Recognition of viruses in the cytoplasm by RLRs and other helicases—how conformational changes, mitochondrial dynamics and ubiquitination control innate immune responses

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Abstract

Mammalian cells possess multiple sensors for recognition of broad range of microbes. This recognition occurs through specific molecular signatures found across various pathogens. Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs) are the major cellular PRRs responsible for this recognition. TLRs are transmembrane sensors, whereas other PRRs mainly localize in the cytoplasm for the activation of type I IFNs and pro-inflammatory cytokines. Among these PRRs, RLRs are well known for its indispensable role in sensing the invasion of RNA viruses. This review summarizes recent advances in viral recognition by RLRs and their signalling pathways, and introduces newly emerging RNA helicases involved in innate immune responses.

Introduction

Interferon system is well known for its essential role in mounting the first line defence against infectious pathogens for the past decades. This system can be divided into two types—type I and type II. Type I interferons comprised of several members; one of the example would be interferon α/β superfamily, while type II interferon only consists of interferon-γ. These cytokines were firstly identified as the key proteins to suppress viral replication. They are produced by viral infected cells through the initiation of the first round of signalling cascade, activating several major transcription factors such as ATF/c-jun, IRFs and NF-κB family members, and eventually lead to the production of mature interferon proteins. These proteins will be secreted to the circulation system and bound interferon receptors on the cell surface, which in turn triggered a series of dimerization and phosphorylation derived modifications, initiating the secondary signalling cascade known as JAK-STATs pathways, activating the transcription of numerous interferon-stimulated genes (ISGs).

Investigation of the underlying biochemical mechanisms responsible for interferon production, from transmitting signals after pathogens infections to the activation of transcription factors that drives interferon expression within the nucleus, has been a major focus in this field. It was believed that within cells there exist sensors or receptors that can recognize distinct molecular structure that are broadly shared by pathogens (1). In the past few decades, numerous efforts have been invested to identify these pathogen-recognition receptors (PRRs). To date, major PRRs that have been successfully identified include Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs). NLRs are mainly responsible for intracellular inflammatory responses, while CLRs are mainly responsible for sensing the C-type lectin-like domain that is mainly found in fungi and bacterial (2,3). RLRs mainly functions as cytoplasmic sensors for broad range of viruses, whereas well characterized TLRs are known to detect broad range of pathogens.

Recent studies have also provided us important insights about the mechanism whereby these PRRs regulate both innate and adaptive

immune responses in distinct type of cells; for example, TLR-mediated control of immune responses is mainly orchestrated by immunological cells such as macrophages and dendritic cells (DCs). Despite their dispensable role in plasmacytoid DCs (pDCs), RLRs are still the core molecule responsible for activation of both pro-inflammatory cytokine genes and cytokine-stimulated genes in a broad range of cells, including myeloid DCs (mDCs; also known as conventional DCs), coordinating the first-line of host systemic defences.

In the following article, we will discuss recent advances in our knowledge of the underlying molecular mechanisms whereby the interferon system and other cytokines are stimulated by RLRs. In parallel, some comparisons with TLRs will be depicted through illustrations and tables. Detail advances in TLRs and other PRRs such as NLRs have been reviewed elsewhere (4,5) and will not be discussed in detail in the text below.

The family of RIG-I-like receptors

The discovery of RLRs

In the year 2004, RIG-I was identified by Yoneyama, M *et al*. (6) as a novel cytoplasmic viral sensor through a series of complementary DNA libraries screening in polyI:C stimulated cells (polyI:C is a variable length synthetic analogue of double-stranded RNA). Colonies showing significantly enhanced responses towards an IRF3-based reporter (p55C1B-Luc) were selected for sequencing and 'blasted' for potential candidates. Among the $1x10^5$ clones, RIG-I was identified (6). Gene-targeting of the endogenous RIG-I in mouse-embryonic fibroblasts severely abrogated type I IFN production and ISG activation and, hence, potentiate viral replication. These extensive studies, combined with numerous biochemical analyses indicated that RIG-I plays a key role in mounting the first line defence against RNA viral invasion and, in part, against DNA viruses within cytoplasm through eliciting type I IFN production.

RIG-I (which is encoded by the DDX58 gene) and its closely related RLR-family members—melanoma differentiation associated factor 5 (MDA5; encoded by the IFIH1 gene) and laboratory of genetics and physiology 2 (LGP2; encoded by the DHX58 gene)—belong to the family of DExD/H (Asp-Glu-x-Asp/His) box RNA helicases. Such helicases move along the nucleic-acid phosphodiester backbone, altering the secondary structure and using ATP for energy. All three RLR-family members contain a central helicase domain with ATPase catalytic activity, which is essential for viral double-stranded RNA (dsRNA) binding (6); and a repressing domain (RD) in the C-terminal domain (CTD), which is essential for autoregulation and binding with the 5'-triphosphate end of the viral genome (6,7). Despite sharing these similarities, they do have some differences (Figure 1): both RIG-I and MDA5 possess two tandem CARD domains at the N-terminal region, which is responsible for downstream signal transduction and activation of type I IFN genes after the recognition of non-self RNA (6); on the other hand, LGP2 lacks this signalling domain, suggesting that LGP2 is more likely to be a regulatory molecule rather than functioning as a signal inducer.

RLR-mediated sensing of viruses and the specificity of viral PAMPs

RLRs are known to confer recognition of various types of RNA and DNA viruses. Substantial progress has been made in defining the specific RNA signatures recognised by each RLR. In RIG-I-deficient fibroblast cells, little cytokine is produced in response to Paramyxoviruses such as Newcastle disease virus (NDV) and Sendai virus (SeV), rhabdoviruses such as vesicular stomatitis virus (VSV) (8,9), flaviviruses such as hepatitis C virus (HCV) and Japanese encephalomyocarditis virus (JEV) (10,11), and orthomyxoviruses such as influenza A and B (12,13). In contrast MDA5, despite being closely related to RIG-I, mainly recognizes Picornaviruses such as encephalomyocarditis virus (EMCV), poliovirus, Theiler's murine encephalomyelitis virus (TMEV) and Mengo viruses (11). In addition, west Nile virus (WNV) and dengue virus are recognized by both receptors. In the case of chemically defined oligonucleotides, RIG-I mainly recognizes double-stranded blunt-ended dsRNA and single-stranded RNA bearing 5'-triphosphate. Removing all three phosphates

groups through phosphatase treatment, or using chemically synthesized di- or mono-phosphate ssRNA does not activate RIG-I-dependent signalling. Based on previous studies, RIG-I could also triggered IFN-gene activation in response to RNA products reverse-transcribed from the genome of

DNA viruses through cytoplasmic RNA polymerase III (14,15). By using chemically synthesized polydA:dT, these studies proposed this novel role of RIG-I as an 'indirect' DNA sensor. In addition, the 'panhandle' structure found in the influenza genome, RNAs cleaved by RNase-L (an interferon-induced ribonulease) and polyU/UC-rich RNA (e.g. the 3'UTR of HCV) were also reported to be ligands for RIG-I (13, 16-17). Strikingly, Luthra *et al*. (18) provide evidence that viral mRNA from parainfluenza 5 viruses could activate IFN production through an MDA5–RNase-L pathway. Unlike previous observations that used chemically synthesized oligonucleotides, this is the first evidence reporting a *bona fide* single-stranded ligand that could activate MDA5; moreover, it's a viral mRNA with guanosine capped at the 5' terminal.

Since RNase-L is essential for the activation of IFN production by MDA5–RNase-L, the catalytic processing activity of RNase-L is inevitably involved, and some modifications as a result of cleavage by RNase-L might contribute. Since the exact RNA signature was not well characterized in this study, further analysis in this direction might revealed a brand new activation mode of MDA5. Long, synthetic dsRNAs such as polyI:C are known to be MDA5 ligands, whereas short polyI:C molecules turn out to be ligands for RIG-I (19). Other potential ligands for RLRs are summarized in Table 1. Substantial progress in structural analysis further confirmed these predictions (20-23). Collectively, the length and the composition and/or defined secondary structures of RNA might determine the differential recognition between these two major RLRs.

Structural analysis of RIG-I

Numerous questions regarding the activation mode of RIG-I remain unsolved, particularly how each domain contributes to the conformational change of RIG-I upon engagement with viral RNA. Efforts in deciphering the structure of RIG-I have been tremendous. Here, we will discuss some of the newly proposed structural models of RIG-I activation.

Previously, it has been proposed that the basic structural mode of RIG-I remains in a 'closed' conformation and undergoes conformational changes after binding to its RNA ligand, exposing the CARD domains for signalling and eventually alerting the cell about viral invasion (23) (Figure 2A).

In the same issue of *Cell* in 2011, Luo *et al*. (24) and Kowalinski *et al*. (25) independently revealed some new structural insights into RIG-I activation. In these reports, both groups concomitantly resolved the structure of

several key RIG-I domains-Helicase-1 (Hel1; from human RIG-I [hRIG-I] aa236–455 and duck RIG-I [dRIG-I] aa242–456) and Helicase-2 (Hel2; from hRIG-I aa456–795 and duck RIG-I aa458–794).

Hel2 further includes two subdomains, known as Hel2 insertion domain (Hel2*i*) and the 'pincher/bridging' domain. Through crystallization of human truncated RIG-I mutants, Luo *et al*. (24) showed that the RNA binding activity of RIG-I is regulated through multiple domains in an orchestrated way. For example, they showed that Hel2*i* specifically detects dsRNA, whereas the pincher/bridge domain possesses a V-shaped structure and mainly functions in controlling both Hel1–Hel2 and the CTD upon binding with RNAs, pinching them into a central groove with a 'closed clamping' structure. This model suggests that the pincher/bridge domain could act as the 'messenger' to communicate between the CTD and Helicase domains.

In addition, Luo *et al*. (24) demonstrated that the oligomerization of RIG-I is dependent on RNA length, with a minimal length of 18bp of dsRNA being necessary to significantly induce the oligomerization of RIG-I, and

that ATPase activity is dispensable for this aspect. In their experiment, the maximum length of dsRNA used was only a 22-mer, unlike previously published work carried out *in vivo*, in which dsRNA with a length up to 1 kbp could still activate RIG-I (19). Solving the structural changes of RIG-I using RNAs with different lengths and sequence composition might be interesting.

In conclusion, Luo *et al*. (24) successfully highlighted the functional importance of several previously undefined subdomains in coordinating the conformational changes of RIG-I. Their experimental observations are still, however, insufficient to define the exact sequential changes in RIG-I architecture from RNA sensing to signalling, as crystallization of full-length human RIG-I poses a daunting challenge.

In the same issue of *Cell*, Kowalinski *et al*. (25) made a remarkable step in answering this question of how RIG-I transmit sensing to signalling. By using intense X-ray beams, the authors further verified that in the quiescent state, instead of the 'closed' conformation as previously anticipated, RIG-I possesses a 'partially open' conformation. The CARDs are sequestered together with Hel2*i*, with a linker sterically hindering the

binding of viral RNA to the helicase and hindering the ubiquitination of CARD by tripartite-motif-containing 25 (TRIM25). In biochemical pulldown assays, purified dRIG-I CARD is able to bind to Hel2*i*. These findings further rationalized our understanding that the CTD domain is not essential for the autorepressing function but the CARD domains are crucial in maintaining RIG-I in a non-active state. In contrast, the CTD region is flexibly exposed, with no strong chemical interactions with the other domains, and can thus fulfill a sensing role (25).

On the basis of these observations, Kowalinski *et al*. (25) proposed a sequential RIG-I activation model — upon binding with viral 5'-triphosphate dsRNA by the CTD, signals triggered the pinching of both the helicase domains and the CTD–dsRNA complex by the V-shaped pincher/bridging domain; the helicase domain is flexibly adapted to fit in the viral dsRNA after ATP hydrolysis. This cooperative binding results in a strong and stable V-groove closed-clamping complex, and eventually promotes the expulsion of the CARD regions, leaving them exposed for interaction with MAVS (Figure 2B). Despite their novelty, it should be noted that these observations were conducted using dRIG-I, with only

53% homology to hRIG-I. The hRIG-I activation mechanism might differ; thus complementary studies comparing the human equivalent are definitely required. In general, these reports further advanced our understanding of the molecular architecture of RIG-I during signal activation.

The RLRs-mediated signalling

Events that follow RLR recognition of viral PAMPs

Upon virus infection, RIG-I recognizes dsRNA with 5`-triphosphate as the PAMP, being distinct from self-RNA. In the quiescent state, both RIG-I and MDA5 remain in a partially closed conformation; after binding with its molecular PAMPs, RIG-I undergoes conformational changes by exposing its CARD-domain and forming a translocon complex with a chaperone molecule called 14-3-3ε (26). This chaperone mainly functions as a scaffold for stabilizing TRIM25-mediated ubiquitination and facilitates the translocation of RIG-I–ligand complexes to mitochondria to form homotypic interactions with the CARD region of its adapter, MAVS, which is predominantly located on mitochondria, for downstream signal

transduction (27). This interaction further induces the assembly of numerous proteins to form complexes; examples of these proteins are STING (stimulator of IFN genes; also known as MITA [mediator of IRF3 activation], ERIS [endoplasmic reticulum interferon stimulator] or MYPS [a motif from DNA polymerase]), TRADD, FADD, RIP1, caspases and TRAF2/3/6 (28-35). These complexes further induce the activation of both NF-κB and IRF3 through IKKα–IKKβ and TBK1–IKKγ complexes, respectively, and eventually trigger the transcription of type I IFN genes and other pro-inflammatory cytokines (Figure 3).

The role of LGP2

LGP2, a member of the RLR family, has been proposed to have a regulatory function for both RIG-I and MDA5. *In vivo* studies using LGP2-deficient cells revealed that cytokine production was impaired after viral infection (36,37), whereas *in vitro* overexpression of LGP2 inhibits IFN-β production (9). Since LGP2 lacks a CARD domain, major functional analyses, such as the RNA binding activities and ATPase activity, mainly focus on its CTD and helicase domains. Biochemical and structural analyses demonstrate that the CTD of LGP2 could bind to both

ssRNA and dsRNA with higher affinity than RIG-I and MDA5 do (38). In a system in which it is overexpressed, LGP2 might therefore act as a potent negative regulator because sequestration of these ligands through competitive binding could prevent the activation of RIG-I and/or MDA5.

Satoh *et al*. (37) demonstrated that ATPase activity is essential, using an LGP2 mutant that had lost its ATP-catalytic activity; expressing this mutant in LGP2-deficient cells failed to restore IFN production. Other *in vitro* analyses indicated, however, that ATPase is dispensable for its functions (39). Hence, the precise role of LGP2 is still unclear, with different functions being variously affected by various physiological conditions such as ATPase activities, RNA binding, subcellular localization, expression levels and involvement of unknown third-party components.

In a recent report, the complement component C1qA was found to be essential in RIG-I-mediated IFN-β gene activation via TBK1 (40). Overexpression of C1qA enhanced RIG-I and MAVS-mediated IFN-β gene activation and, hence, efficiently suppressed viral replication in human 293T cells. C1qA was initially reported to be mainly produced by

macrophages or related cell lines such as THP1; concomitantly, proteomic analyses from the above report (40) also indicate the strong association between C1qA and MAVS in the THP1 lineage. Further examination on the role of C1qA in RIG-I-mediated signalling in macrophages could be potentially interesting. In addition, FAK (focal adhesion kinase), a large protein complex that bridges the cytoplasm to the extracellular matrix through the cytoskeleton, was also shown to be essential for RIG-I-mediated signalling through interactions with MAVS (41).

Apart from these proteins, other major regulatory molecules of RLRs can be classified within the mitochondrial and ubiquitination machinery. In the following sections, we will discuss recent progress regarding how these two classes of machinery stringently control the downstream signalling pathways.

Mitochondrial dynamics in RLRs antiviral signalling

In 2005, MAVS (also known as IPS-1 [IFN-β promoter stimulator-1], Cardif [CARD adapter inducing IFN-β] and VISA [virus-induced signalling adaptor]), the central adaptor for RLR-mediated signalling, was identified by four independent laboratories (42-45). This protein contains a CARD domain (amino acids 10–77), which is essential for signal transduction through homotypic interaction with CARD of RIG-I and MDA5; a proline-rich region (amino acids 107–173); and a transmembrane domain (amino acids 514–535), which is essential for anchoring this protein on the outer membrane of mitochondria for antiviral signal transduction. The discovery of this novel RLR adaptor led to an avalanche of analyses focusing on mitochondrial proteins. Indeed, subsequent articles have further enlightened our understanding of how mitochondria play an essential role as a central signalosome downstream of RLR-dependent IFN gene activation.

To date, numerous mitochondrial proteins have been suggested to have a positive or negative regulatory role in MAVS-mediated RLR signalling.

The first example is TOM70, a mitochondrial import receptor encoded by the *TOMM70A* gene. As reported by Liu *et al*. (46), TOM70, an outer mitochondrial membrane protein, positively regulates RLR-mediated signalling by recruiting TBK1 and IRF3 to MAVS, resulting in the formation of a supramolecular complex for signal transduction. Ectopic expression of TOM70 successfully enhanced IFN production and effectively suppressed viral replication. These analyses were further verified through endogenous depletion of TOM70, which abrogated IFN production in response to SeV infection.

Although Liu and colleagues (46) succeeded in demonstrating the mechanistic link of MAVS–TBK1–IRF3 complex being dependent on cytosolic chaperone Hsp90, the hierarchical role of TOM70 in the proposed pathway remains unclear. In a recent report, Kasama *et al*. (47) demonstrated that exogenous expression of TOM70 failed to rescue RLR-mediated IFN-signalling in hepatocytes after HCV infection. It was known that HCV could impair RLR-mediated signalling through the cleavage of MAVS by its NS3/4A protease (43). In the report by Kasama *et al*. (47),

abrogation of the IFN-β response because of cleavage of MAVS by NS3/4A in the TOM70-overexpression system was still observed, suggesting that TOM70 works downstream of MAVS.

Despite the proteomics analyses performed by Liu and colleagues (46), who demonstrated a strong physical association between TOM70 and MAVS, the mechanistic aspects of how TOM70 communicates with MAVS still remain unclear. Addressing these could further strengthen our understanding on the role of TOM70 as well as other mitochondrial adaptor proteins in RLR-mediated innate immunity. The mitochondrial network is highly dynamic even when the cell is in a quiescent state. Both fusion and fission processes occur regularly to maintain healthy mitochondrial morphology for optimum cellular metabolism. Mitofusin proteins are known to be essential regulators in these events. Recently, several reports concomitantly demonstrated that mitofusin proteins play a vital role in RLR-mediated signalling. Mitofusin 1 (Mfn1) regulates fusion events by tethering neighbouring mitochondria and was shown to be a positive regulator in RIG-I signalling (48,49). The study by Onoguchi *et al*.

(48) undertook two approaches to verify the involvement of Mfn1: 1) The effect of ectopic expression; and 2) loss-of-function analyses. Overexpression of Mfn1 enhanced IFN-β gene activation in response to both NDV infection and stimulation with RNAs containing 5'-triphosphates; moreover, both using mouse embryonic fibroblasts deficient in Mfn1 and endogenous depletion of Mfn1 using siRNAs severely impaired IFN-β gene activation.

Mutagenesis analysis further indicated that GTPase domain of Mfn1 is essential to facilitate efficient signal transduction, suggesting that mitochondrial dynamics mediated by Mfn1 could be the key in modulating the MAVS-containing central signalosome. Indeed, distinct MAVS aggregation was observed using FLAG-tagging (in which an octapeptide tag is added using recombinant DNA technology) of cells that stably express MAVS; endogenous depletion of Mfn1 blocked this aggregation. On the basis of these results, Onoguchi *et al*. (48) proposed a model in which MAVS aggregation is essential for downstream antiviral signal transduction and this aggregation is mediated by Mfn1 through

mitochondrial fusion processes. These findings were subsequently partially verified by Castanier *et al*. (49). These authors demonstrated that siRNA knockdown of both Mfn1 and OPA1 (optic atrophy 1) perturbed normal mitochondrial morphology and, hence, abrogated NF-κB and IRF3-dependent antiviral responses.

How MAVS is aggregated upon virus infection is a particularly interesting area of research. In conjunction with the finding that MAVS are present on peroxisomes (50), reallocation of MAVS from peroxisomes to mitochondria might not be improbable; since both are metabolically linked organelles and evidence about dual redistribution of proteins under constitutive dynamics and interactions between both organelles is well established (51). In a recent report, a distinct organelle compartment called MAM (mitochondria-associated membrane), which connects the ER to mitochondria and tethering with peroxisomes, was shown to be essential for RIG-I functions (52), providing us some insights that the close inter-relationship among these membranous organelles regulates RLRs in an

orchestrated way, which includes cross-talk, and a mitochondria-peroxisomes-ER vesicular trafficking pathway.

Overproduction of inflammatory cytokines might trigger severe systemic diseases that might cause a high mortality and morbidity rate for the host; therefore stringent control exerted over various stages throughout the entire line of cytokine production is indispensable. In the past few decades, numerous negative regulators that reside on mitochondria and that specifically target MAVS-containing signalosomes were identified. Mfn2, the homologous protein of Mfn1, was found to inhibit RLR-mediated signalling (53). In this report, the authors demonstrated that Mfn2 sequestered MAVS, resulting in a stable complex with a higher molecular weight when cells were in a quiescent state. Depletion of Mfn2 further enhanced RLR-mediated antiviral responses and effectively suppressed viral replication.

There are, however, several contradictions within the reports about mitochondria dynamism; we do not know what exactly causes these

discrepancies. Admittedly, the proposed model was almost entirely based on *in vitro* immunostaining experiments combined with fundamental lossof-function analyses, so we have no idea on what exactly happens to MAVS under physiological stimulus *in vivo*. A report published in 2011 by Chen and *et al*. (54) once again drew considerable attention on MAVS aggregation. The authors employed elegant *in vitro* biochemical experiments and, remarkably, demonstrated that endogenous MAVS assembles into prion-like aggregates for efficient signal transduction, and these aggregates were dependent on linkage to the K63 (Lys63) residue of ubiquitin chains. The authors did not, however, further address whether prion protein (PrP)-like aggregates of MAVS are dependent on mitochondria dynamism, leaving the proposed model unsupported by a strong biochemical experimental system.

There are several other RLR-related negative regulators that localize on mitochondria. Polo-like kinase1 prevents the interaction between MAVS and TRAF proteins (55). Poly(rC)-binding protein 2 inhibits RLR-dependent signalling through proteasomal degradation of MAVS (56).

NLRX1 prevents CARD–CARD homotypic interactions between RLRs and MAVS (57). The receptor for the globular head domain of complement component C1q (gC1qR) has a similar function to Mfn2, sequestering MAVS and inhibiting its signalling (58). In addition to all these regulators, the modulation RLR signalling was also performed, in part, through the ubiquitination system, as we will describe in the following section.

Essential role of ubiquitination in RLRs-mediated signalling

Ubiquitination mediates an indispensable role in RLR-mediated signalling. Upon viral infection, central core components in RLR-mediated signalling, including RIG-I, and their binding partners will undergo robust ubiquitination. Activation, conjugation and ligation of ubiquitin are mediated, respectively, through various E1-, E2- and E3-family proteins. TRIM25 is a member of the tripartite motif family, members of which have kinds of domain: a RING finger region; one or two B-box zinc-finger domains; and a coiled-coil domain. TRIM25 also contains a SPRY domain with E3-ligase function that was first identified to be essential for RIG-I ubiquitination (59). Gack *et al*. (60) demonstrated that lysine at position 172 of the N-terminal CARD of RIG-I is the main target for TRIM25. Inhibition of RIG-I signalling through TRIM25 sequestration by influenza NS1 further highlighted the importance of TRIM25 in this signalling.

Further screening analysis revealed that Riplet, also known as RNF135 or REUL (RIG-I E3 ubiquitin ligase), is another RIG-I-binding partner, which positively regulates RIG-I signalling through K63-linked

polyubiquitination (61-63). Loss-of-function analyses using Riplet-deficient fibroblast further underscored the importance of this molecule in antiviral signal transduction. However, there were several discrepancies regarding the ubiquitination site of this molecule (63), further clarification is required. Other E3-family members, including cIAP1, cIAP2 and TRAF-family members such as TRAF3, TRAF2, TRAF5 and TRAF6, were demonstrated to be essential in controlling the downstream signalling pathways (31, 64-66). Both cIAP1 and cIAP2 were shown to regulate RIG-I through K63-linked polyubiquitination of RIP1. The TRAF-family E3 ligases mainly possess MAVS-binding motifs, interacting with MAVS upon viral infection and subsequently facilitating downstream IRF3 and/or NF-κB activation predominantly through K63-linked polyubiquitination.

RLR-mediated signalling is stringently controlled at different stages through deubiquitination processes. CYLD is one of the deubiquitinases that interact with the CARD domain of RIG-I to remove the K63-linked ubiquitin chain, shutting down its signal (67). A recent report by Jung *et al*.

(68) demonstrated that another complex, called LUBAC, negatively regulates RIG-I-mediated antiviral function by targeting TRIM25. LUBEC contains two E3-ligases— HOIL-1L (heme-oxidised IRP2 [iron regulatory protein 2] ubiquitin ligase 1) and HOIP. In this report, the authors demonstrated that LUBAC promotes K48-linked polyubiquitination of TRIM25, leading to proteasomal degradation. Of note, LUBAC, particularly its HOIL-1L E3, can act independently from HOIP to compete with TRIM25 for interaction with RIG-I. These suggested that there are two separate, independent mechanisms exerted by LUBAC to negatively regulate RIG-I signal transduction. How these two distinct inhibition pathways works might need further clarification.

Deubiquitinating enzyme A (DUBA) [also known as ovarian tumour domain (OTUD5)], negatively regulates RIG-I signalling by interfering with the function of TRAF3, especially positive regulation with TBK1. TRAF3 is also targeted by Triad3A, which is an E3 protein that mainly catalyzes K48-linked polyubiquitin chains for degradation (69). Two negative deubiquitinating regulators — AIP4 (atrophin-1-interacting

protein 4) and RNF5 (56,70) —are reported to promote the proteasomal degradation of MAVS and STING, respectively.

Newly emerging RNA helicases in innate immune system

Recently, several newly emerging RNA helicases were substantially reported to be either sensors of non-self RNA or regulators of known PRRs such as RIG-I. In 2010, Oshiumi *et al*. (71) demonstrated that the helicase DDX3 regulates RIG-I signalling through MAVS. A year later, the same group reported that the helicase DDX60 is vital to promote RIG-I activities (72). Liu and colleagues (73,74) further showed that the complex of helicases DDX1–DDX21–DHX36 and the helicase DHX9 sense dsRNA in mDCs, whereas the helicase DDX41 is essential for sensing cytoplasmic DNA (75). DDX1–DDX21–DHX36 could only function as a complex whereas DHX9 could work independently for this sensing process. Liu and colleagues (76) also demonstrated that the helicases DHX9 and DHX36 are essential for sensing DNA in pDCs and transducing downstream signals via MyD88, the downstream adaptor for TLRs, suggesting that there is a cross-talk between these two endosomal

and cytoplasmic PRRs. Previous reports have, moreover, clearly shown that TLR9-independent but MyD88-dependent DNA sensors might exist in pDCs (77,78); hence, these two helicases could be the missing parts of the puzzle.

Taken together, all these reports also concomitantly proposed the notion that the existence of multilayer defensive lines against viruses could function in cell-type-specific, (Figure 4) and/or a time-dependent manners. Continuing effort is, however, required to delineate the underlying mechanisms such as the potential cross-talk among PRRs and their putative downstream adaptors. Since pDCs are extremely efficient in detecting viral infections for the initiation of both innate and adaptive immune responses, multiple sensors might not be improbable and at least some redundancy seems likely to occur *in vivo*. In keeping with this notion, RNA helicases are well known for their association with a diverse range of cellular activities, especially in RNA metabolism where they affect factors such as RNA stability, splicing, export and maintenance of secondary

structure (79). Most conclusions from these reports are mainly derived from observations obtained after deleting the endogenous gene of interest; admittedly, the observed impairment in cytokine production might therefore be due to an unknown partial defect in an RNA metabolic pathway, rather than elimination of a genuine viral sensor. In-depth and more-advanced experimental systems are required to verify these considerations.

Conclusion and Future Perspective

Since the discovery of RIG-I as a novel cytoplasmic viral sensor, various efforts have been made to further decipher the antiviral functions of RLRs, especially RIG-I. There are still, however, many fundamental questions that remain poorly understood; for example, little is still known about either MDA5 or LGP2. As noted above, the functional role of LGP2 as a regulatory molecule for RIG-I and MDA5 is still controversial, so more studies are needed to precisely assess its contribution. In conclusion, RLRs possess an indispensable role in mounting defenses against non-self RNA, greater understanding of the underlying molecular mechanisms of RLR activation and regulation could open new doors for the design of therapeutic strategies against viral infection.

On the other hand, previous efforts to generate RIG-I-knockout mice posed daunting challenges because RIG-I deficiency causes embryonic lethality in certain genetic backgrounds, suggesting that there are diverse cellular functions of RIG-I distinct from antiviral defense, probably in the

developmental processes. Indeed, in a recent report, Liu *et al*. (80) revealed a new role for RIG-I in the aging process, and this phenotype was mediated through a well-known anti-aging factor known as Klotho (81). Liu *et al*. (80) showed that Klotho functions as an anti-aging factor by suppressing RIG-I-mediated inflammation. This report has opened a new direction of research on RIG-I, other than IFN-mediated antiviral response, with perhaps, new therapies to combat aging over the horizon.

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FIGURE 1

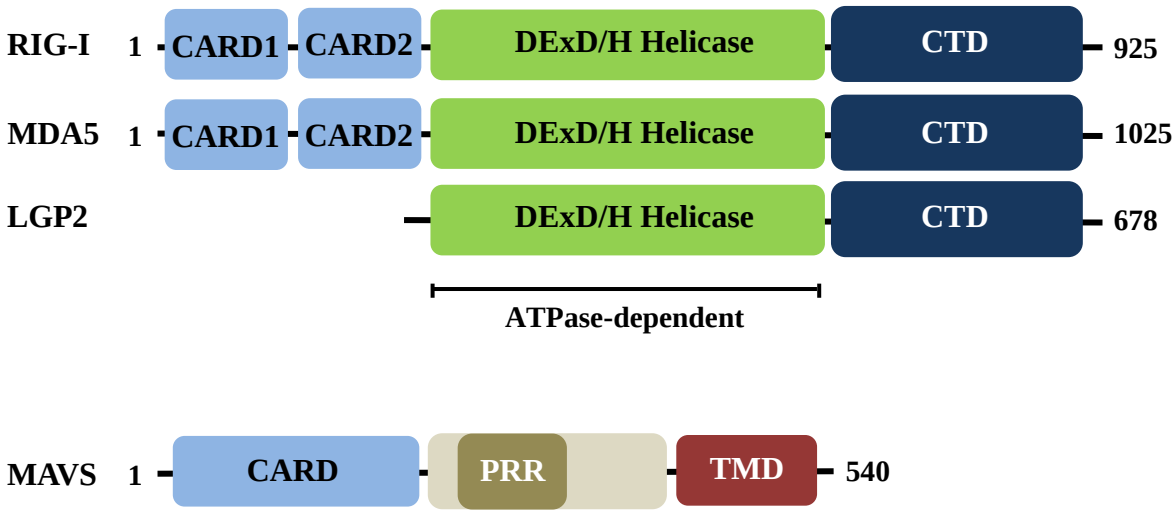
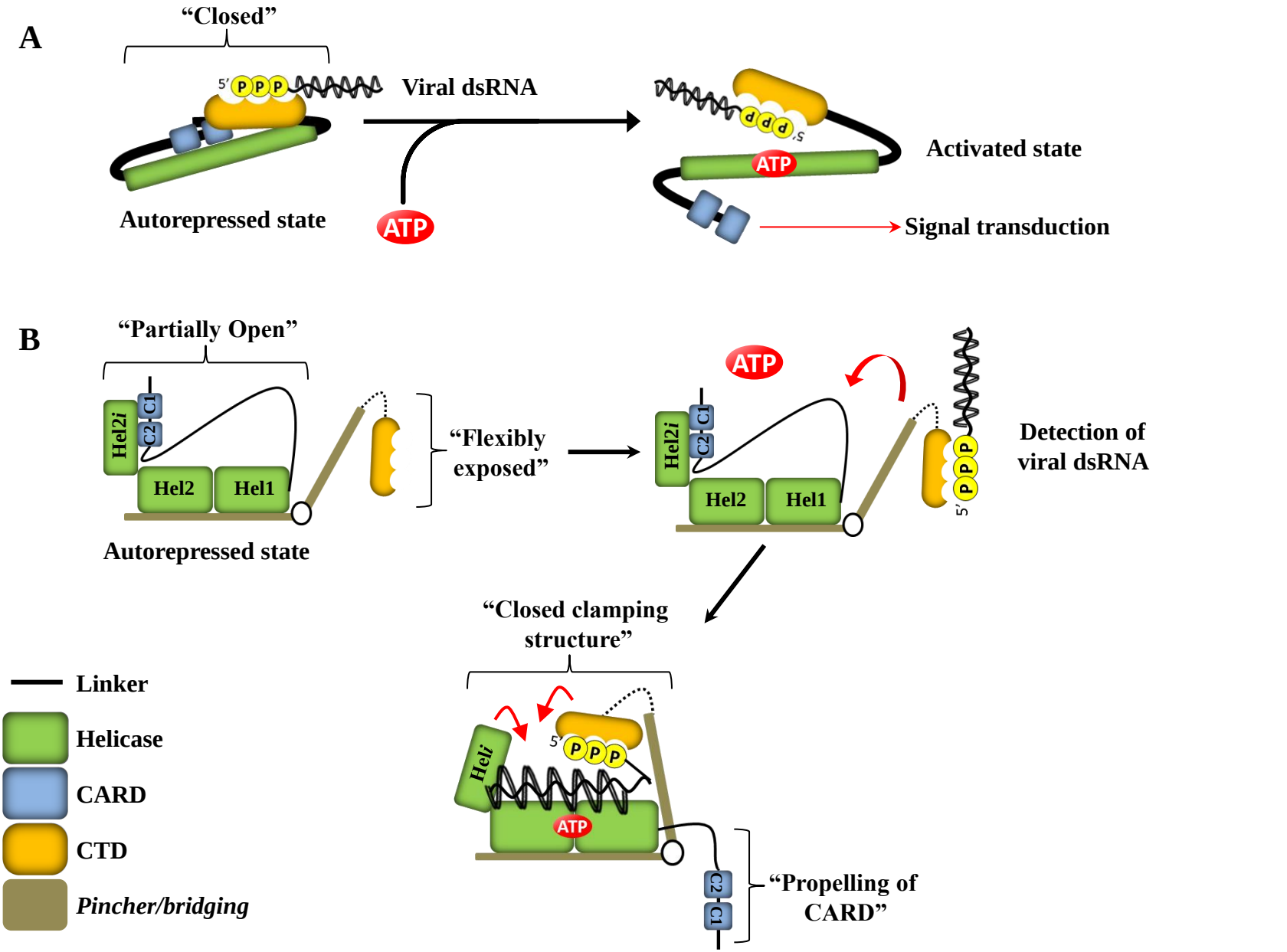

RIG-I
1
CARD1
CARD2
DExD/H Helicase
CTD
925
MDA5
1
CARD1
CARD2
DExD/H Helicase
CTD
1025
LGP2
DExD/H Helicase
CTD
678
ATPase-dependent
MAVS
1
CARD
PRR
TMD
540

FIGURE 2


A
"Closed"
5' P P P
Viral dsRNA
Autorepressed state
ATP
d d d 5'
ATP
Activated state
Signal transduction
B
"Partially Open"
Hel2i
C1
C2
Hel2
Hel1
"Flexibly exposed"
Autorepressed state
ATP
Hel2i
C1
C2
Hel2
Hel1
5' P P P
Detection of viral dsRNA
"Closed clamping structure"
Heli
5' P P P
ATP
C2
C1
"Propelling of CARD"
Linker
Helicase
CARD
CTD
Pincher/bridging

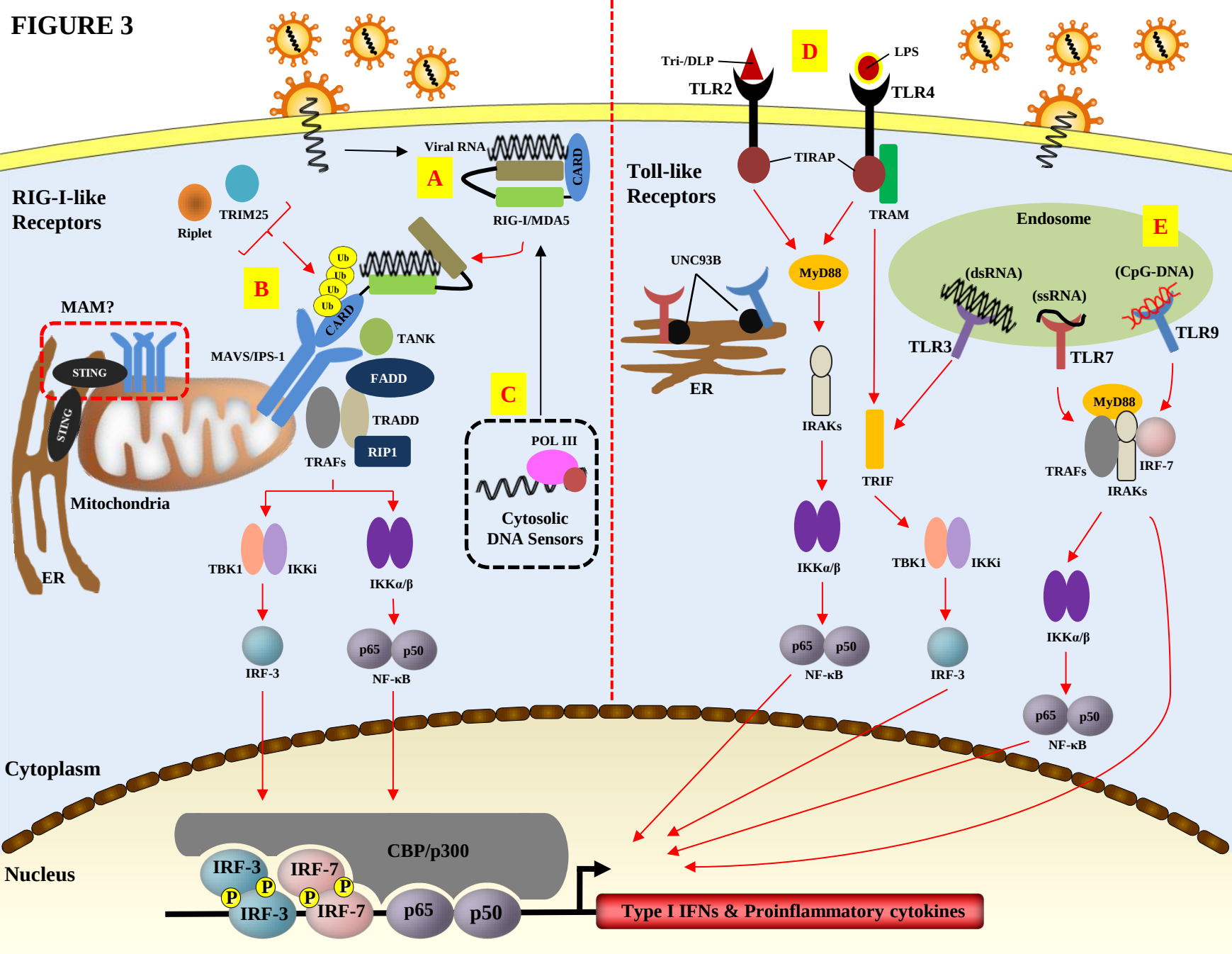


FIGURE 3
RIG-I-like Receptors
Viral RNA
A
CARD
RIG-I/MDA5
TRIM25
Riplet
B
Ub
CARD
MAM?
STING
STING
MAVS/IPS-1
TANK
FADD
TRADD
RIP1
TRAFs
Mitochondria
ER
C
POL III
Cytosolic DNA Sensors
TBK1
IKKi
IKKα/β
IRF-3
p65
p50
NF-κB
Toll-like Receptors
D
Tri-/DLP
TLR2
LPS
TLR4
TIRAP
TRAM
UNC93B
ER
MyD88
IRAKs
TRIF
TBK1
IKKi
IKKα/β
IRF-3
p65
p50
NF-κB
E
Endosome
(dsRNA)
(ssRNA)
(CpG-DNA)
TLR3
TLR7
TLR9
MyD88
TRAFs
IRF-7
IRAKs
IKKα/β
p65
p50
NF-κB
Cytoplasm
Nucleus
CBP/p300
IRF-3
IRF-7
P
IRF-3
IRF-7
p65
p50
Type I IFNs & Proinflammatory cytokines

FIGURE 4

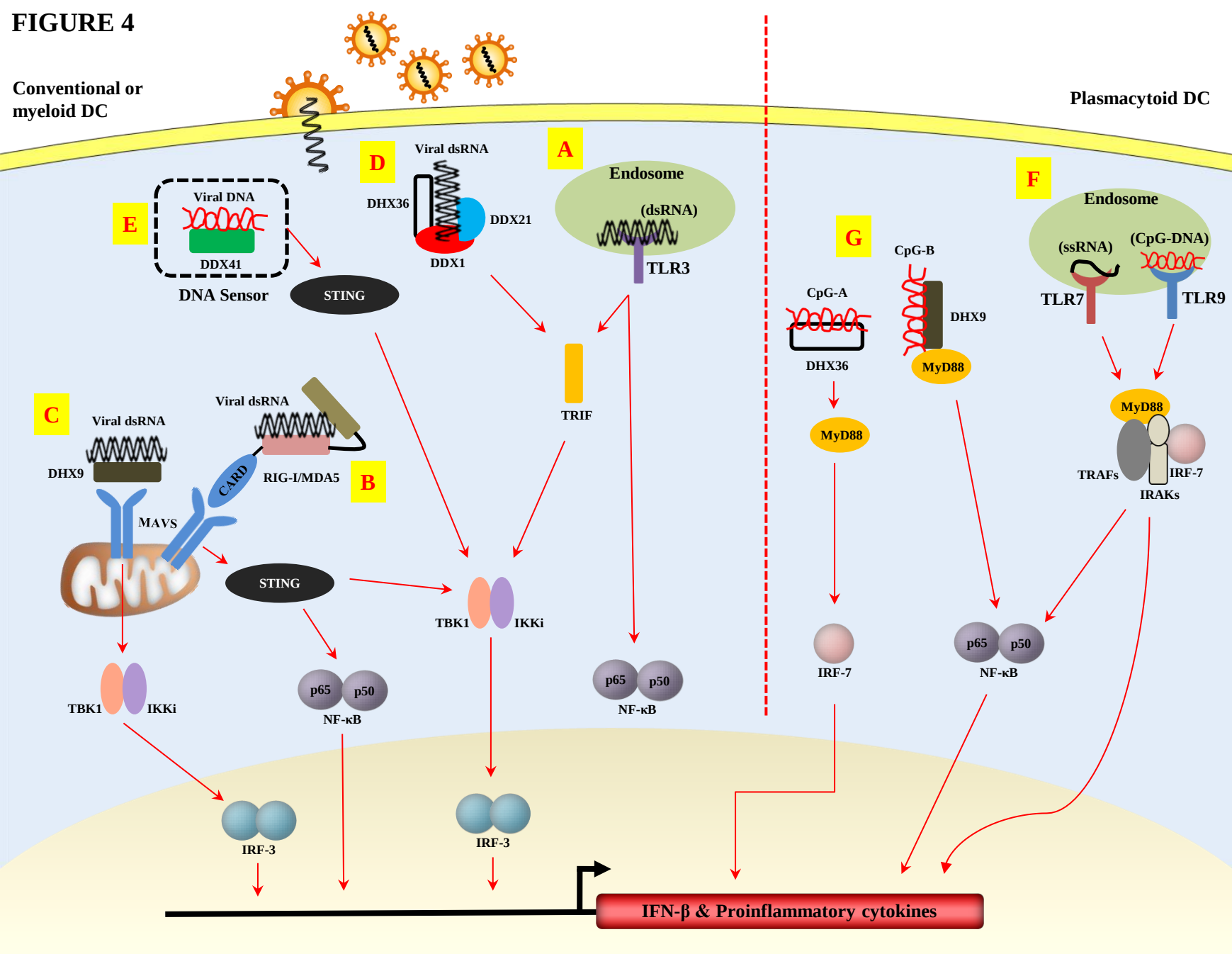

Conventional or myeloid DC
Plasmacytoid DC
A
Endosome
(dsRNA)
TLR3
TRIF
B
Viral dsRNA
RIG-I/MDA5
CARD
MAVS
STING
TBK1
IKKi
p65
p50
NF-κB
IRF-3
C
Viral dsRNA
DHX9
D
Viral dsRNA
DHX36
DDX21
DDX1
E
Viral DNA
DDX41
DNA Sensor
STING
F
Endosome
(ssRNA)
(CpG-DNA)
TLR7
TLR9
MyD88
TRAFs
IRF-7
IRAKs
G
CpG-A
DHX36
MyD88
IRF-7
CpG-B
DHX9
MyD88
p65
p50
NF-κB
IFN-β & Proinflammatory cytokines

FIGURE 1 Schematic structural representation of RIG-I-like family members and their signaling adaptor MAVS.

The RLR family consists of RIG-I, MDA5 and LGP2. All RLRs contain a DExD/H-box helicase domain containing ATPase activity; a C-terminal domain (CTD) for functional repression or autoregulation, and CARD domains at the N-terminus of both RIG-I and MDA5 but not LGP2. These RLRs signal through a common adaptor MAVS/IPS1, which contains a transmembrane domain (TMD) at the C-terminal region, a proline-rich region (PRR) and a homologous CARD at the N-terminus.

FIGURE 2. Schematic models of the structure of RIG-I before and after ligands detection

The CTD is essential for recognising the 5'-triphosphate of viral PAMPs. (A) Because the CTD is also known to possess an RD, in the initially widely accepted model the CTD was proposed to maintain RIG-I in a quiescent state. Upon viral detection there would be a conformational change that exposes the CARD domains and allows signalling. (B) Following recent structural analyses, a new model was proposed. RIG-I remains in an autorepressed state with its two tandem CARD repeats binding to the helicase Hel2*i*, preventing the helicase region from binding viral dsRNA and other interacting proteins; whereas the CTD is flexibly exposed for detection of dsRNA. Upon binding 5'-triphosphate, the CTD is flexibly repositioned to allow dsRNA binding at the helicase region. Coupled with ATP binding, this cooperative binding induces the formation of a high-affinity and stable helicase–dsRNA closed-clamping structure, sterically propelling the CARD domains outwards and exposing them for interaction with MAVS.

FIGURE 3. Comparisons of majors cellular PRRS (RLRs, TLRs) for first line antiviral defense.

(A) The cytosolic viral sensors RIG-I and MDA5 detect invaded viral dsRNA. Upon binding, both RIG-I and MDA5 undergo conformational change and (B) interact with MAVS localized on the mitochondrial membrane, inducing the recruitment of various regulatory components and activating both NF-κB and IRF3 for production of type I interferons. Riplet and TRIM25 mediate RIG-I ubiquitination. STING/MITA/ERIS/MYPS, a positive regulator of RLR signaling is reported to localize on either ER or outer-membrane of mitochondria. Since ER is tightly juxtaposed to mitochondria, the term for 'MAM' is proposed for this area. (C) Cytosolic RNA polymerase III was identified as a DNA sensor, generating RNA templates from the viral DNA that act as ligands for RLRs. (D) TLRs are the major transmembrane PRRs. Upon PAMP detection, TLR2 and TLR4 will recruit the downstream adaptor molecule TIRAP and subsequently MyD88, whereas (E) endosome-localized TLR3, TLR7 and TLR9 will be re-compartmentalized through an ER protein, UNC93B. TLR2, 7 and 9 activate the transcription of inflammatory cytokines and IFNs through MyD88-dependent pathways, whereas TLR4 and 3 activate through a TRIF/TICAM-dependent pathway.

FIGURE 4. Antiviral recognition of viruses by PRRs in dendritic-cell populations.

(A) Endosome-localized TLR3 and (B) the cytosolic viral sensors of the RLR family are well-known sensors for viral dsRNA in mDCs. Upon detection of PAMPs, RLRs will interact and transmit downstream signals through the MAVS signalosome, whereas TLR3 recruits TRIF/TICAM for both IRF3 and NF-κB activation. (C) The RNA helicase DHX9 and (D) the complex of three helicases DDX1–DDX21–DHX36 were identified as vital PRRs for recognition of viral dsRNA in mDCs. DHX9 works independently and pairs with MAVS for signal transduction upon detection. The DDX1–DDX21–DHX36 complex activates production of cytokines and pro-inflammatory cytokines through TRIF/TICAM — a common downstream adaptor with TLR3. (E) DDX41 was shown to have an essential role in detecting viral DNA in cDCs. This molecule signals through STING and activates IRF3 via TBK1. (F) In pDCs, endosome-localized TLR7 and TLR9 are well known for detection of viral RNA and DNA, respectively. (G) Two cytosolic DNA sensors in pDCs have been identified: DHX36 and DHX9. DHX36 detects cytidine-phosphate-guanosine-A (CpG-A) and recruits MyD88 as the downstream adaptor, transmitting through IRF7 activation; DHX9 also recruits MyD88 upon recognition with cytidine-phosphate-guanosine-B and activates cytokine production through NF-κB.

Table 1 PAMPs for RLRs and TLRs

Type of receptors	Molecular structure of PAMPs	Example of species and/or microorganisms	Ref.
TLRs			
TLR2/1	a) Triacyl lipopeptides b) Soluble lipoproteins	a) Mycobacteria b) Bacteria, eg. *Neisseria meningitides* c) Synthetic compound, eg. Pam_3CSK_4	3, 83, 86
TLR2	a) Peptidoglycan b) lipoteichoic acid c) Lipoprotein d) lipoarabinomannan e) zymosan	a) Gram (+) and (-) bacteria b) Mycobacteria c) Yeast cell wall	3, 83, 84, 86
TLR3	a) dsRNA b) synthetic polyI:C (<8000 bp)	dsRNA viruses	3, 83, 85, 86
TLR4	LPS, flavolipin	a) Gram-negative bacteria, eg, *Flavobacterium meningosepticum*	3, 83, 86
TLR5	Flagellin	Bacteria with flagellum, eg. *Salmonella typhimurium*	3, 83, 86
TLR6/2	Diacyl lipopeptides	*a) Mycoplasma fermentans* b) yeast zymosan from *S. cerevisiae*, c) lipoteichoic acid from group B *Streptococci* and *Staphylococci*	3, 83, 86
TLR7 and TLR8	a) ssRNA b) Nucleoside analogs	a) RNA viruses b) Vaccine adjuvant-R848 c) Synthetic ss-poly U rich RNAs	3, 83, 85, 86
TLR9	a) specific unmethylated CpG oligonucleotides (ODN) motif; b) Hemozoin in parasite c) DNA	a) *Plasmodium* b) DNA virus eg. HSV c) CpG motif in bacteria and protozoa	3, 83, 85, 86
RLRs			
RIG-I	a) Short double stranded synthetic RNAs (<1000 bp); b) PolyU/UC rich RNA; c) 5'-triphosphate RNAs; d) RNaseL RNA fragments e) RNAs with panhandle structure	a) ssRNA viruses, eg. HCV poly U/UC rich segment; IAV strain A and B; VSV, SeV, NDV, RSV, MV, NiV; b) dsRNA virus, eg. reovirus.	11, 13, 16, 17, 19
MDA5	a) Long synthetic polyI:C (>1000 bp) with mono- and/or diphosphate ends; b) RNaseL-cleaved self RNAs with monophosphate ends; c) High-order of RNA web	a) Picornaviruses, eg. EMCV, TMEV, poliovirus, mengo virus b) DNA virus, eg. Vaccina virus. c) dsRNA virus, eg. reovirus.	11, 16, 19, 82