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The Kinase Complex Responsible for IRF-3–Mediated IFN-β Production in Myeloid Dendritic Cells (mDC)

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Type I interferons (IFN) IFN-α and -β play a central role in the induction of antiviral immunity, which involves up-regulation or activation of a large number of IFN-inducible genes in host immune competent cells. Initial events in the antiviral response may occur in myeloid dendritic cells (mDCs), and the proteins expressed provoke early responses to cope with concomitant infection in the host. The participation of transcription factors IRF-3/7, AP1 and NF-κB in IFN-β promoter activation in mDCs is well established. An initial trigger of this event is a viral dsRNA that is recognized by proteins with an RNA-binding motif. Toll-like receptor (TLR) 3 on membranes and RIG-Iin the cytoplasm are molecules with dsRNA-recognition ability. Our main aim in the present review is to describe how IRF-3 and/or NF-κB are activated through the initial recognition of dsRNA by these pattern-recognition receptors. By analogy to the trimolecular complex of IKKγ, IKKα and IKKβ, thus far, IRF-3–activated kinases have been reported to be kinase complexes with trimolecular assembly. Two kinases, TBK1 and IKKε, are thought to be linked to regulatory subunit TANK or NAP1 with no kinase activity like IKKγ. The TLR3 and RIG-I pathways converge upstream of IRF-3, possibly at NAP1, the regulatory subunit of IRF-3-activating kinase. Thus, a novel function of the regulatory subunit has emerged. These proteins are involved in the TLR3 and RIG-I pathways, and act as adapters bridging on the dsRNA-recognition unit and IRF-3–activating kinases in addition to their kinase-regulatory function. Here, we summarize the properties of regulatory subunits NAP1 and TANK, and the mode of activation of NF-κB and IRF-3 in conjunction with the unique properties of the TLR3 function.

Key words: Toll-like receptor, type I interferon, viral infection.

Abbreviations: dsRNA, double-stranded RNA; FADD, Fas-associated protein with the death domain; IFNAR, interferon-α/β receptor; IKK, IκB kinase; IRF, interferon-regulatory factor: mDC, myeloid dendritic cell; NAP, NAK-associated protein; pDC, plasmacytoid dendritic cell; RIP, receptor-interacting protein; STAT, signal transducers and activation of transcription; TBK, TANK-binding kinase; TICAM, Toll/IL-1 receptor homology domain–containing molecule; TLR, Toll-like receptor; TRAF, tumor necrosis factor receptor–associated factor; VAK, virus-activated kinase.

TLR is a type I transmembrane protein consisting of extracellular leucine-rich repeats (LRRs) and an intracellular Toll-IL-1 receptor homology domain (TIR). Humans possess a family of TLRs consisting of ten TLR proteins. Homologous or heterologous combinations of them recognize a variety of microbial pattern molecules (*1*, *2*). These TLRs are expressed in myeloid cells, particularly in mDCs. The recognition of multifarious microbial patterns by primary pattern–recognition receptors in a manner unique to TLRs is the first defense for host immunity against various pathogens in these cells. Many microbial pattern molecules have been reported, most of which serve as agonists for TLRs.

Poly(I:C), a representative dsRNA, has been reported to be a potent type I IFN inducer (*3*), but its mechanism remained unknown until the discovery of the function of TLR3. Human and mouse TLR3 acts as receptors for poly(I:C) in mDCs and some epithelial cells (*4*, *5*). The unique IFN-inducing property of TLR3 is in part characterized by its specific selection of TLR adapter TICAM-1 (also called TRIF) (*6*). Of the four adapters that have the Toll-IL-1β homology (TIR) domain, TICAM-1 binds directly to TLR3 and indirectly to TLR4 (*7*). Subsequently, TICAM-1 allows the selection for the pathway to activation of IRF-3 followed by IFN-β promoter activation (*8*). In myeloid dendritic cells (mDCs), this pathway is pivotal for the induction of type I IFN. Another adapter, MyD88, induces IFN-α only in plasmacytoid DC (pDCs) by activating IRF-7 through a different molecular interaction (*9*, *10*). Two additional adapters, Mal/TIRAP and TICAM-2 (TRAM), essentially function as bridging adapters, as delineated in Fig. 1 (*11*).

A trimolecular complex consisting of IKKγ, IKKα and IKKβ has been well characterized, but a complex of IRF-3-activated kinases has not yet been clearly demonstrated. However, recent reports suggested that the putative trimolecular kinase complex TANK, IKKε and TBK1 participates in activation of not only NF-κB but

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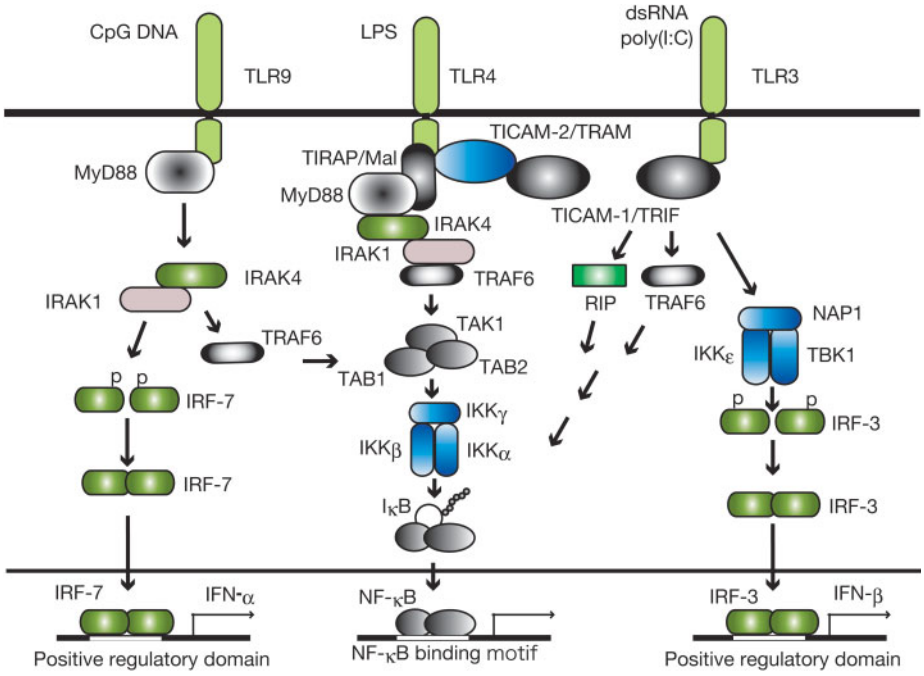
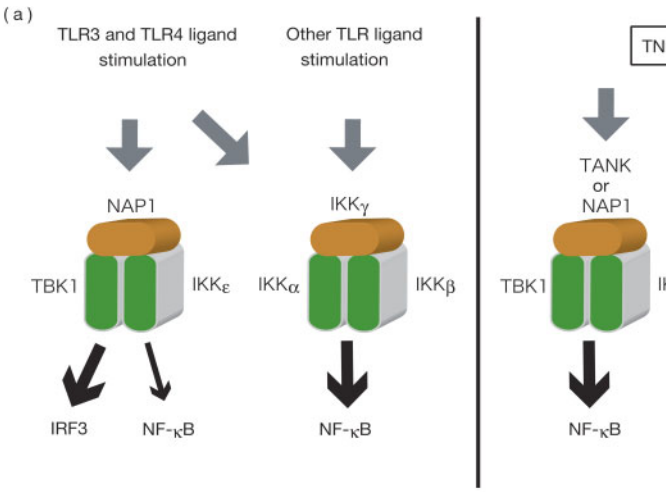


Fig. 1. **Possible signaling pathways coupling TLRs to IFN induction.** TLR9 (also possibly TLR7) can signal exclusively via MyD88 to activate IRF-7 and NF-κB. IRF-7 activation activates the IFN-α promoter in pDCs. TLR3 uses TICAM-1 but not MyD88 in mDCs to induce activation of IRF-3 and the IFN-β promoter. TICAM-1 can recruit RIP1, which together with TRAF6 activates NF-κB. TLR4 can signal via both MyD88 and TICAM-1 in mDCs. Mal/TIRAP and TICAM-2 are adapters involved in coupling TLR4 to MyD88 and TICAM-1, respectively. Either MyD88 or TICAM-1 promotes activation of NF-κB and MAPKs, leading to transcription of cytokine genes. TICAM-1 in the TLR4 pathway also activates IRF-3, allowing weak IFN-β production.



(b)

% identity	TANK	IKKγ
NAP1	18.2	20.3
TANK	—	17.1

Fig. 2. **Trimolecular interactions of two kinases and their regulatory subunits in the TLR pathway.** IKKγ, TANK and NAP1 do not act as kinases, but act as regulatory subunits for kinase complexes. TBK1 and IKKε (or IKKi) assemble with NAP1 or TANK to activate IRF-3 and to a lesser extent NF-κB. IRF-3 is preferentially activated in the TLR pathway. IKKγ couples with IKKα and IKKβ, which allows the activation of NF-κB. IRF-3/7 are not activated via this complex. The three non-kinase subunits are structurally similar to one another.

also IRF-3 (*12*, *13*). Judging from their similarity and analogy, we here hypothesize that the two kinase complexes similarly assemble, at least in a certain situation. On infection, viruses activate type I IFN, possibly *via* so-called virus-activated kinase (VAK) involving IKKε and TBK1, and IRF-3 and IRF-7 (*14*). We will focus on how the TLR3-TICAM-1 pathway links the kinases that activate IRF-3 during viral infection, and then summarize the structure-function relations of the family of molecules that bridge TICAM-1 and IRF-3-activating kinases.

Downstream of TICAM-1—TICAM-1 is an essential molecule for IFN-β production through TLR3 and TLR4 (*15*). We recently reported that NAP1 interacts with TICAM-1 (*16*). It has been reported that NAP1 directly interacts with TBK1 (also called NAK) and triggers NF-κB activation through TBK1 (*17*). TICAM-1 recruits NAP1, either directly or indirectly, to its N-terminal region. NAP1 forms a trimolecular complex with IKKε and TBK1, and this complex mainly targets IRF-3. Besides IRF-3 activation, TICAM-1 induces NF-κB and MAPK activation. There are three molecules that reportedly participate in NF-κB activation through TICAM-1. Firstly, the NAP1 complex binds TICAM-1 to activate NF-κB in the TLR3 pathway (*16*) in a manner similar to in the TNF-α-inducing pathway that involves the TBK1/IKKε complex (*17*). Secondly, TRAF6 interacts with the N-terminal region of TICAM-1 and then mediates NF-κB activation (*18*). TRAF6 binds IRAK1/4, activates the TAK/TAB1/TAB2 complex, and subsequently activates the IKKα/β/γ complex on TLR4-ligand stimulation. This trimolecular complex degrades IκB and liberates NF-κB. It is notable that IRAK1 and IRAK4 are not involved in

TLR3-mediated signaling. After ubiquitination, TRAF6 may interact with other molecules besides IRAK1/4 and form a complex with IKKα/β/γ (*19*). Thirdly, RIP1 binds TICAM-1 *via* a RIP homotypic interaction motif (PHIM) domain located in its C-terminal region (*20*). RIP1 associates with TRADD, leading to activation of the IKKα/β/γ complex following TNF-α stimulation, and leading finally to NF-κB activation. Thus, two different kinase complex properties effectively function downstream of TICAM-1 in the NF-κB activation pathway. The reported kinase complex consisting of TANK, IKKε and TBK1 should therefore be present in addition to the complex of NAP1, IKKε and TBK1. However, the role and function of the former complex downstream of TICAM-1 remain to be elucidated.

The Properties of the Kinase Complex—The three molecules NAP1, TANK and IKKγ show significant structural similarities, suggesting that they comprise a protein family with similar functional characteristics (Fig. 2a). In most cell types, for activation of TBK1 and IKKε leading to IRF-3-mediated IFN-β induction, NAP1 plays the main role in TBK1/IKKε-driven signaling. Similar to NAP1, TANK has been reported to assemble with TBK1 and IKKε (*21*, *22*). However, TANK does not form a molecular complex with TICAM-1 in HEK293 or other cells (*16*). Thus, the functional properties of the TANK-TBK1-IKKε complex, if any, have not been well characterized. It is likely that this complex is placed downstream of other adapter or DNA/RNA-binding proteins.

Structurally, NAP1 is 20.3% similar to IKKγ and 18.2% similar to TANK (Fig. 2b). NAP1 consists of 391 a.a. (*17*), TANK of 426 a.a. (*21*), and IKKγ of 419 a.a. (*23*). Their TBK1-binding regions have been reported to reside within 158-270 in NAP1 and 1-190 in TANK. The IKKα/β-binding region is within 44-85 in IKKγ (Fig. 3). Comparison of the a.a. sequences of these three proteins suggested that

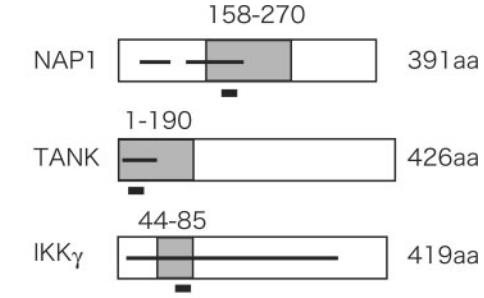


Fig. 3. **Seqence similarity between the three regulatory subunits.** NAP1 consists of 391 a.a. and binds TBK1 in the region of 158-270 a.a. (17). TANK is a 426 a.a. protein with binding capacity as to TBK1 at 1-190 a.a. (21). IKK-γ comprises 419 a.a., and its IKKβ-binding region has been identified as 44-85 a.a. (23). Clustal W alignment analysis suggested the presence of a highly conserved region in these family proteins. This conserved region lies within the predicted coiled-coil region in each protein. Solid bars, coiled coil regions; gray boxes, reported TBK1- or IKKβ-binding regions; thick underlines, the highly conserved regions in the TBK1 and IKKγ-binding sites, the sequences of which are shown below the picture.

there are sequence-conserved regions in the TBK-binding regions of these proteins (Fig. 3). The conserved regions are located within the expected coiled-coil domain, supporting the previous notion that these three molecules bind kinase molecules to regulate downstream signaling (*17*, *21*, *23*). The mechanism underlying differential selection of kinase complexes by each regulatory protein, however, remains undetermined.

IFN-β Induction via Virus Infection—Virus infection generally causes IFN-α/β production. This has been confirmed using various species of RNA viruses and some DNA viruses. The TLR3-TICAM-1 pathway is crucial in mCMV infection (*24*). This pathway may participate in Westnile virus and influenzavirus infections (*25*, *26*). Interestingly, TICAM-1 is a substrate of NS3/4A protease of HCV (*27*). Poly(I:C) as well as dsRNA appear to activate IRF-3 at least in part via the TLR3-TICAM-1 pathway. Such activation occurs even when they are exogenously added. However, poly(I:C) introduced into the cytoplasm of mDC induces strong activation of IRF-3 and type-I IFN induction even without TLR3 (*28*). Thus, the TLR3-independent pathway for activation of IRF-3 should be present inside the cells.

Recently, a CARD domain-containing helicase named RIG-I was reported to participate in signaling for TLR3-independent IFN-β-induction. RIG-I recognizes dsRNA in the helicase domain and induces activation of IRF-3 in the CARD domain (*29*). Similar results are obtained with another RIG-I family protein, MDA5, in Sendai and some other virus species (*30*). PKR might participate in dsRNA-mediated IFN-α/β induction (*31*). Thus, it remains unclear if RIG-I represents the TLR3-independent IRF-3 activation pathway. In the TLR3-TICAM-1 pathway, NAP1 downstream of TICAM-1 participates in poly(I:C)-dependent intracellular activation of IRF-3. The RIG-I pathway was examined using several viruses and polyI:C, and the NAP1-IKKε/TBK1-IRF-3 pathway was found to participate in TLR3-independent IFN-β induction (*32*). In the model proposed by Nakanishi *et al.* (*33*), NAP1 assembles with TBK1 and IKKε kinases to activate NF-κB. This and a recent report (*32*) suggest that the NAP1-TBK1-IKKε complex is placed downstream of both TICAM-1 and RIG-I/MDA5, resulting in IFN-β production. This kinase complex, called virus-activated kinase (VAK), acts *via* both intra- and extra-cellular routes to activate IRF-3 in response to poly(I:C) (Fig. 4). Which of the pathways mainly takes part in IRF-3 activation, what molecule associates with TICAM-1 and NAP1 or RIG-I and NAP1, and whether or not NAP1 is involved in the targeting of viral factors for activation of VAK in any given cell line remain unknown. However, the current literature in this area suggests that the two pathways (TLR3-dependent and -independent), converge on the trimolecular kinase complex in mDCs (*34*, *35*).

Perspectives—The TLR3-TICAM-1 pathway is intriguing since dsRNA is an essential prerequisite for its activation. TLR3 resides in putative endosomes whereas dsRNA is generated in the cytoplasm during viral replication. How they interact in mDCs and virus-infected cells remains to be investigated. An important issue to be resolved is why this pathway is conserved in mDCs. These cells participate mainly in antigen-presenting and TLR3-mediated IFN-β production. Furthermore, they are

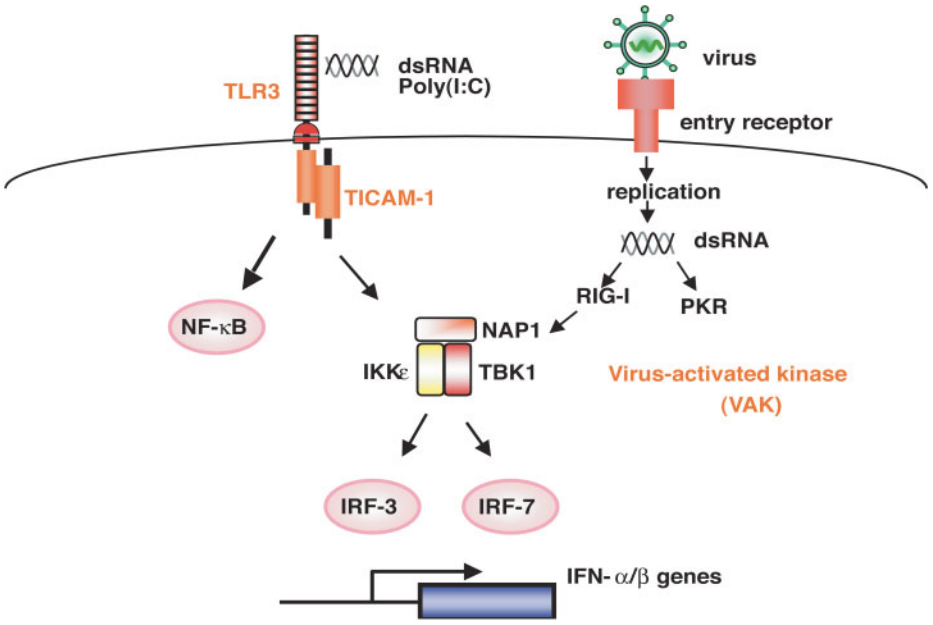


Fig. 4. **The TLR3-TICAM-1 pathway converges with the RIG-I pathway.** The trimolecular complex of NAP1, TBK1 and IKKε is presumed to be virus-activated kinase (VAK), and to be activated via TLR3 and RIG-I in response to dsRNA. TLR3 recognizes dsRNA in certain endosomes in mDCs, whereas RIG-I recognizes it in the cytoplasm (see the text for the differential roles of TLR3 and RIG-I in mDC-mediated antigen-presentation). IRF-7 is an IFN-inducible gene in mDCs (although constitutive in pDCs) and enhances type I IFN production. IFN-β once produced then signals in an auto- or para-cline manner via IFNAR and STAT1/STAT2/IRF-9 to increase up-regulation of CD80/CD86 and CD40. Hence, strong induction of type I IFN and sufficient antigen presentation are accomplished through TICAM-1 signaling in mDCs.

unequivocally involved in cross-priming, an pivotal event for endocytosed antigen presentation via MHC class I in mDCs. Systemic type I IFN production is governed by pDCs via the TLR7/9-MyD88-IRF-7 pathway. Hence, the role of the TLR3-TICAM-1 pathway cannot be simply meant for type I IFN induction alone. Virus-infected apoptotic cells or cell debris may contain viral antigens as well as dsRNA, which can be phagocytosed by mDCs. In such situations, endosomal TLR3 may recognize dsRNA and MHC class II presents the antigen in the phago-endosome. Although the exact molecular mechanism remains to be determined, the endosomal uptake antigen could be followed by class I-restricted CTL induction if co-existing dsRNA activates endosomal TLR3 in mDCs. In fact, OVA-specific $CD8^+$ CTLs are induced via cross-presentation in a TLR3-dependent manner by mouse mDCs ($CD8^+$ splenic DC), which phagocytose poly(I:C) and apoptotic cells (*36*). This, together with a recent review (*37*), allows us to hypothesize that TLR3 and TICAM-1-mediated IRF-3 activation is assigned with cross-priming for class II/class I switching, which might augment antigen presentation in mDCs. This could be the reason for conservation of this pathway in mDCs. Precise comparison of class I antigen presentation and the gene-inducing sequential process in mDCs would allow us to elucidate the role of the TLR3-TICAM-1 pathway in the immune process and its unique function in comparison with the RIG-I pathway. Through such studies, the role of participating cells in infectious lesions, particularly those of mDCs and pDCs, and their subsets, will be clearly revealed. Furthermore, the differential roles played by these agents in a cell-specific manner will be determined at the molecular level.

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