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RESEARCH HIGHLIGHT

The NLRP4-DTX4 axis: a key suppressor of TBK1 and innate antiviral signaling

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The innate immune system provides the first line of defense for protection against pathogenic bacteria or viruses. The host 'senses' pathogen infection by recognition of pathogen-associated molecular patterns *via* germ-line-encoded pattern-recognition receptors including Toll-like receptors, RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and DNA sensors. Upon receptor engagement, signaling cascade events culminate in the production of the type I interferons (IFNs) IFN-α and IFN-β that restrict virus replication. RIG-I and MDA5 are the key RLRs that recognize viral genomic RNAs and transmit downstream signals to the mitochondrial adaptor molecule MAVS (also known as IPS-1, VISA or Cardif).[1] MAVS nucleates a signaling complex anchored to the mitochondria containing the E3 ubiquitin ligase TRAF3, the non-canonical IκB kinases (IKKs) TBK1 and IKKi (also known as IKK epsilon) and the adaptor molecules NEMO (also known as IKK gamma) and TANK.[1] TBK1 and IKKi phosphorylate the interferon regulatory factor 3 (IRF3) transcription factor to trigger IRF3 dimerization and nuclear localization to activate type I IFN transcription.[2] TBK1 and IKKi are both regulated by posttranslational modifications that occur during virus infection including lysine 63 (K63)-linked polyubiquitination by TRAF3 or other E3 ubiquitin ligases.[3,4] TBK1/IKKi K63-linked polyubiquitination is linked to their activation and may be involved in recruiting signaling complexes containing ubiquitin-binding domains to transmit downstream signals. Dysregulation of type I IFN signaling, characterized by overproduction of IFN-α and IFN-β, has been implicated in numerous autoimmune diseases such as systemic lupus erythematosus.[5] Therefore, the production of IFN is subject to tight regulatory control by numerous mechanisms to maintain immune homeostasis. In a recent article in *Nature Immunology*, Cui *et al.*[6] have reported that the NLR protein NLRP4 functions as a negative regulator in the RLR pathway by targeting TBK1 for proteasomal degradation.

NLRs represent a family of innate immune regulatory proteins that consist of a nucleotide-binding and oligomerization (NOD) domain, a leucine-rich repeat region and a variable amino-terminal effector domain. NLRs may function as either activators or negative regulators of the RLR signaling pathway.[7] NLRP4, an NLR family member, has been reported to inhibit the nuclear factor-κB signaling pathway; however, its role in the RLR pathway was not previously examined.[8] The authors initially performed an overexpression screen using vectors encoding various NLRs and identified NLRP4 as an inhibitor of RLR signaling.[6] NLRP4 inhibited the induction of IFN-β and ISRE luciferase reporters by poly(I:C) (double-stranded RNA mimic), poly(dA:dT) (double-stranded DNA mimic) and vesicular stomatitis virus-GFP infection. Knockdown of NLRP4 by RNA interference potentiated the activation of IFN-β and ISRE luciferase reporters in 293T cells. Furthermore, the authors used more physiologically relevant immune cells including monocytic THP-1 cells and peripheral blood mononuclear cells to demonstrate that knockdown of NLRP4 enhanced the expression of interferon stimulated genes generated during virus infection.

To identify the target(s) of NLRP4, the authors overexpressed key signaling molecules in the RLR/Toll-like receptor pathways (RIG-I, MDA5, MAVS, TRIF, TBK1 and IKKi) to activate IFN-β and ISRE luciferase reporters and found that NLRP4 inhibited activation by all of the expression vectors except for IKKi.[6] Therefore, NLRP4 inhibited RLR signaling at a downstream step, likely at the level of TBK1. Indeed, NLRP4 interacted specifically with overexpressed TBK1, but not IKKi or IRF3, as shown by coimmunoprecipitation experiments. However, an interaction between endogenous NLRP4 and TBK1 proteins was signal-dependent and was observed only upon virus infection, suggesting that NLRP4 interacts specifically with activated TBK1. Consistent with these observations, NLRP4 interacted with the kinase domain, but not the ubiquitin-like domain or coiled-coil domains, of TBK1. Interestingly, NLRP4 was not recruited to TBK1 until 8–10 h after virus infection, suggesting that NLRP4 participates in the later phases of the termination of RLR signaling. It was previously demonstrated that the regulatory molecules A20, TAX1BP1 and ABIN1 inhibit RLR signaling by suppressing K63-linked TBK1 polyubiquitination at an early phase after virus infection (4 h).[4,9] Thus, TBK1 appears to be targeted by multiple inhibitory proteins in distinct phases after virus infection.

It has been previously reported that TBK1 is conjugated by K63-linked polyubiquitin chains by the E3 ubiquitin ligase TRAF3.[10] The authors conducted a kinetic analysis of TBK1 ubiquitination during virus infection that revealed that K63-linked ubiquitination of TBK1 occurred early after virus infection (4 h), whereas K48-linked ubiquitination of TBK1 was observed at later times (8 h) after virus infection correlating with NLRP4 and TBK1 binding.[6] Indeed, the authors found that NLRP4 promoted K48-linked

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polyubiquitination of TBK1 which triggered its degradation by the proteasome. NLRP4 required its NOD domain, but not the pyrin or leucine-rich repeat domains, to interact with and promote the degradation of TBK1. Taken together, it appears that TBK1 is inactivated by a ubiquitin-editing mechanism and NLRP4 is essential for the K48-linked polyubiquitin conjugation step.

Since NLRP4 lacks E3 ligase activity, the authors hypothesized that NLRP4 was functioning as an adaptor molecule for an E3 ligase to trigger TBK1 ubiquitination and degradation. Using a sublibrary of RING domain E3 ligase shRNAs, the authors identified the E3 ubiquitin ligase DTX4 as an inhibitor of TBK1-mediated activation of an ISRE luciferase reporter.[6] Furthermore, knockdown of DTX4 using shRNA prevented the inhibition and degradation of TBK1 by NLRP4. Since DTX4 was also dependent on NLRP4 to ubiquitinate and degrade TBK1, it was likely that NLRP4 served as an adaptor molecule for DTX4. Consistent with this notion, NLRP4 and DTX4 formed an inducible protein complex in response to virus infection and both proteins interacted with TBK1 at the precise time when TBK1 was conjugated with K48-linked polyubiquitin chains. These data suggest that both NLRP4 and DTX4 cooperatively induce the ubiquitination and degradation of TBK1 to restrict the induction of virus-induced type I IFN.

The authors next used a bioinformatics strategy to identify lysine 670 (K670) within the coiled-coil domain of TBK1 as the residue that was ubiquitinated by DTX4.[6] Mutation of K670 to arginine (K670R) prevented NLRP4-mediated ubiquitination and

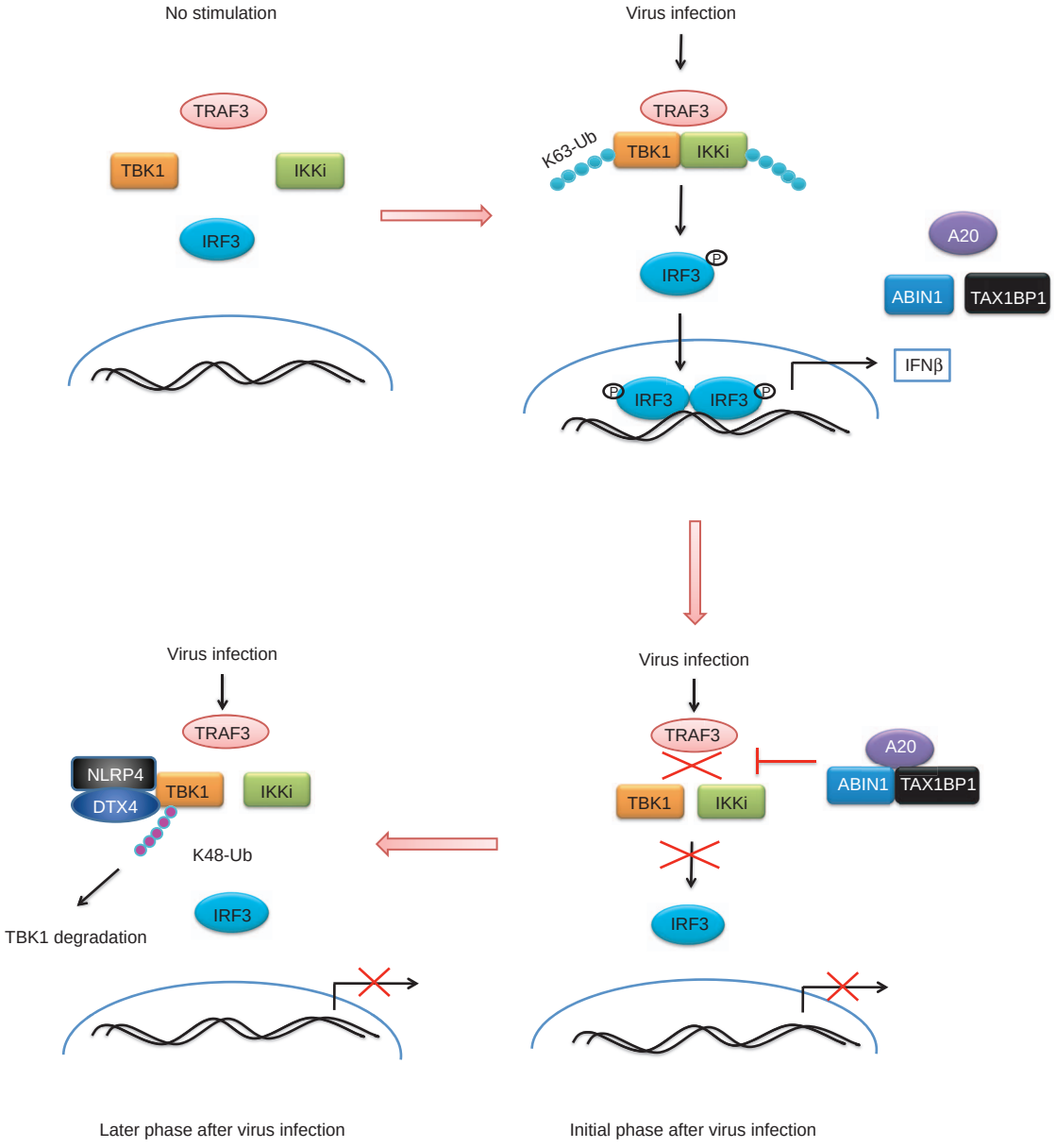

Figure 1 Model of the negative regulation of RLR signaling. The kinases TBK1 and IKKi undergo TRAF3-dependent K63-linked polyubiquitination during activation by virus infection. A20, TAX1BP1 and ABIN1 disrupt the interactions between TRAF3 and TBK1-IKKi to block TBK1 K63-linked polyubiquitination during the initial phases (4 h) of signal termination. At later phases (8 h), NLRP4 and DTX4 promote the K48-linked polyubiquitination of TBK1 to trigger its proteasomal degradation. Therefore, TBK1 is targeted by multiple inhibitory protein complexes for ubiquitin editing to suppress RLR signaling. RLR, RIG-I-like receptor.

degradation of TBK1. In addition, DTX4 knockdown enhanced ISRE reporter activation in the presence of wild-type TBK1 but not with the TBK1 K670R mutant. Finally, the authors demonstrated that NLRP4 was unable to induce the ubiquitination of a TBK1 phosphorylation mutant, suggesting that the activated form of TBK1 was selectively targeted by NLRP4 and DTX4.

In summary, Cui *et al.* have demonstrated that NLRP4 functions as an adaptor for the E3 ubiquitin ligase DTX4 to target TBK1 for K48-linked ubiquitination to trigger its degradation and restrict the induction of type I IFN. Although this study has provided new insight into the mechanisms involved in the inhibition of innate antiviral signaling, there are still key questions that should be addressed in future studies. What is the purpose of multiple inhibitors of TBK1 such as the previously reported A20-TAX1BP1-ABIN1 axis which disrupts the interactions between TRAF3 and TBK1-IKKi to suppress TBK1-IKKi K63-linked ubiquitination? There are likely temporal differences that allow these proteins to suppress TBK1 by ubiquitin editing in a coordinated and highly complementary manner. Whereas A20-TAX1BP1-ABIN1 inhibits TBK1 at an earlier phase after virus infection (4 h), NLRP4-DTX4 triggers TBK1 degradation at later times (**Figure 1**). This two-step mechanism of substrate inactivation was first described for A20 inhibition of RIP1 in the tumor necrosis factor receptor 1 pathway.[11] In future studies, it will be interesting to determine if NLRP4-DTX4 inhibition of TBK1 is dependent on the prior suppression of TBK1 K63-linked polyubiquitination by the A20 complex. This can be addressed by examining the ubiquitination and degradation of TBK1 by NLRP4-DTX4 in A20, TAX1BP1 or ABIN1-deficient cells. Furthermore, a recent study has implicated the E3 ubiquitin ligase TRAF-interacting protein in the degradation of TBK1 to limit virus-induced IFN-β production.[12] Does TRAF-interacting protein function together with NLRP4 and DTX4 in a protein complex to promote K48-linked ubiquitination and degradation of TBK1? If so, why are multiple E3 ubiquitin ligases involved in TBK1 degradation? The answers to these questions may elucidate the complexities of homeostatic control of innate immune signaling and provide new insight into the underlying causes of certain autoimmune diseases.

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