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Antiviral Signaling Through Pattern Recognition Receptors

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Viral infection is detected by the host innate immune system. Innate immune cells such as dendritic cells and macrophages detect nucleic acids derived from viruses through pattern recognition receptors (PRRs). Viral recognition by PRRs initiates the activation of signaling pathways that lead to production of type I interferon and inflammatory cytokines, which are important for the elimination of viruses. Two types of PRRs that recognize viral nucleic acids, Toll-like receptors (TLR) and RIG-I-like RNA helicases (RLH), have been identified. Of the TLRs, TLR3 recognizes viral double-stranded (ds) RNA, TLR7 and human TLR8 identify viral single-stranded (ss) RNA and TLR9 detects viral DNA. TLRs are located in endosomal compartments, whereas RLH are present in the cytoplasm where they detect viral dsRNA or ssRNA. Here we review the role of TLRs and RLHs in the antiviral innate immune response.

Key words: innate immunity, virus infection, Toll-like receptor, RIG-I-like RNA helicase, signal transduction.

The innate immune system is an evolutionally conserved mechanism that protects the host from invading microbial pathogens including viruses (*1–3*). Within the host, antigen-presenting cells such as dendritic cells (DCs) and macrophages, express a variety of pattern recognition receptors (PRRs) which recognize specific pathogen-associated molecular patterns (PAMPs) within microbial structures such as nucleic acid (*4*). Signaling via PRRs leads to the production of a variety of cytokines including inflammatory cytokines, type I interferon (IFN; both α and β), chemokines and IL-12, and to increased surface expression of co-stimulatory molecules to support the proliferation of T cells and differentiation into Th cells. Type I IFNs play a central role in the induction of antiviral responses as they lead to transcription of many IFN-inducible genes, which influence protein synthesis, growth arrest and apoptosis. Type I IFN also enhances DC maturation, natural killer (NK) cell cytotoxicity, and differentiation of virus-specific cytotoxic T lymphocytes, thus providing a link between innate and adaptive immune responses (*3*).

PRRs are classified into several families. The Toll-like receptor (TLR) family consists of more than 10 members which enable innate immune cells to respond to a variety of PAMPs (*1*). TLR3, TLR7, TLR8 and TLR9 represent a TLR subfamily that recognizes viral nucleic acid and has the ability to induce type I IFN (*2*). More recently, it has become apparent that viral RNA is also detected by members of the RIG-I-like RNA helicase (RLH) family such as RIG-I and Mda5 (*1–3*). TLR and RLH differ in their cellular localization, ligand specificity and downstream signaling pathways, which suggests that host cells have multiple defense mechanisms against viral infection.

RECOGNITION OF NUCLEIC ACID BY TLRS

Double-stranded (ds) RNA is synthesized during the replication of many viruses, which includes RNA and DNA viruses, and is a potent activator of innate immune cells that induce type I IFN. Polyinosinic acid:cytidylic acid (poly IC), which has been extensively used to mimic dsRNA, is recognized by TLR3 (*5*). TLR3 is also implicated in recognizing viral dsRNAs derived from dsRNA viruses such as reovirus or ssRNA viruses such as respiratory syncytial virus, EMCV and West Nile viruses (*6, 7*). TLR7 was initially identified as a receptor able to recognize imidazoquinoline derivatives with antiviral activity, such as imiquimod and resiquimod (R-848), and guanine analogues such as loxoribine (*8, 9*). Subsequently, guanosine or uridine rich single-stranded (ss) RNA, such as that derived from the human immunodeficiency virus (HIV) and the influenza virus, was identified as a natural ligand for TLR7 (*10–12*). In addition, TLR7 recognizes synthetic poly U RNA and certain small interfering RNAs (*11, 13*). TLR8 is phylogenetically similar to TLR7. Human TLR8 preferentially mediates the recognition of HIV-derived ssRNA and R-848 (*9, 10*). In contrast, TLR8-deficient mice respond normally to these molecules, suggesting that mouse TLR8 may not be functional (*10*). TLR9 recognizes unmethylated 2′-deoxyribo (cytidine-phosphate-guanosine) (CpG) DNA motifs that are present at high frequencies in pathogens such as viruses and bacteria but are rare in vertebrates (*14*). For example, genomic DNA purified from DNA viruses such as Herpes Simplex Virus (HSV)-2 or synthetic CpG oligonucleotides can activate immune cells via TLR9 (*15, 16*).

TLR3, TLR7, TLR8 and TLR9 are exclusively localized to intracellular compartments like endosomes, suggesting that these intracellular TLRs recognize nucleic acids following the internalization and lysing of viruses (*1*). When the transmembrane and cytoplasmic region of

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TLR9 are replaced by that of TLR4, this chimeric protein is expressed on the cell surface and cells respond to self DNA, suggesting that the endosomal location of TLR9 is important for prevention of an immune response to self DNA (*17*).

TLRs are expressed on macrophages and DC which play a central role in innate immunity. In mice, conventional DC (cDC) are divided into several populations depending on whether they express CD4 and/or CD8. TLR3 is expressed on a CD4-CD8+ subset of cDC which have high phagocytic activity. The apoptotic bodies of virus-infected or dsRNA-loaded cells are taken up by CD8 + DC and TLR3 recognizes the dsRNA within these cells. This process triggers cross-presentation, a pathway important for the development of CD8 cytotoxic T cell response against viruses that do not directly infect DC. Thus, the TLR3-dependent pathway mediates cross-presentation of CD8 + DC (*6*).

TLR7 and TLR9 are highly expressed on plasmacytoid DC (pDC; also known as IFN-producing cells), which are a subset of DC that have a plasmacytoid morphology and primarily secrete vast amounts of type I IFN in response to viral infection (*18, 19*). In TLR7-deficient mice, IFNα production by pDC is impaired after infection with influenza virus or vesicular stomatitis virus (*11, 12*). Moreover, IFNα production by pDC in response to DNA viruses such as murine cytomegalovirus, HSV-1 and -2 is dependent on TLR9 (*15, 16, 20*). Inactivated HSV-2 or genomic DNA purified from this virus triggers IFNα secretion in pDC in a TLR9-dependent manner (*16*). In summary, pDC rely on TLR7 and TLR9 to detect viral infection. However, the viral detection by pDC does not seem to require viral replication within the cells.

RECOGNITION OF NUCLEIC ACID BY RLHS

TLRs that are localized to endosomes are unable to sense viruses once they have entered the cytosol (either from the cell exterior or from endosomes) and initiated replication to produce dsRNA. Numerous studies have demonstrated the existence of TLR-independent mechanisms for the detection of viral infection. For example, induction of IFNβ following intracellular administration of poly IC or infection with RNA viruses is normally observed in fibroblasts or cDC derived from TLR3 deficient mice, suggesting that host cells have an intracellular mechanism to sense actively replicating viruses in the cytoplasm (*21–24*). In this regard, RIG-I, a member of the RNA helicase family of PPRs, was identified. RIG-I contains a DExD/H box RNA helicase and two caspase recruiting domain (CARD)-like domains (*21*). The helicase domain interacts with dsRNA, whereas the CARD-like domains are required for activating downstream signaling pathways. Furthermore, two additional RIG-I-like RNA helicases (RLH) have been identified: Mda5 and LGP2 (*25, 26*). Mda5 contains two CARD-like domains and a helicase domain, however, LGP2 lacks the CARD-like domains and is thought to negatively regulate RIG-I and Mda5.

Studies of RIG-I- and Mda5-deficient mice have revealed that RIG-I is essential for the recognition of a set of ssRNA viruses, which includes Flaviviruses, Paramyxoviruses, Orthomyxoviruses and Rhabdoviruses, whereas Mda5 is required for the recognition of a different set of RNA viruses that includes Picornaviruses (*27–29*). Furthermore, Mda5 and RIG-I detect poly IC and long dsRNA, respectively, indicating these RNA helicases perform different roles during the detection of viruses. Notably, it was recently shown that RIG-I-mediated detection of RNA depends on the 5′-triphosphate end of RNA that is generated by viral polymerases (*30, 31*).

While cDC, macrophages and fibroblast cells derived from RIG-I- or Mda5-deficient mice show impaired type I IFN induction after RNA virus infection, the production of type I IFN is still observed in pDCs derived from these mice (*27, 28*). It is notable that the TLR system is required for pDCs to induce the antiviral response (*27*). Collectively, these observations indicate that the TLR system plays a pivotal role in the detection of viruses by pDC.

TLR3 SIGNALING

TLR is a type I membrane protein characterized by an ectodomain composed of leucine rich repeats that are responsible for ligand recognition, and a cytoplasmic domain known as the TIR domain that is required for recruiting downstream signaling molecules. In response to stimulation by poly IC, TLR3 recruits a TIR-domain containing adapter protein called TRIF, which indirectly activates several transcription factors, including IRF3, NF-κB and AP-1 (*32, 33*) (Fig. 1). IRF3 controls the expression of genes for type I IFN, while NF-κB and AP-1 transcription factors regulate the expression of genes that encode the inflammatory cytokines. TRIF recruits non-canonical IKKs, TBK1 (also known as T2K or NAK) and IKKi (also known as IKKε) to its N-terminal domain, enabling them to phosphorylate IRF3 (*34, 35*). Phosphorylated IRF3 forms a dimer that translocates to the nucleus to induce expression of IFNβ (*36*).

TRIF also recruits TRAF6, a K63-linked ubiquitin E3 ligase, to the same N-terminal region (*37*). TRAF6 activates TAK1, a member of the MAPKKK family in a ubiquitin-dependent manner (*38*). TAK1 in turn activates canonical IKKs (IKKα and IKKβ), which lead to the phosphorylation and subsequent degradation of IκBs, allowing NF-κB to translocate to the nucleus. TAK1 simultaneously activates the MAPK (JNK, p38 and ERK) pathway, leading to phosphorylation and activation of members of the AP-1 family of transcription factors. On the other hand, the C-terminal region of TRIF contains RHIM, which mediates the interaction with RIP1 that is involved in TNFR-mediated NF-κB activation (*39*). RIP1 is inducibly polyubiquitinated and forms a complex with both TRAF6 and TAK1 (*40*). Thus, TRIF's recruitment of both RIP1 and TRAF6 is likely to facilitate TAK1 activation, resulting in maximal NF-κB and MAPK activation.

TBK1/IKKi-mediated phosphorylation of the C-terminal serine and threonine clusters in IRF3 is required for transcriptional activity, however, phosphorylation of Ser339 by an unknown protein kinase is linked to IRF3 destabilization. The cytoplasmic peptidyl-prolyl-isomerase Pin1, which catalyzes the cis-trans isomerization of

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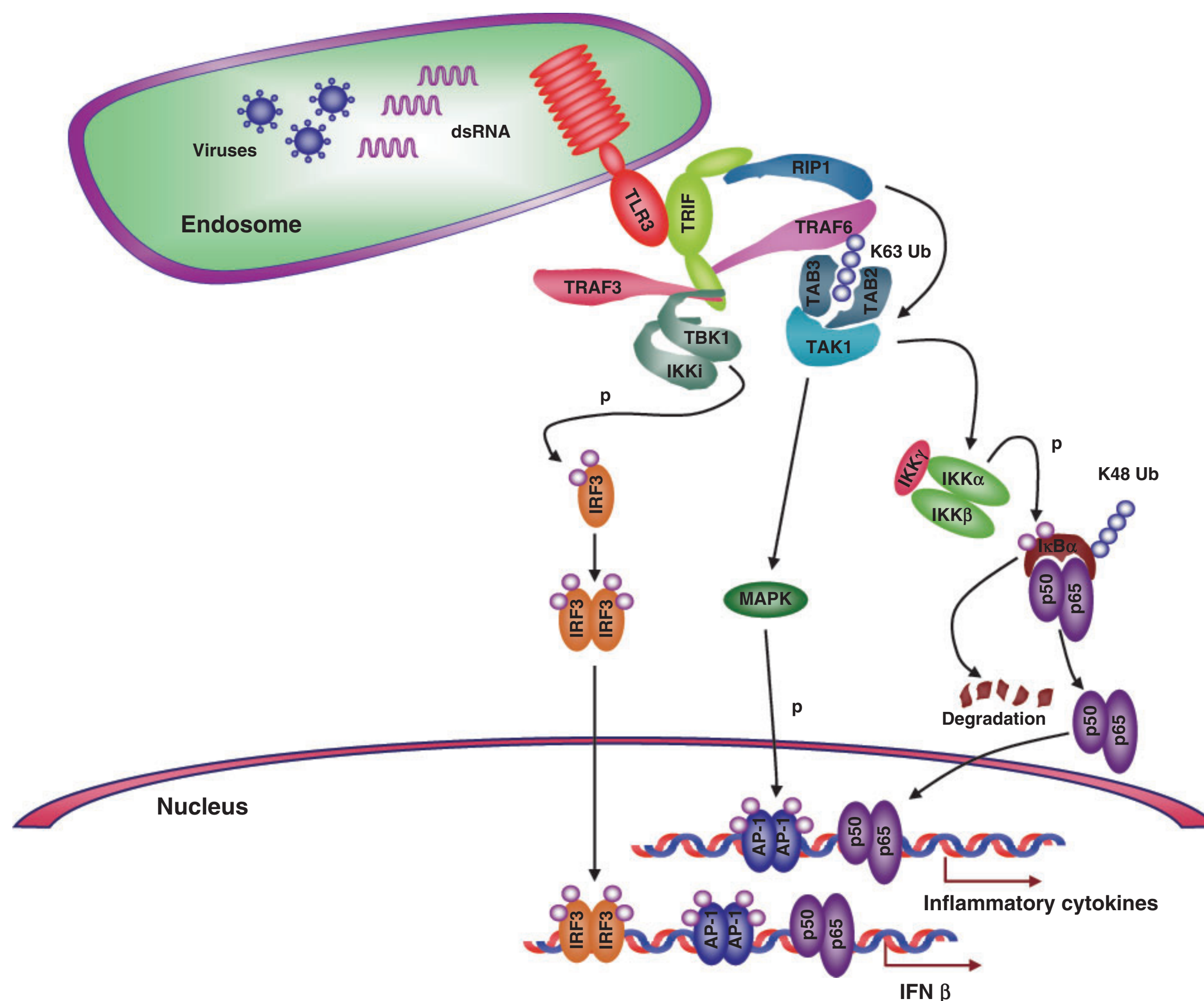

Fig. 1. **TLR3 signaling pathway**. TLR3 recruits TRIF, which interacts with TRAF3, TBK1 and IKKi. TBK1 and IKKi mediate phosphorylation of IRF3 (P). Phosphorylated IRF3 dimerizes and translocates to the nucleus where it binds DNA and induces expression of IFNβ. TRIF also interacts with TRAF6 and RIP1 to mediate NF-κB activation. TRAF6 activates the TAK1/TAB2/TAB3 complex via K63-linked ubiquitination (Ub). The activated TAK1 complex then activates the IKK complex consisting of IKKα, IKKβ and IKKγ/Nemo, to catalyze phosphorylation of IκBs (P). IκBs are destructed by the proteasome pathway, allowing NF-κB to translocate to the nuclei. TAK1 simultaneously activates the MAP kinase pathway, which results in phosphorylation (P) and activation of AP-1. NF-κB and AP-1 control inflammatory responses by inducing proinflammatory cytokines.

peptide-bonds located N-terminal to a proline residue, binds IRF3 when it is phosphorylated at Ser339, triggering ubiquitination and subsequent degradation of IRF3 to terminate the IFN response (*41*).

TLR7 AND TLR9 SIGNALING

Following nucleic acid recognition, TLR7 and TLR9 recruit a TIR-domain containing adapter called MyD88, which is universally utilized by all TLRs, with exception of TLR3 (*1*). MyD88 forms a complex with members of IRAK family (IRAK1 and IRAK4) and TRAF6, which in turn activates TAK1 and results in the activation of NF-κB (Fig. 2).

In mice, both pDC and cDC have the ability to induce IFNβ and inflammatory cytokine production in response to TLR7- and TLR9-ligands, whereas pDC, but not cDC, are able to induce IFNα, suggesting that pDC contain a unique pathway that mediates IFNα induction. Type I IFN induction in response to TLR7- and TLR9-ligands has been shown to be independent of IRF3, suggesting that other IRF members are involved in type I IFN induction (*42*). IRF7 is structurally the most similar to IRF3, and potently activates the promoters of the IFNβ and IFNα genes. Unlike IRF3, which is ubiquitously expressed, IRF7 expression is weak but is induced in response to viral infection in most cell types. However, IRF7 is constitutively expressed in pDC, suggesting that IRF7 may play a specialized role in pDC. It was shown that IRF7 is able to form a signaling complex with MyD88, IRAK1, IRAK4 and TRAF6, and that it translocates to the nucleus in response to CpG DNA (*43–45*) (Fig. 2). Mice deficient in MyD88, IRAK4 or TRAF6 have defects in both IRF7- and NF-κB-activation with a

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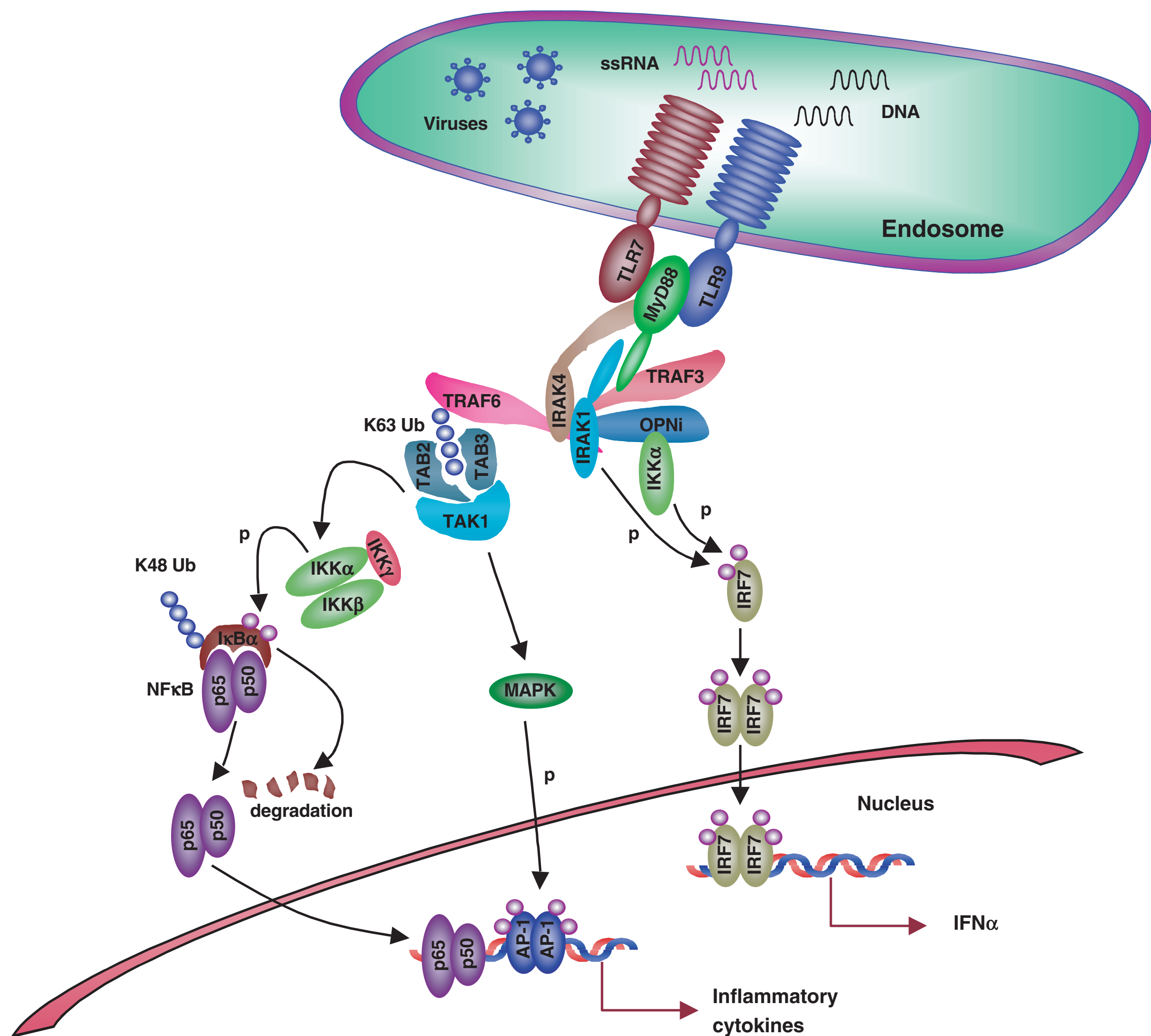

Fig. 2. **TLR7 and TLR9 signaling pathway**. TLR7 and TLR9 recruit MyD88 to induce type I IFN and inflammatory responses through the activation of IRF7 and NF-κB, respectively. In pDC, MyD88 form a signaling complex with IRAK1, IRAK4, IKKα, TRAF3, TRAF6, OPN-i and IRF7. In response to ligand stimulation, IRF7 is phosphorylated by IRAK1 and IKKα, and translocates to the nuclei to regulate expression of type I IFN genes, especially IFNα. TRAF3, IRAK1, IKKα and OPN-i are indispensable for IRF7 activation and dispensable for NF-κB activation, however, IRAK4 and TRAF6 are indispensable for both NF-κB and IRF7 activation.

concomitant impairment in the induction of type I IFN and inflammatory cytokines in response to CpG DNA (*43, 44*). On the other hand, IRAK1 deficiency results in the loss of IRF7 activation without affecting NF-κB activity (*45*). Moreover, IRAK1, but not IRAK4, is capable of phosphorylating IRF7 (*45*). Together, these findings suggest that IRAK1 could be the kinase that phosphorylates IRF7. IKKα has been reported to play a similar role to IRAK1 as it binds and phosphorylates IRF7, however, the functional relationship between IRAK1 and IKKα remain unclear (*46*). IRF8 has also been implicated in the TLR9-mediated viral response (*47*). pDCs derived from IRF8-deficient mice display loss of type I IFN and inflammatory cytokine induction by TLR9. NF-κB DNA-binding activity in response to TLR9-ligands was reported to be severely impaired in the absence of TLR9, suggesting that IRF8 facilitates NF-κB DNA-binding activity or that IRF8 is located upstream of the IKK complex. TRAF3 is a member of the TRAF family that is critical for induction of type I IFN in response to TLR7- and TLR9-signaling. TRAF3 binds MyD88 and IRAK1, activating IRF7 (*48, 49*) (Fig. 2). Furthermore, TRAF3 binds TRIF to facilitate TBK1/IKKi-dependent IRF3 activation (Fig. 1), indicating that TRAF3 is an integral component of IRF7 and IRF3 activation during TLR3-, TLR7- and TLR9-signaling. Osteopontin (OPN) is a protein secreted by osteoblasts that has diverse biological functions, including bone resorption and inflammation. However, a precursor of OPN (OPN-i) was shown to be retained in the cytoplasm. It was demonstrated that OPN-i interacts with and colocalizes with MyD88, and that nuclear

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translocation of IRF7 in response to a TLR9 ligand is diminished in OPN-deficient pDC (*50*). These observations strongly suggest that OPN-i is a component of the MyD88-IRF7 complex in pDC (Fig. 2).

TLR9 signaling pathways are thought to differ between cDC and pDC. cDC derived from IRF1-deficient mice have impaired induction of IFNβ, inducible nitric-oxide synthase and IL-12 p35 in response to a TLR9-ligand while pDC derived from IRF1-deficient mice show normal induction of type I IFN, indicating that IRF1 has an essential role in TLR9-mediated signaling in cDC (*51*). It is well-known that cytokine induction in response to TLR ligands is enhanced by pretreatment with IFNγ. Consistent with the observation that IRF1 expression is increased by IFNγ, the induction of cytokine production following treatment with IFNγ is impaired in IRF1-deficient mice. Thus, following IFNγ-mediated induction of IRF1, IRF1 is recruited by MyD88 to form a complex that translocates to the nucleus in response to TLR stimulation. IRF5-deficient cDC and macrophages exhibit impaired inflammatory cytokine production in response to many TLR ligands while the secretion of type I IFN by pDC is unaffected. IRF5 binds to MyD88 and translocates to the nucleus after phosphorylation, where it binds to ISRE motifs found in the promoter region of genes encoding inflammatory cytokines to stimulate transcription (*52*). On the other hand, IRF4 negatively regulates IRF5 function by competing with IRF5 for MyD88 (*53*).

RLH SIGNALING

Induction of type I IFN following viral infection is impaired in embryonic fibroblast cells derived from mice deficient in both TBK1 and IKKi, suggesting that RIG-I and Mda5 might utilize an adapter molecule bearing a similar CARD structure that physically links these helicases to TBK1/IKKi. In this regard, IPS-1 (also known as MAVS, Cardif or VISA) contains a CARD-like domain, interacts with RIG-I and Mda5 and was identified as a potent activator of the IFNβ promoter (*54–57*) (Fig. 3). Overexpression of IPS-1 leads to activation of IFNα4, IFNα6, NF-κB and IFNβ promoters and results in the production of type I IFN, which then inhibits viral replication. Conversely, small interfering RNA knock-down of IPS-1 blocks IFN production in response to poly IC transfection or viral infection. cDC, macrophages and embryonic fibroblasts derived from IPS-1-deficient mice fail to produce type I IFN and inflammatory cytokines and fail to activate IRF3 and NF-κB when challenged with RNA viruses that are recognized by either RIG-I or Mda5 (*58, 59*). Similarly, cytokine induction in response to poly IC or long dsRNA is also impaired in IPS-1-deficient mice (*58*). IPS-1-deficient mice consistently show a susceptibility to infection with RNA viruses. Collectively, these findings suggest that IPS-1 is an essential adapter utilized by both RIG-I and Mda5, which mediates the antiviral innate immune response. In pDC, however, TLR7 and TLR9 preferentially contribute to type I IFN induction after viral infection, indicating a cell type-specific involvement of TLR and RLH in the induction of antiviral immune responses.

IPS-1 activates IRF3 and IRF7 via TBK1/IKKi, although there is no direct interaction between IPS-1 and TBK1/IKKi. TRAF3 binds both IPS-1 and TBK1/IKKi, and TRAF3 deficiency results in impaired type I IFN induction after viral infection, indicating that TRAF3 provides a link between IPS-1 and TBK1/IKKi (*60*) (Fig. 3). IPS-1 also interacts with FADD, a death domain-containing adapter involved in death receptor signaling (*54*). Cells deficient in FADD display reduced induction of IFNβ and inflammatory cytokines. FADD forms a complex with caspase-10 and caspase-8, and these caspases are cleaved in response to poly IC (*61, 62*). The cleaved fragments of these caspases (which encode a death-effector domain) are capable of activating NF-κB. Cells lacking caspase-8 show reduced activation of NF-κB and reduced induction of inflammatory cytokines, whereas type I IFN induction is unaffected. Thus, caspase-8 and caspase-10 are specifically involved in NF-κB activation downstream of FADD (Fig. 3). A precise mechanism of how these fragments can activate NF-κB remains unclear.

IPS-1 contains a transmembrane domain that is rich in hydrophobic residues in the C-terminal tail and targets IPS-1 to the mitochondria (*55*). Notably, mitochondrial retention of IPS-1 is essential for IRF3, IRF7 and NF-κB activation, suggesting that signaling from mitochondria plays an important role in the antiviral immune response. The NS3/4A serine protease encoded by the hepatitis C virus has been demonstrated to target IPS-1 for cleavage (*56, 63–65*). Using an *in vitro* cell culture infection system to introduce the hepatitis C virus, a putative cleavage site of IPS-1 was located upstream of the transmembrane domain. The cleaved form of IPS-1, which lacks the transmembrane region, failed to activate IFNβ and NF-κB. Collectively, these findings suggest that cleavage of IPS-1 by the hepatitis C virus NS3/4A protease might lead to a switch in the cellular location of IPS-1, from the mitochondria to the cytoplasm, that results in inhibition of type I IFN and inflammatory responses.

FUTURE PERSPECTIVES

Recent progress has revealed that there are multiple defensive mechanisms against viral infection. dsRNA is recognized by two sensors: TLR3 and RLH. TLR3 detects dsRNA that is released into endosomes following phagocytosis of apoptotic bodies from virally infected cells, or TLR3 detects dsRNA viruses that are internalized by receptor-mediated endocytosis. In contrast, Mda5 and RIG-I are expressed in various cell types, and recognize the cytoplasmic dsRNA that is produced during viral replication. Therefore, these helicases detect actively replicating viruses. TLR7 and TLR9 are expressed by pDC and act as sensors for viral ssRNA and DNA, which trigger the production of large amounts of type I IFN. TLR7 and TLR9 use MyD88 as an adapter to induce type I IFN via IRAK1/IKKα-dependent phosphorylation of IRF7. In contrast, TLR3 relies on the adapter TRIF to recruit TBK1/IKKi and induce IRF3 phosphorylation. During RIG-I and Mda5 signaling, IPS-1 functions as an essential adapter that mediates

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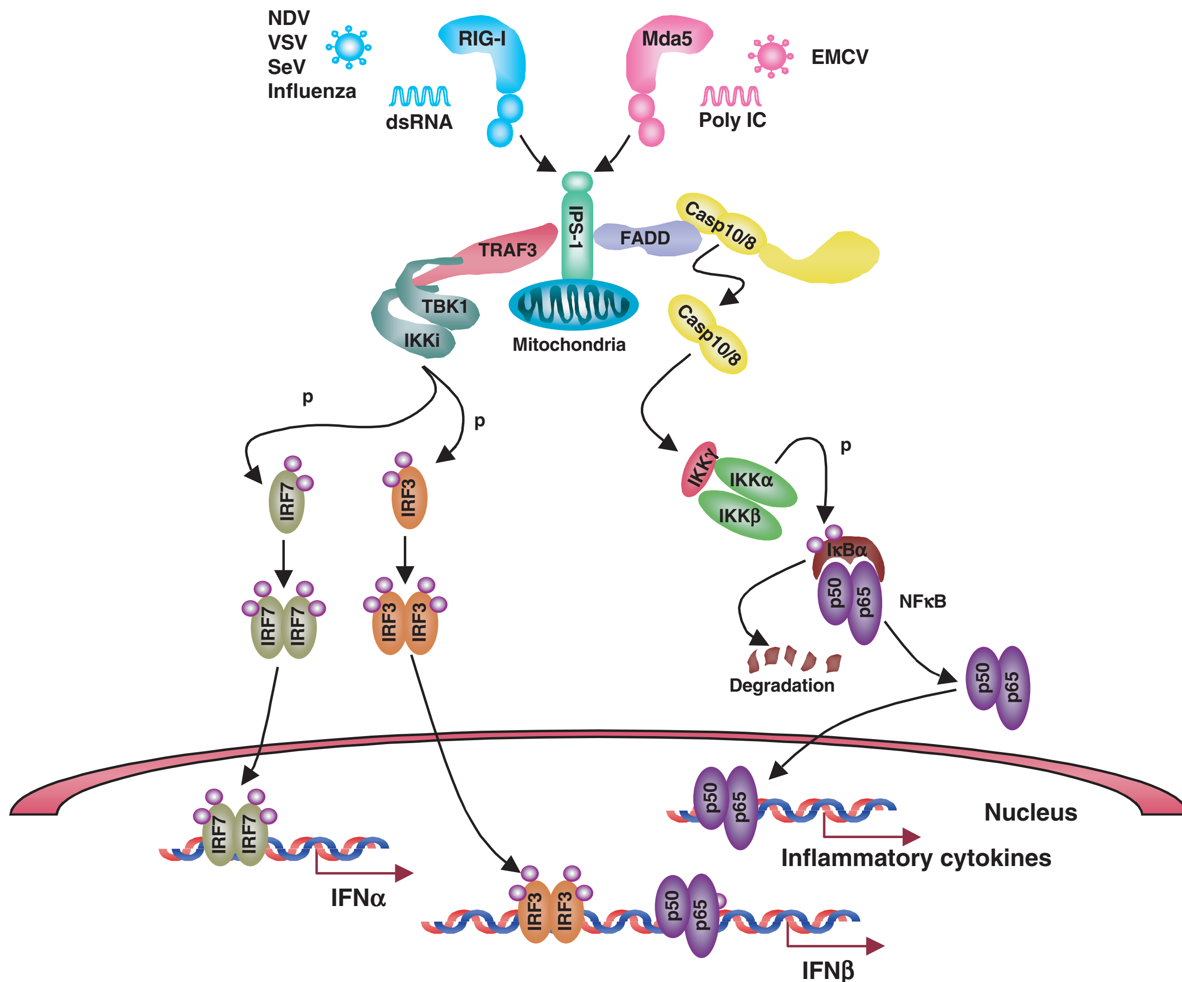

Fig. 3. **RLH signaling pathway**. RIG-I and Mda5 interact with the adapter IPS-1 via CARD-like domains. IPS-1 is localized to mitochondria and initiates intracellular signaling pathways that lead to activation of IRF3/7 and NF-κB via the activation of TBK1/IKKi and IKKα/IKKβ, respectively. A FADD, Caspase-8 and Caspase-10 complex has been implicated in IPS-1-dependent NF-κB activation, whereas TRAF3 provides a physical link between IPS-1 and TBK1/IKKi.

IRF3 and IRF7 activation via TBK1/IKKi. All of the above-mentioned PRRs induce an antiviral immune response, however, they differ in their expression profile, cellular localization and signaling pathways. Conversely, viruses have developed immune evasion mechanisms that target these signaling pathways, for example, the hepatitis C virus protease NS3/4A inactivates both IPS-1 and TRIF by cleavage to inhibit RLH and TLR3 signaling pathways (*66, 67*).

Cells appear to express an as yet, unidentified cytosolic receptor(s) that recognizes right-handed dsDNA released by DNA viruses, bacteria and damaged host cells (*68, 69*). It will be important to identify this DNA sensor and to further our understanding of how PPRs detect nucleic acid and induce antiviral innate immune responses. Such knowledge could improve therapeutic strategies for the treatment of infectious diseases and autoimmune diseases, which are often triggered by viral infections.

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