggers the binding of TAB2 and activation of TAK1, which subsequently phosphorylates IKKβ. IKKβ phosphorylates IκBα, leading to its K48-linked poly-ubiquitination by SCF-βTrCP and proteasome-dependent degradation. In this way, NF-κB (shown as p65/p50 dimers) is set free and translocates to the nucleus to bind the promoters of responsive genes. (*B*) TAK-1-independent/PKC-dependent pathway. Upon IL-1 stimulation, TRAF6 can also interact with p62, leading to activation of atypical PKCs and phosphorylation of IKKβ. (*C*) TAK-1-independent/MEKK-3-dependent pathway. Upon IL-1 stimulation, IRAK-1 and TRAF6 can also interact with MEKK-3, leading to activation of IKKα, which subsequently phosphorylates IκBα in a way that does not trigger its recognition by SCF-βTrCP and proteasome-dependent degradation. Phosphorylated IκBα dissociates from NF-κB, thus allowing NF-κB to translocate to the nucleus.

ulation is impaired [44]. As UbcH7 is also known to mediate K63-linked poly-ubiquitination of TRAF6 [45], there might be redundancy for TRAF6 auto-ubiquitination, but not for IKKγ poly-ubiquitination by TRAF6. Furthermore, RNA interference-mediated knock-down of either Ubc13 or Uev1A abolishes TRAF6-induced NF-κB activation, IKKγ poly-ubiquitination and p65 nuclear translocation upon LPS stimulation [46]. Finally, TAB2, TAB3 and IKKγ also become K63-poly-ubiquitinated by TRAF6 [22, 47–49]. A role for oligomerization of TRAF6 in enhancing its ubiquitinating activity has also been postulated [39,42,43]. In fact, 'TRAF-interacting protein with a forkhead-associated domain' (TIFA) was found to interact with TRAF6, thereby inducing TRAF6 oligomerization and enhancing TRAF6 ubiquitinating activity [50]. Endogenous TIFA constitutively associates with TRAF6, whereas it only interacts with IRAK-1 upon IL-1 stimulation [51]. In addition, overexpression of TIFA induces NF-κB and JNK activation in HEK293 cells [52]. Lysine 124 in TRAF6 was identified as the main ubiquitin acceptor site for auto-ubiquitination, and mutation of this lysine leads to impaired TAK1, IKK and JNK activation [22]. Moreover, complementation of TRAF6-deficient cells with this mutant or a RING finger mutant does not restore TRAF6 auto-ubiquitination, IKKγ poly-ubiquitination, and subsequent IKK activation upon IL-1 stimulation [22], demonstrating an essential role for TRAF6 E3 ligase activity in signaling. To conclude, oligomerization of TRAF6 might lead to auto-poly-ubiquitination of TRAF6, which is necessary for IL-1- and LPS-induced NF-κB activation, whereas TRAF6-induced poly-ubiquitination of NEMO might rather play a role in IL-1-induced JNK activation.

TAB1, TAB2 and TAB3 have been described as adaptors for TAK1 [48, 53]. However, the role of TAB1 in IL-1-induced NF-κB activation has been argued. In TAB1 knockout cells, no effect on IL-1-induced NF-κB activation could be observed, suggesting that TAB1 is dispensable for this [54]. In contrast, although RNA interference-mediated knock-down of TAB1 had no effect on IL-1-induced nuclear localization of p65, the levels of IL-6, IL-8 and GM-CSF upon IL-1 stimulation of these cells was markedly reduced [55]. Furthermore, Mendoza et al. could not observe any IL-1-induced activity of TAK1 in TAB1-deficient cells [56]. These contradictory results might be explained by the existence of TAK1-independent signaling pathways (see further) or possible effects of TAB1 on p65 transactivation. TAB2 and TAB3 contain two ubiquitin-binding domains that are required for their NF-κB activating function [49, 57]: an N-terminal CUE domain and a C-terminal nuclear protein localization 4 zinc finger (NZF) [49, 53]. Although the CUE domain interacts with ubiquitin in a yeast two-hybrid system, it is dispensable for TRAF6 binding [57]. The NZF domain, in contrast, is involved in binding to poly-ubiquitin chains of TRAF6 [49]. Therefore, TAB2 and TAB3 are proposed to function as adaptor proteins linking TRAF6 to TAK1 [48, 58]. However, *TAB2*$^{-/-}$ murine embryonic fibroblasts exhibit normal IL-1-induced NF-κB activation [59], probably due to redundancy with TAB3. Indeed, RNA intereference-mediated knockdown of both TAB2 and TAB3 abrogates IL-1-induced NF-κB and JNK activation [48]. In contrast, Kishida et al. demonstrated that IL-1-induced TRAF6 poly-ubiquitination and TAK1 auto-phosphorylation are abolished in TAB2-deficient murine embryonic fibroblasts [57]. In addition, TAB2 was shown to facilitate the interaction between TRAF6 and the IKK complex. In contrast to TAB2 and TAB3, TAB1 does not recognize poly-ubiquitin chains, but is implicated in the regulation of the kinase activity of TAK1 [60]. TAK1 kinase activity is only detected in complex III [27], and TAK1 becomes activated upon poly-ubiquitin binding of TAB2 or TAB3. In fact, TAB2 activates TAK1, and subsequently the IKK complex, only in the presence of ubiquitination components, including E1, Ubc13/Uev1A (E2), TRAF6 (E3) and ubiquitin [39], depending on its NZF domain [49]. Likewise, the NZF domain of TAB3 is indispensable for TAK1 and IKK complex activation. Activation of TAK1 leads to auto-phosphorylation and phosphorylation of TAB1, whereas TAB2 becomes phosphorylated at the membrane, probably by an upstream protein kinase, as TAK1 kinase activity is only detected in complex III [27, 58]. Finally, activated TAK1 is able to phosphorylate IKKβ on serines 177 and 181 in its activation loop, thus activating the IKK complex [39]. RNA interference directed against TAK1 abolishes IL-1-mediated NF-κB activation [61], demonstrating its important role in IL-1-induced activation of the NF-κB pathway. In addition, murine embryonic fibroblasts expressing an inactive form of TAK1 are impaired in IL-1-mediated NF-κB activation, although IL-1-induced NF-κB activation is not completely abrogated in these cells [62]. Similarly, B-cells expressing this TAK1 mutant show impaired IκBα degradation and NF-κB DNA binding in response to LPS. Shim et al. revealed that TAK1-deficient murine embryonic fibroblasts are defective in IL-1-induced NF-κB activation, but LPS-mediated NF-κB activation is only partially reduced in these cells [54]. This partial reduction in signaling in TAK1-deficient cells upon IL-1 and LPS triggering suggests the existence of yet to be fully characterized TAK1-independent pathways.

One good candidate could be mitogen-activated protein kinase kinase kinase 3 (MEKK3), as MEKK3-deficient cells show impaired NF-κB activation upon IL-1 signaling. In addition, IL-1-induced IκBα phosphorylation is only completely abolished when both TAK1 and MEKK3 are impaired [63]. In this context, Yao et al. proposed the existence of two independent signaling pathways to NF-κB upon IL-1 stimulation. The TAK1-dependent pathway leads to IKKβ phosphorylation and activation through the classical pathway, resulting in IκBα phosphorylation and degradation. The TAK1-independent, MEKK3-dependent pathway involves IKKγ phosphorylation and IKKα activation. Remarkably, the latter results in NF-κB activation through IκBα phosphorylation and dissociation from NF-κB, but is not associated with IκBα degradation [63]. Indeed, IL-1-induced IKKα and IKKβ phosphorylation was shown to be reduced in TAK1-deficient cells, whereas IKK kinase activity was intact. In addition, IKKγ was phosphorylated upon IL-1 stimulation of these cells. TAK1 inhibition abolishes IL-1-induced IκBα phosphorylation in IKKα-deficient cells, but not in IKKβ-deficient cells. In the TAK1-independent pathway, IκBα phosphorylation by IKKα is not recognized by βTrCP E3 ligase, so no IκBα degradation occurs. Still, NF-κB is dissociated and translocates to the nucleus. Interestingly, Solt et al. also demonstrated the existence of an IKKα/IKKγ-dependent, but IKKβ-independent, IL-1-mediated signaling pathway to NF-κB [64]. Furthermore, MEKK3 interacts with IRAK-1 and TRAF6 upon IL-1 stimulation. The existence of this alternative pathway could be demonstrated in primary colon epithelial cells upon IL-1 and LPS stimulation [63].

Protein kinase C (PKC) is also a likely candidate for substituting TAK1. Indeed, depletion of atypical PKCs or p62, an atypical PKC-interacting protein, inhibits NF-κB, but not JNK activation in response to IL-1 or TRAF6 overexpression [65]. In addition, TRAF6 specifically interacts with p62 upon IL-1 stimulation. The fact that p62 noncovalently interacts with mono- and poly-ubiquitin in a yeast two-hybrid system [66] suggests that p62 binds ubiquitinated TRAF6.

Finally, ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) is another TRAF6-interacting protein that was shown to be involved in LPS- and IL-1-induced signaling [67]. Overexpression of a dominant-negative mutant or RNA interference-mediated knock-down of ECSIT expression abolishes LPS- and IL-1-mediated NF-κB activation. The exact function of ECSIT, is however, still unclear.

Although TLR-4 and IL-1RI have a related intracellular TIR domain that initiates downstream signaling, TLR-4 adds a little variation to the general theme (Fig. 2). In contrast to the IL-1RI complex, recruitment of MyD88 to TLR-4 is dependent upon TIR-domain-containing adaptor protein (TIRAP), also known as MyD88 adaptor like (Mal). Indeed, Mal-deficient mice do not produce inflammatory cytokines in response to LPS [68, 69]. Recently, it was shown that Mal contains a phosphatidylinositol 4,5-bisphosphate (PIP2)-binding domain, targeting Mal to discrete regions in the plasma membrane. MyD88 is then recruited to the plasma membrane to interact with Mal via their TIR domains. Mutations of the PIP2-binding domain result in an impairment of Mal and subsequent MyD88 recruitment to the plasma membrane. As bypassing Mal requirement by endowing MyD88 with a PIP2-binding domain restores TLR-4 signaling in Mal-deficient cells [70], it has been suggested that Mal only functions to recruit MyD88 to the PIP2-containing membrane part. Such a bridging function for Mal was also demonstrated using MAPPIT, a mammalian two-hybrid system [71]. Furthermore, upon LPS stimulation, Mal becomes tyrosine phosphorylated by Bruton's tyrosine kinase (Btk) [72]. Tyrosine phosphorylation of Mal is necessary for NF-κB activation by LPS, but the mechanism remains unknown. Possibly, tyrosine phosphorylation of Mal induces conformational changes that unmask the PIP2-binding domain. However, no experimental evidence for this is available yet.

Secondly, a MyD88-independent signaling pathway originates from TLR-4. Although MyD88-deficient cells fail to express several inflammatory cytokines upon LPS stimulation, LPS still induces a delayed activation of NF-κB and JNK [73]. Furthermore, induction of type I interferons (IFNs) is not impaired [74], pointing to the existence of a second signaling pathway from TLR-4 upon LPS stimulation. This MyD88-independent signaling pathway involves the recruitment of TRIF-related adapter molecule (TRAM, also known as TICAM2) and TIR domain-containing adapter-inducing IFNβ (TRIF, also known as TICAM1). Recently, TLR-4 was shown to activate the MyD88-dependent and TRIF-dependent signaling pathways sequentially in a process organized around endocytosis of the TLR-4 complex [75]. More specifically, TLR-4 first induces Mal-MyD88 signaling at the plasma membrane and is then endocytosed and activates TRAM-TRIF signaling from early endosomes. TRIF associates with TRAF6 and receptor-interacting protein 1 (RIP1) to induce NF-κB activation, while activation of the IKK-related kinases TANK-binding kinase 1 (TBK1) and IKKε by TRIF results in dimerization and phosphorylation of the transcription factor IFN regulatory factor 3 (IRF3), which binds and activates type I IFN promoters in the nucleus (for overview see

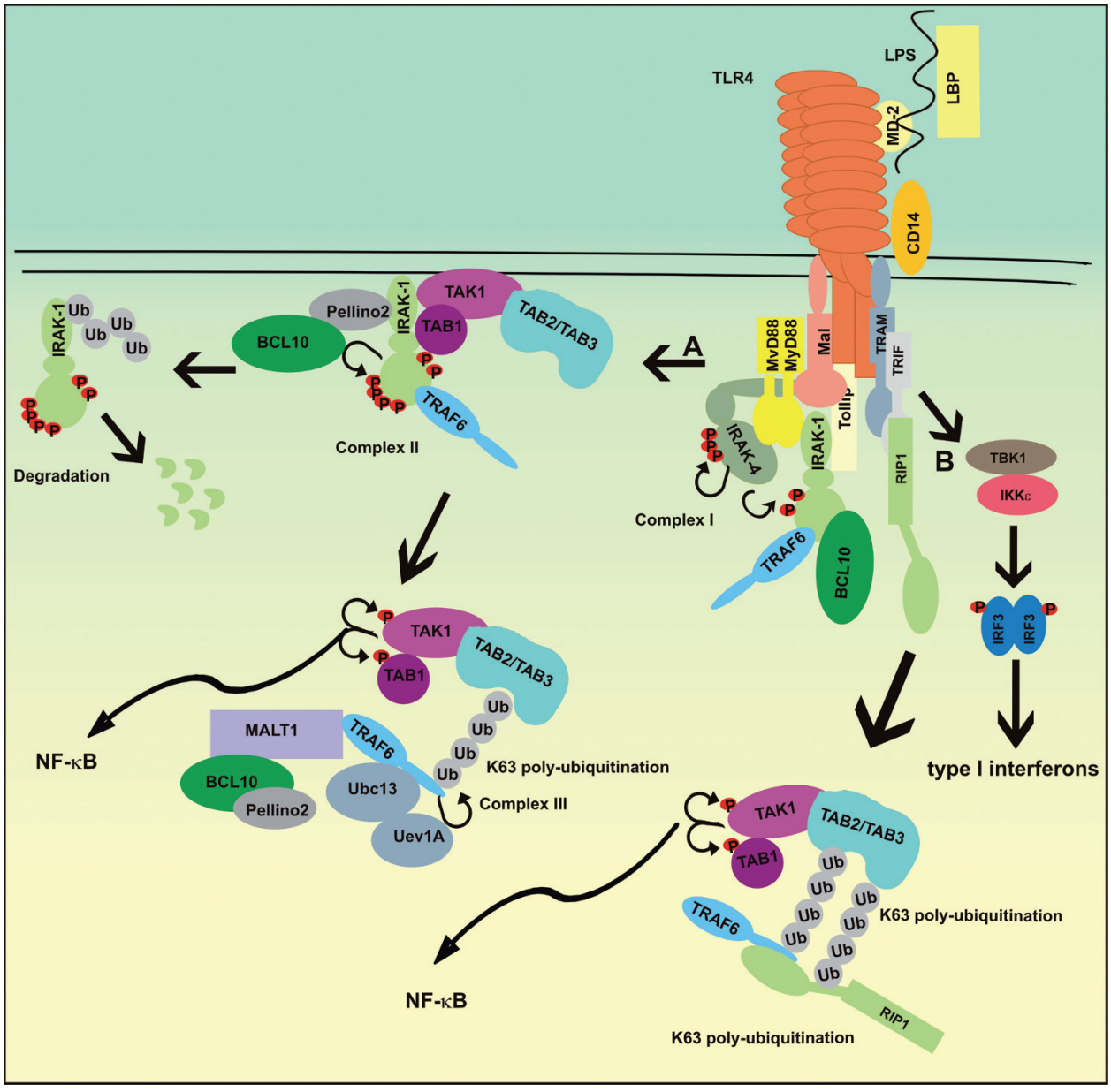

Figure 2. LPS signaling pathways to NF-κB**.** LPS in complex with LPS-binding protein (LBP) binds CD14 on the cell membrane, which transfers LPS to MD-2 and TLR-4. (*A*) In contrast to IL-1RI signaling, MyD88 is recruited to the receptor complex via its interaction with Mal, which is attached to the membrane by its PIP2-binding domain. Complex I, II and III formation proceeds as described in Figure 1 for IL-1R1 signaling, but in addition, IRAK-1 interacts with BCL10 in complex I. BCL10 binds to Pellino2 in complex II and with MALT1 in complex III. MALT1 further contributes to TRAF6 activation. (*B*) Upon LPS stimulation, the adaptor proteins TRAM and TRIF are also recruited to TLR-4 (TRIF and MyD88 are here shown in the same TLR-4 complex, but recent evidence indicates their sequential recruitment, with TRAM and TRIF being recruited on endosomal TLR-4). TRIF associates with RIP1 to induce a late-phase NF-κB activation. To this end, poly-ubiquitinated RIP1 associates with poly-ubiquitinated TRAF6 and the TAB-TAK1 complex. In addition TRIF initiates a pathway leading to the activation of TBK1 and IKKε and subsequent IRF3 activation by phosphorylation, a step required for type I IFN induction.

[76]). Similar to MyD88-deficient cells, TRAF6-deficient macrophages also demonstrate a delayed activation of NF-κB and JNK upon LPS stimulation, but unaffected IFN production, suggesting that TRAF6 is essential for MyD88-dependent, but not TRIF-dependent signaling [77].

Upon LPS stimulation, the scaffolding protein B-cell leukemia/lymphoma 10 (BCL10) is recruited into the TLR-4 receptor complex via its interaction with IRAK-1 [78]. Furthermore, IRAK-1 oligomerization stimulates BCL10 oligomerization, which is necessary for LPS-induced NF-κB activation. The BCL10-interacting protein mucosa-associated lymphoid tis-

sue 1 (MALT1) is also an essential intermediate in the LPS-signaling cascade towards NF-κB since RNA interference against MALT1 abolishes LPS-induced NF-κB activation. Moreover, TRAF6 auto-ubiquitination is markedly reduced upon LPS stimulation of MALT1-deficient macrophages [78]. LPS stimulation effectively induces BCL10-MALT1 and MALT1-TRAF6 interactions, indicating that the BCL10-MALT1-TRAF6 signaling cascade to NF-κB is not only important in T-cell receptor signaling but also in the TLR-4 pathway. Upon LPS stimulation, MALT1 can only be detected in the cytosolic TAK1 complexes, whereas BCL10 is also found in TLR-4 and TAK1 complexes at the membrane. In addition, no IRAK-1 can be detected in the cytosolic TAK1 complex, suggesting that BCL10 dissociates from IRAK-1 before binding to MALT1 in the cytosolic TAK1 complex [78]. Pellino-2 was also found to interact with BCL10 in both the membrane-bound and cytosolic TAK1 complex, and its knock-down inhibits the recruitment of BCL10 to the cytosolic TAK1 complex. Recently, a role for the cysteine protease caspase-1 in TLR-4 signaling to NF-κB was described [79]. Caspase-1 binds and cleaves the adaptor protein Mal. In addition, LPS signaling is impaired in caspase-1-deficient cells, and a non-cleavable Mal mutant acts as a dominant-negative inhibitor towards TLR-4 signaling. Finally, also human caspase-4, which does not have a murine ortholog, has been implicated as an essential mediator of LPS-induced NF-κB activation via its interaction with TRAF6 [80]. The underlying mechanism by which caspase-4 mediates NF-κB activation is, however, still unclear.

TNF-R signaling to NF-κB

TNF exerts its function by binding, as a trimer, to either TNF-R1 or TNF-R2. Both TNF receptors belong to the so-called TNF receptor superfamily, with several of its members playing a pivotal role in the development and function of the immune system [81]. All TNF receptor family members are characterized by the presence of one to six cysteine-rich domains in their extracellular portion. They can be further divided based on the presence of specific signaling motifs or domains in their cytoplasmic tails: a first subgroup for which TNF-R1 is representative shares a death domain (DD); a second subgroup, which includes most TNF-R family members including TNF-R2 contains a consensus motif that allows binding to TRAF signaling proteins; and a third subgroup does not contain any known signaling motifs. TNF-R2 expression and biological responses are mainly restricted to T cells. We will therefore in this review focus on TNF-R1 as a universal receptor for TNF.

Similarly as discussed above for the sequential formation of distinct TLR-4 signaling complexes at the plasma membrane and endosomal membrane, respectively, TNF-R1 signaling also involves the formation of two sequential signaling complexes [82–84]. Upon activation of TNF-R1 at the plasma membrane, the TNF-R1 DD serves as a docking site for the DD-containing adaptor protein TRADD through homotypic DD interactions. TRADD, in turn, recruits TNF receptor-associated factor 2 (TRAF2) and the serine/threonine kinase RIP1 [85, 86], which rapidly signal NF-κB activation (Fig. 3). At later time points, TRADD, RIP1 and TRAF2 dissociate from TNF-R1, and endosomal TNF-R1 recruits the DD-containing adaptor protein FADD, which binds itself to caspase-8, forming a cytoplasmic complex that is implicated in signaling to apoptosis [86]. In addition to TRAF2, TRAF5 has also been implicated in TNF-induced NF-κB activation, as in contrast to the single TRAF2 or TRAF5 knockout cells, TRAF2/TRAF5 double-knockout cells show impaired NF-κB activation upon TNF stimulation [87, 88]. Unlike TRAF2, TRAF5 only interacts with RIP1, but not with TRADD in co-immunoprecipitation assays [87]. TRAF2 becomes K63-linked poly-ubiquitinated upon TNF stimulation, which is dependent on its RING and zinc finger domains and which requires Ubc13/Uev1A. Similarly to TRAF6, TRAF2 has therefore been proposed to be an E3 ubiquitin ligase [89, 90]. *In vitro*, TRAF2 can generate K63-linked poly-ubiquitin chains via an E2 Ub-conjugating enzyme Ubc13/Uev1A-dependent mechanism [38]. The necessity of the E3 ligase activity of TRAF2 for TNF-induced NF-κB activation remains controversial, as TRAF2 translocation and poly-ubiquitination was demonstrated to be required for JNK, but not NF-κB activation upon TNF stimulation [89, 90]. However, the RING finger structure of TRAF2 is necessary for TNF-induced NF-κB activation [85]. Also, a naturally occurring splice variant of TRAF2, called TRAF2A, containing a mutated RING finger domain, is unable to activate NF-κB but remains a potent activator of the JNK pathway [91]. The fact that depletion of TRAF2 is sufficient to abolish TNF-induced JNK activation, whereas depletion of both TRAF2 and TRAF5 are necessary for abrogating NF-κB activation [87], suggests possible redundancy between TRAF2 and TRAF5 for TNF-induced NF-κB activation. However, no E3 ligase activity has been demonstrated yet for TRAF5. Furthermore, an intact RING finger structure is required for NF-κB activation, but not for JNK activation, by TRAF5 [91].

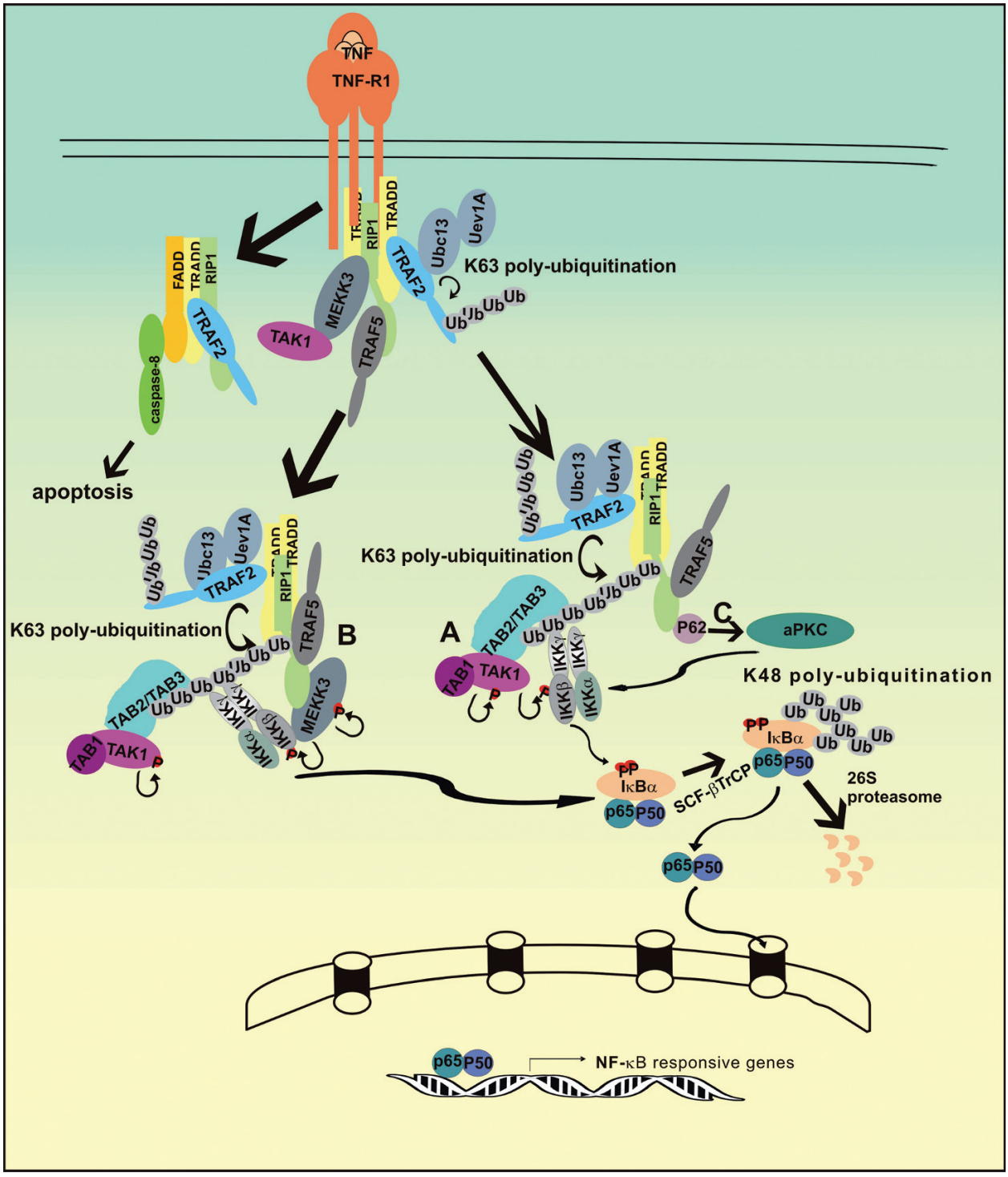

Figure 3. TNF signaling pathways to NF-κB. Upon TNF stimulation, plasma membrane-associated TNF-receptor 1 (TNF-R1) trimerizes and recruits via its death domain TRADD, which binds itself TRAF2 and RIP1, which also interacts with TRAF5. Upon TNF-R1 endocytosis, TRADD dissociates from TNF-R1 and associates with FADD, leading to recruitment of caspase-8, which initiates pro-apoptotic signaling. TRAF2 undergoes auto-poly-ubiquitination and ubiquitinates RIP1 via K63-linked poly-ubiquitin chains. (*A*) RIP1 poly-ubiquitination leads to the recruitment of TAK1 and IKKβ via respectively TAB2 and IKKγ, which binds to the poly-ubiquitin chain of RIP1. Finally, TAK1 phosphorylates IKKβ, leading to the activation of the IKK complex as described for Figure 1. (*B*) Upon TNF stimulation, RIP1 also recruits MEKK3, which phosphorylates IKKβ, leading to activation of the IKK-complex. TAK1 is necessary for the regulation of the kinase activity of MEKK3. (*C*) RIP1 can also interact with p62, leading to activation of aPKCs and the TAK1-independent activation of the IKK complex.

RIP1 and TRAF2 can be recruited to the TNF-R1 complex independent of each other, as RIP1 and TRAF2 were shown to be recruited to the TNF-RI complex upon TNF stimulation in TRAF2-deficient and RIP1-deficient cells, respectively [92, 93]. Upon TNF stimulation, TNF-R1, together with TRADD, TRAF2 and RIP1, are relocated to lipid rafts, which are microdomains in the membrane that are enriched in sphingolipids and cholesterol [94]. Moreover, TNF-R1 and RIP1 become poly-ubiquitinated in lipid rafts upon TNF stimulation [93, 94]. RIP1-deficient cells fail to activate NF-κB upon TNF-stimulation, demonstrating the essential role for RIP1 in TNF signaling to NF-κB [95]. In addition, RIP1 plays a role in the recruitment of TAK1, as TAK1 fails to translocate to the TNF-R1 complex upon TNF stimulation of RIP1-deficient Jurkat cells [96]. In response to TNF, RIP1 becomes K63-poly-ubiquitinated at lysine 377 in its intermediate domain. This is indispensable for IKK activation upon TNF stimulation, as mutation of lysine 377 abolishes the ability of RIP1 to rescue IKK activation in RIP1-deficient cells [97, 98]. Although previous reports indicated that TRAF2 is necessary for recruitment of the IKK complex and that RIP1 mediates IKK activation [85, 92], RIP1-deficient cells stably transfected with a RIP1 lysine 377 mutant fail to recruit TAK1 and IKKγ to the TNF-R1 upon TNF stimulation [9, 97, 98]. So, poly-ubiquitination of RIP1 on lysine 377 is not only necessary to recruit TAK1, via binding to TAB2, but is also needed to directly recruit IKKγ [97]. Binding of IKKγ via its NEMO ubiquitin-binding domain to K63-poly-ubiquitinated RIP1 [7, 8] is necessary for IKK activation upon TNF stimulation, as mutations in the NEMO ubiquitin-binding domain abolish TNF-induced NF-κB activation [8, 97]. As IKKγ interacts with K63-linked poly-ubiquitin chains *in vitro* [97], and also binds the intermediate domain of RIP1 [84, 99], IKKγ most likely interacts directly with the ubiquitin chains on RIP1 via its NEMO ubiquitin-binding domain. *In vivo*, endogenous IKKγ only co-immunoprecipitated with RIP1 after TNF stimulation, and RIP1 was immunoprecipitated as a ladder of higher molecular weight bands, which represents poly-ubiquitinated forms of RIP1 [8]. In addition, binding of IKKγ to poly-ubiquitinated RIP1 protects the latter from proteasome-mediated degradation [8], probably by preventing de-ubiquitination of K63-poly-ubiquitinated RIP1 and subsequent K48-linked ubiquitination by A20 [100]. Similar to IRAK-1 in the IL-1 and LPS signaling pathway, the kinase activity of RIP1 is also dispensable for IKK activation and RIP1 poly-ubiquitination [93]. TRAF2 might be the E3-ligase for RIP1, as no poly-ubiquitination of RIP1 occurs in TRAF2-deficient cells upon TNF stimulation [93]. Moreover, overexpression of TRAF2 in HEK293T cells induces K63-poly-ubiquitination of RIP1 [100]. Ubc13 also plays an essential role in TNF-induced NF-κB activation, as macrophages and splenocytes isolated from *Ubc13*$^{+/-}$ mice stimulated with TNF show impaired IκBα degradation [43]. Furthermore, siRNA directed against either Ubc13 or Uev1A abolishes TRAF2-mediated signaling to NF-κB [46]. In contrast, Yamamoto et al. demonstrated that Ubc13-deficient murine embryonic fibroblasts show normal activation of NF-κB and JNK in response to TNF [44].

The TAK1/TAB1/TAB2/TAB3 complex is also implicated in TNF signaling. As with IL-1 and LPS signaling, TAB2 and TAB3 are redundant for TNF-induced NF-κB activation. Indeed, while TAB2-deficient murine embryonic fibroblasts still exhibit normal TNF-induced NF-κB activation [59], RNA interference mediated knock-down of both TAB2 and TAB3 abrogates TNF-mediated signaling [48]. Upon TNF stimulation, poly-ubiquitinated RIP1 associates with TAB2, but poly-ubiquitinated TRAF2 does not [49]. In contrast, Ishitani et al. could show an interaction between TAB2 or TAB3 and TRAF2 upon TNF stimulation [48]. Unlike TRAF6, co-expression of TRAF2 with TAB2 or TAB3 does not lead to poly-ubiquitination of TAB2 and TAB3. In contrast to IL-1 stimulation, however, RNA interference-mediated downregulation of TAB1 had no effect on p65 nuclear localization and expression of IL-6, IL-8 and GM-CSF, demonstrating a differential role for TAB1 in IL-1 and TNF signaling pathways [55]. However, stimulation of TAB1-deficient cells with TNF did not lead to activation of TAK1 [56]. The essential role of TAK1 in TNF signaling was demonstrated by the use of RNA interference directed against TAK1 and the generation of TAK1-deficient murine embryonic fibroblasts, which both lead to an impaired NF-κB activation upon TNF stimulation [49, 54, 62, 96, 97].

Furthermore, an important role for another MAP kinase kinase kinase, MEKK3, in TNF-induced NF-κB activation has been revealed by the generation of MEKK3-deficient murine embryonic fibroblasts, which are impaired in TNF signaling [101]. MEKK3 can be recruited to the receptor complex upon TNF-triggering via RIP1 [101, 102]. In addition, MEKK3 associates with and phosphorylates IKKβ *in vitro* [102]. TAK1 kinase activity was demonstrated to regulate the kinase activity of MEKK3 [96], but MEKK3 is not a direct substrate for TAK1 [97]. In addition, knock-down of TAK1 blocks RIP1-induced NF-κB activation, but not MEKK3-induced NF-κB activation, suggesting that TAK1 acts downstream of RIP1, but upstream of MEKK3. Remarkably, the

kinase activity of MEKK3 is indispensable for TNF-mediated NF-κB activation [101]. Moreover, TAK1-TAB1 overexpression can still activate NF-κB in MEKK3-deficient cells [96], suggesting that similar to IL-1 signaling, two independent TNF signaling pathways might exist, a TAK-1-dependent and a TAK1-independent/MEKK3-dependent pathway. In this regard, Di et al. demonstrated very recently an important role for TAB1 in MEKK3 and TAK1 signaling upon TNF stimulation [103]. They could show a physical interaction between MEKK3 and unphosphorylated TAK1, leading to inhibition of MEKK3 phosphorylation and NF-κB activation. However, TAK1 becomes phosphorylated upon co-expression of TAB1 and dissociates from MEKK3, leading to activation of NF-κB. These results also demonstrate the existence of two signaling pathways upon TNF stimulation, which regulate each other's activation and explain the indirect dependence of MEKK3 on the kinase activity of TAK1.

Like TRAF6 in the IL-1 signaling pathway, RIP1 interacts with p62 upon TNF stimulation [104]. In addition, overexpression of dominant-negative forms of atypical PKCs or knock-down of p62 abolishes RIP1-induced, but not TRAF2-induced NF-κB activation, suggesting that p62 might represent an independent signaling pathway towards NF-κB activation upon TNF stimulation.

Conclusions

Signaling in response to TLRs, IL-1RI and TNF-R1 shows an ever-growing complexity of molecules and networks. Because of their related intracellular TIR domain, TLR-4 and IL-1R signaling share a common MyD88-dependent signaling pathway that is involved in NF-κB activation, but inclusion of an MyD88-independent pathway has provided unique functions to TLR-4. Moreover, it is not clear why TLR-4 needs Mal to recruit MyD88, whereas IL-1RI does not. The extra recruitment of Mal might not only recruit MyD88 to the membrane but also provide an additional platform for the recruitment of other signaling molecules still to be discovered. Because TNF-R1 contains an intracellular DD instead of a TIR domain, other signaling molecules are recruited. However, similar to the homotypic TIR-TIR interactions in the recruitment of adaptor proteins to TLR/IL-1R, homotypic DD-DD interactions mediate the recruit of different adaptor proteins to the TNF-R1. Downstream signaling involves the activation of kinases and ubiquitin ligases, of which some are receptor-specific and others are not. IRAK-1 and IRAK-4 mediate TLR/IL-1R signaling, and their counterparts in TNF-R signaling are RIP1 and most likely RIP4 [105]. However, RIP1 seems to be a more universal player, which can also take part in TLR signaling and many other receptor specific signaling pathways to NF-κB [106]. TLR/IL-1R as well as TNF-R signaling does not depend on the catalytic activity of the above-mentioned kinases, although they are functionally active. The exact role of their catalytic activity still needs to be identified, but a role in the fine-tuning of NF-κB activation is most likely. Similar to the use of distinct kinases, TLR/IL-1R and TNF-R signaling depends on distinct members of the TRAF family, TRAF6 and TRAF2/TRAF5, respectively. Originally thought to be simple adaptors, TRAF molecules are now recognized to function as ubiquitin ligases, leading to K63-linked auto-poly-ubiquitination and the K63-linked poly-ubiquitination of other signaling proteins, thus creating novel docking sites for the binding of other ubiquitin-binding proteins. Alternatively, K48-linked poly-ubiquitination of signaling proteins results in their proteasome mediated degradation. In this context, both RIP1 and IRAK-1 are modified in both ways, with K48-poly-ubiquitination leading to their degradation and the ending of signaling. For RIP1, this negative regulatory mechanism has been attributed to A20, whereas for IRAK-1 the identity of the E3-ligase remains unclear, although Pellino proteins might be good candidates. TLR/IL-1R and TNF-R signaling downstream of RIP1 and IRAK-1 converges on TAK1, which functions as an activating kinase for IKKβ. In addition to this phosphorylation-dependent activation step, the IKK complex is also activated by induced oligomerization, which is mediated by the K63-poly-ubiquitin-dependent recruitment of the IKK adaptor protein IKKγ to K63-poly-ubiquitinated RIP1 and IRAK-1 in the TNF-R and TLR/IL-1R signaling pathway, respectively. It is clear that early signaling of these receptors follows many common themes but involving distinct signaling molecules. The use of different molecules exerting similar functions allows the cell to specifically regulate the effect of for example TLR stimulation, leaving the effect of TNF-R1 signaling intact. Several examples of negative regulatory molecules interfering with NF-κB signaling in a receptor-specific way are already known, such as A20 [107], MyD88s [108, 109] and TAX1BP1 [110, 111].

Anti-TNF therapies (e.g. adalimumab, a fully human monoclonal antibody; etanercept, a soluble receptor construct; and infliximab, a chimeric monoclonal antibody) have already found their way to the clinic for the treatment of autoimmune diseases, such as rheumatoid arthritis, Crohn's disease and psoriasis. Similarly, anti-IL-1 therapy (anakinra, a recombinant form of a naturally occurring IL-1RI-binding mole-

cule) has been approved to manage rheumatoid arthritis patients but seems to be less effective than anti-TNF therapy. Anti-TNF and anti-IL-1 therapies are associated with an increased risk of infection as well as other potentially serious side effects and are therefore only used for patients that are refractory to more conventional forms of treatment [112]. More recently, also manipulating the activity of TLRs to modulate immune responses for therapeutic purposes has initiated intense activity in the pharmaceutical industry. The focus of these activities has been largely on the use of TLR agonists or antagonists in the areas of infectious diseases, cancer, allergic diseases and vaccine adjuvants [113]. Although initial clinical trials for infectious diseases and cancer showed early promise, subsequent longer-term trials have been disappointing, and more research is required to find strategies that balance efficacy with acceptable side-effect profiles. In this context, inhibition of the NF-κB signaling pathway to prevent the expression of pro-inflammatory mediators or to sensitize tumor cells to anti-cancer treatment has been proposed, and clinical trials with several IKK inhibitors are ongoing. However, NF-κB inhibition at the level of IKK is a double-edged sword. NF-κB signaling pathways initiated from distinct receptors all converge at the level of IKK, implying that IKK inhibitors block signaling in response to multiple receptors that play crucial roles in maintaining health and immune surveillance. It is therefore likely that IKK inhibitors will also have significant side-effects, particularly if they are used for a long time. More promising would be inhibitors that target the NF-κB pathway initiated by a specific receptor whose deregulated activity is linked to a certain disease, leaving NF-κB activation in response to other receptors intact. An increased understanding of the mechanisms that regulate NF-κB activation in response to TNF-R, IL-1R and TLRs can therefore be expected to open new avenues for therapeutic drug development.

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