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HIGHLIGHTS

REVIEW

Post-translational regulation of antiviral innate signaling

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The innate immune system initiates immune responses by pattern-recognition receptors (PRR). Virus-derived nucleic acids are sensed by the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) family and the toll-like receptor (TLR) family as well as the DNA sensor cyclic GMP-AMP (cGAMP) synthase (cGAS). These receptors activate IRF3/7 and NF-κB signaling pathways to induce the expression of type I interferons (IFNs) and other cytokines firing antiviral responses within the cell. However, to achieve a favorable outcome for the host, a balanced production of IFNs and activation of antiviral responses is required. Post-translational modifications (PTMs), such as the covalent linkage of functional groups to amino acid chains, are crucial for this immune homeostasis in antiviral responses. Canonical PTMs including phosphorylation and ubiquitination have been extensively studied and other PTMs such as methylation, acetylation, SUMOylation, ADP-ribosylation and glutamylation are being increasingly implicated in antiviral innate immunity. Here we summarize our recent understanding of the most important PTMs regulating the antiviral innate immune response, and their role in virus-related immune pathogenesis.

Keywords: Antiviral immunity · Interferons · Phosphorylation · Post-translational modifications · PRR · Ubiquitination

Introduction

Infectious diseases, especially virus infections, are still a serious threat to humanity and the host innate immune system represents a critical defense against invading viruses. Host cells can initiate these innate immune responses by detecting viral DNA and RNA with a set of pattern recognition receptors (PRRs) including the toll-like receptor (TLR) family and the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) family, as well as cytosolic DNA sensors such as cyclic GMP-AMP (cGAMP) synthase (cGAS), IFI16 and DDX41 [1, 2]. After recognition of viral nucleic acids, these PRRs trigger the production of proinflammatory cytokines, chemokines and type I interferons (IFNs), which subsequently induce synthesis of antiviral proteins, death of infected cells and activation of the adaptive immune response [3, 4]. These antiviral signals must be spatially and temporally orchestrated to achieve an optimal outcome for the host and much attention has been raised to understanding the signaling pathways and regulatory factors in the antiviral innate immunity.

Toll-like receptors, including TLR3, TLR7, TLR8 and TLR9, sense endosomal nucleic acids derived from the enclosed microbes and infected apoptotic cells. While TLR9 detects unmethylated CpG DNA species, TLR3 and TLR7/8 recognize double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA), respectively [5] (Fig. 1). Following ligand binding, the TLRs form a signaling platform in which distinct Toll/interleukin-1 receptor (TIR) domain-containing adaptors are engaged. For instance, TLR3 signals via TIR-domain-containing adaptor protein inducing interferon beta (TRIF), and TLR7/8/9 rely on myeloid differentiation factor-88 (MyD88) [6]. For the TRIF-dependent pathway, the ubiquitin E3 ligase TNF receptor-associated factor 3(TRAF3) is recruited and hence activates TANK-binding kinase-1 (TBK1) and Inhibitor-κB kinase ε (IKKε) [7]. Activated TBK1 and IKKε then phosphorylate IFN regulatory factor (IRF) transcription factors IRF3 and IRF7 to

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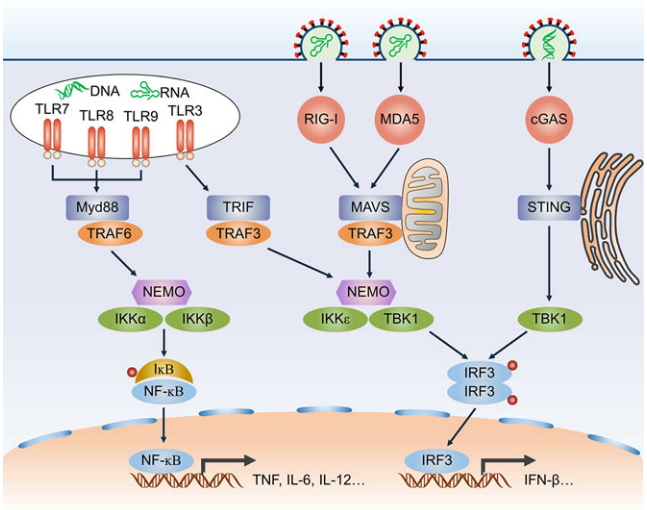

Figure 1. Antiviral signaling pathways. PRRs (red) are activated by endosomal and cytosolic viral RNA and DNA species. TLR7, TLR8 and TLR9 recruit MyD88 and MyD88 in turn activates TRAF6. TLR3 recruits TRIF and subsequently activates TRAF3. TRAF6 and TRAF3 then induce the formation of NEMO-IKKα/β and NEMO-IKKε/TBK1 complex respectively. IKKα/β activate the transcription factor NF-κB and IKKε/TBK1 phosphorylates the transcription factor IRF3. NF-κB and IRF3 then translocate into the nucleus and drive proinflammatory cytokines and type I IFNs expression. For the RLR pathway, RIG-I and MDA5 activate TRAF3-TBK1 axis through the mitochondria-located adaptor MAVS. For the cGAS pathway, cGAS recognize cytosolic DNA and activate the ER-located adaptor STING, which then translocates to and activates TBK1. Red circle, phosphorylation.

drive the expression of type I IFNs [8]. For the MyD88-dependent pathway, TRAF6 is engaged to the MyD88 signal platform, leading to the activation of the kinase complex composed of IKKα and IKKβ. Activated IKK complex induces NF-κB activation, which induces the expression of proinflammatory cytokines such as TNF, IL-6 and IL-12 [6] (Fig. 1). In addition, Myd88 also facilitates IFN-α production by promoting IKKα-dependent IRF7 phosphorylation, which is particularly important for the antiviral activities of plasmacytoid dendritic cells [9, 10].

Aside from endosomal sensing by TLRs, viral RNA in the cytosol is also recognized by RLRs, including Retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [11]. Notably, the RLRs initiate antiviral immune responses in most cell types, which is in contrast to TLR3 and TLR7, which mainly show phagocyte-restricted expression [12, 13]. Upon sensing pathogen-specific molecular features of viral RNA such as 5′-triphosphate (5′-ppp) for RIG-I and long double-stranded segments for MDA5, these RLRs translocate to mitochondria and interact with the mitochondrial antiviral-signaling protein (MAVS) [14, 15] (Fig. 1). This interaction causes the aggregation of MAVS to form a huge prion-like protein complex for TRAF3 and TRAF6 engaging, which transmit signals to TBK1-IRF3 and IKKα/β- NF-κB pathways [3, 16] (Fig. 1).

The immunostimulatory effect of foreign DNA was reported more than 50 years ago, but only recently the sensors that link foreign DNA to type I IFNs have been identified. Cytosolic viral DNA is mainly recognized by cyclic GMP-AMP (cGAMP) synthase (cGAS) that contains a nucleotidyltransferase (NTase) domain. Following DNA binding, cGAS synthesizes a second messenger molecule, cyclic GMP-AMP (cGAMP), which then activates the stimulator of interferon genes (STING) [17, 18]. STING is an ER-located adaptor protein that plays an essential role upstream of TBK1 in the cytosolic viral DNA sensing pathway [19]. Although some other proteins such as IFI16, DDX41 and Mre11 are also reported to be receptors mediating DNA-induced IFN-β production in a STING-dependent manner, only cGAS, which enzymatically generates cGAMP as a second messenger that activates STING, provides a clear molecular mechanism for DNA-stimulated IFN-β production [20] (Fig. 1).

Post-translational modifications (PTMs), involving the covalent linkage of new functional groups to amino acid chains, greatly expand the function of proteins and thus play crucial roles in numerous physiological activities. PTMs control immune responses via regulating protein folding, stability, location and interaction with other molecules [21, 22]. The best characterized PTMs, phosphorylation and ubiquitination, as well as other PTMs such as SUMOylation, methylation and acetylation, have been reported to control antiviral signaling via reversible post-translational modification of virus sensors and downstream signaling molecules [21, 23]. Moreover, emerging studies have shown that different PTMs mutually interact, indicating a precise and elaborate PTMs' regulatory network in the antiviral immunity [24, 25]. In this review, we focus on our recent knowledge how individual PTMs regulate antiviral innate immune responses and their functions in virus-related immune pathogenesis and disorders.

Phosphorylation

In eukaryote, phosphorylation is the kinase-catalyzed conjugation of a phosphate group to the serine (Ser), threonine (Thr) or tyrosine (Tyr) residues of proteins. In this process, introducing a single phosphoryl group with a -2 charge at physiological pH always results in significant protein conformation change, which can influence protein properties and/or create binding motifs for other molecules. Nevertheless, this rigid PTM can be reversed through enzyme-catalyzed hydrolysis by specific phosphatases [26].

In the TLR pathway, Bruton's tyrosine kinase (BTK), EGFR and Src have been shown to phosphorylate tyrosine residues in the cytoplasmic domain of TLR3 to enhance IFN-β and proinflammatory cytokine production in macrophages in response to poly (I:C) and LPS stimulation [27–29]. Moreover, in the late phase of infections, the iNOS/Src signal axis retains TLR3 activation through Src-induced Tyr759 phosphorylation to support sustained IFN-β transcription [30] (Fig. 2). However, whether and which kinases might phosphorylate TLR7, TLR8 or TLR9 is not known, though tyrosine phosphorylation of the TLR8 cytosolic domain is reported to be essential for downstream signal transduction based on a site-directed mutation study [31]. TRAF3 and TRAF6

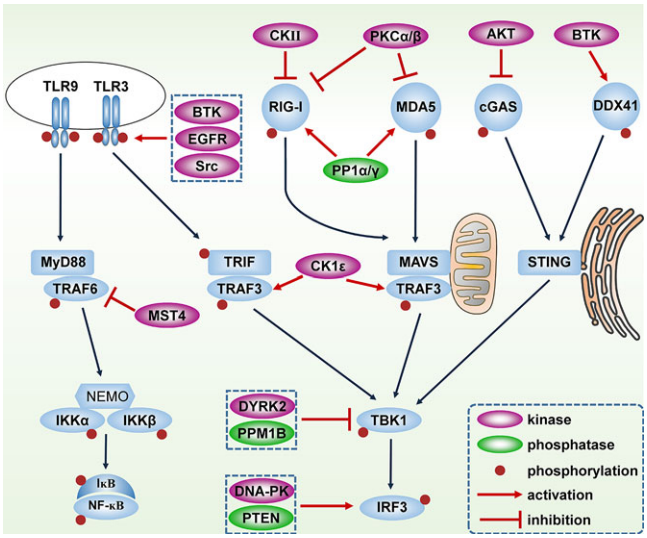

Figure 2. Regulation of antiviral signaling pathways by phosphorylation. For the TLR pathways, TLR3 cytoplasmic domain is phosphorylated and activated by BTK, EGFR and Src. TRAF6 is phosphorylated and inhibited by MST4 while TRAF3 is phosphorylated and activated by CK1ε. TBK1 is inhibited by DYRK2-mediated phosphorylation and PPM1B-mediated dephosphorylation. IRF3 is activated by DNA-PK-mediated phosphorylation and PTEN-mediated dephosphorylation. For the RLR pathways, CKII- and PKCα/β-mediated phosphorylation inhibit RIG-I and MDA5 activation. PP1α/γ dephosphorylate and activate RIG-I and MDA5. For the cytosolic DNA sensing pathways, BTK phosphorylates and activates DDX41 while AKT inhibits cGAS by phosphorylation.

possess important functions in antiviral signaling, but only recently have studies revealed that phosphorylation regulates TRAF3 and TRAF6 activation. In particular, the casein kinase CK1ε directly phosphorylates TRAF3 at Ser349 upon VSV and HSV infections in macrophages, which promotes TRAF3 K63-linked ubiquitination and ultimately enhances IFN-β production [32] (Fig. 2). However, TRAF6 was found to be suppressed by phosphorylation. Mechanistically, the mammalian STE20-like protein kinase 4 (MST4) catalyzes TRAF6 Thr463 and Thr486 phosphorylation in THP1 cells, leading to attenuated oligomerization and ubiquitination of TRAF6, as well as reduced production of IL-6 and TNF-α [33] (Fig. 2). TBK1 undergoes self-association and autophosphorylation at Ser172 after being recruited to the TRAF3 complex upon RNA virus infections, a process in which glycogen synthase kinase 3B (GSK3B) functions as an important physical partner [34, 35]. By contrast, the phosphatase PPM1B removes TBK1 autophosphorylation to eliminate downstream IRF3 activation and IFN-β production in HEK293T cells [36] (Fig. 2). In addition, the dual-specificity tyrosine-phosphorylation regulated kinase 2 (DYRK2) negatively regulates antiviral responses by inducing TBK1 Ser527 phosphorylation and subsequent K48-linked ubiquitination and degradation in HEK293 cells [37] (Fig. 2). Activated IKKε and TBK1 directly phosphorylates IRF3, leading to IRF3 dimerization and nuclear translocation (Fig. 1). Multiple phosphorylated Ser and Thr residues on IRF3 were detected in HEK293T cells by in vitro kinase assay and Ser386 phosphorylation is suggested to be crucial for IRF3 activation [8, 38, 39]. Recently, a study demonstrated that PTEN, a known tumor suppressive phosphatase, unexpectedly promotes antiviral immunity by reversing IRF3 phosphorylation at Ser97, thereby driving IRF3 nuclear transport [40] (Fig. 2). In addition, the DNA-dependent protein kinase (DNA-PK) binds with and phosphorylates IRF3 on Thr135, causing IRF3 to be retained in the nucleus and extending the half-life of IRF3 transcriptional activity in HEC1B cells [41] (Fig. 2). Other signaling molecules, such as MAPKs, IKKα, IKKβ and IkBα, are also regulated by phosphorylation and we note that several excellent reviews on this subject have been published [42, 43].

Multiple phosphorylation-dependent processes are also critical for type I IFN production in the RLR pathway. Phosphorylation of RIG-I by protein kinase C α (PKCα) and PKCβ keeps RIG-I silent in resting cells thus avoiding premature activation of RIG-I signaling [44] (Fig. 2). The casein kinase II (CK II) constitutively phosphorylates RIG-I and hence maintains a quiescent state of RIG-I in HEK293T cells, however, when cells are stimulated with viral RNA, RIG-I is rapidly dephosphorylated [45]. Through phosphatase RNAi screening, PP1α and PP1γ were identified as primary phosphatases responsible for RIG-I and MDA5 dephosphorylation in HEK293T and NHLF cells [46] (Fig. 2). Furthermore, it is reported that IKKε negatively regulates antiviral signaling by phosphorylating RIG-I at Ser855 in HEK293T and MEF cells [47]. MAVS occupies a central position within the RLR signaling cascade and Tyr9 on N-terminal is proved as an essential phosphorylation site for the recruitment of TRAF3 and TRAF6, as well as IFN-β production. However, a definite kinase responsible for Tyr9 phosphorylation has yet to be identified [48].

Among the cytosolic viral DNA signaling, both DDX41 and cGAS are regulated by phosphorylation. Phosphorylation of Tyr414 on DDX41 by BTK was found to be essential for dsDNA recognition and recruitment of STING in BMDMs, HEK293T and MEF cells [49] (Fig. 2). By contrast, Akt phosphorylates cGAS on Ser291 (for mouse) and Ser305 (for human), which inhibits cGAS enzymatic activity in synthesizing cGAMP, leading to a moderate immune responses for the host [50] (Fig. 2).

Notably, a general mechanism of IRF3 activation was recently proposed in which different adaptor proteins (including TRIF for TLR3, MAVS for RIG-I and STING for cGAS) are phosphorylated by cognate downstream IKKε and TBK1 at conserved motif-pLxIS (x for any amino acid, S is phosphorylation site) [51]. These negatively charged motifs then attract the positive charged surface of IRF3, leading to efficient IRF3 phosphorylation by TBK1. Phosphorylated IRF3 then dissociates from the adaptors and forms a homodimer that enters the nucleus to turn on type I interferon transcription [51]. In line with this, protein phosphatase magnesium-dependent 1A (PPM1A) was reported to dampen the phosphorylation cascade by catalytically dephosphorylating MAVS, STING and TBK1 [52, 53]. This model suggests a general phosphorylation-mediated signal transduction in three distinct innate immune pathways, which is impressively similar to the activation mechanism of TGF-β/Smad and JAK/STAT pathways, indicating an intrinsic unity of signal transduction. A detailed list about discussed phosphorylation of the target proteins, enzymes and signaling functions are included in Table 1.

Table 1. Phosphorylation of PRR signaling pathways

Signaling protein	Enzymes	Functions	Ref.
TLR3	BTK	Phosphorylating TLR3 at Tyr759 and promoting the activation of MAPKs, NF-κB and IRF3.	[27]
TLR3	EGFR	Promoting the induction of antiviral genes	[28]
TLR3	Src	Inducing and maintaining TLR3 activation	[30]
TLR8	unknown	TLR8 phosphorylation promoting its interaction with PI3K	[31]
TRIF	TBK1, IKKε	Phosphorylating TRIF at conserved motif-pLxIS to recruit IRF3	[51]
TRAF3	CK1ε	Phosphorylating TRAF3 at Ser349 and promoting its K63-linked ubiquitination	[32]
TRAF6	MST4	Phosphorylating TRAF6 to prevent its activation	[33]
TBK1	PPM1B	Remove TBK1 autophosphorylation	[36]
TBK1	DYRK2	Inducing TBK1 Ser527 phosphorylation and K48-linked ubiquitination	[37]
IRF3	PTEN	Depleting IRF3 Ser97 phosphorylation and promoting its nuclear translocation	[40]
IRF3	DNA-PK	Phosphorylating IRF3 on Thr135 and maintaining IRF3 activation	[41]
RIG-I	PKCα, PKCβ	Phosphorylating RIG-I to keep it silent before viral RNA stimulation	[44]
RIG-I	CK II	Maintaining a quiescent state of RIG-I before viral RNA binding	[45]
RIG-I	PP1α, PP1γ	Removing RIG-I phosphorylation and initiating RIG-I activation	[46]
MDA5	PP1α, PP1γ	Removing MDA5 phosphorylation and initiating MDA5 activation	[46]
RIG-I	IKKε	feedback inhibiting RIG-I activation	[47]
MAVS	unknown	MAVS Tyr9 phosphorylation is essential for TRAF3 and TRAF6 recruitment	[48]
MAVS	TBK1, IKKε	Phosphorylating MAVS at conserved motif-pLxIS to recruit IRF3	[51]
cGAS	AKT	abrogate cGAS activity	[50]
DDX41	BTK	Promoting DDX41 activation	[49]
STING	TBK1, IKKε	Phosphorylating STING at conserved motif-pLxIS to recruit IRF3	[51]

Ubiquitination

Ubiquitination involves the covalent and reversible addition of a 76 amino acid protein named ubiquitin to lysine or other residues in target proteins. A step-wise catalyzation by the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3) is needed for this PTM. Ubiquitin can undergo ubiquitination itself at its seven lysine residues (K6/K11/K27/K29/K33/K48/K63) or its amino-terminal methionine, which generates different types of ubiquitin chains with distinct functions. Ubiquitin chains can be topologically classified into four types of architectures: homogeneous chains, multiple chains in which one substrate is separately modified by distinct chains, mixed chains in which a tandem chain contains two linkage types, and branched chains [54, 55]. Different ubiquitin chains have distinct functions, for instance, K48-linked ubiquitin chain often targets protein for protease degradation while K63-linked ubiquitin chain mediates protein–protein interaction [54, 55].

In the TLR pathway, several TLRs including TLR3 and TLR9 are K48-linked ubiquitinated by Triad3A, which induces protease degradation of these receptors [56] (Fig. 3). Whether other E3 ligases could modify TLRs directly, and whether the remaining TLRs are also regulated by ubiquitination, is still unknown. However, in contrast to the limited data available for the direct ubiquitination of TLRs, their downstream signaling proteins are reported to be extensively ubiquitinated. Myd88 is K48-linked ubiquitinated by the E3 ligase Nrdp1 in the TLR7 pathway, leading to attenuated TNF production by macrophages in response to VSV infection [57]. TRIF is targeted for K48-linked ubiquitination by the E3 ligase WWP2, and the abolition of WWP2 boosts IFN-β, TNF-α, and IL-6 expression after TLR3 agonist poly (I:C) stimulation in 293-TLR3 and BMDM cells [58] (Fig. 3).

K63-linked ubiquitination of TRAF6 and TRAF3 is required for NF-κB and IRF3 activation. Mechanistically, following activation by TLR signal, TRAF6 not only catalyzes its own K63-linked ubiquitination, but also generates unanchored K63-linked ubiquitin chains, both of which serve as scaffolds for TAB2/TAB3-TAK1 and NEMO-IKKα/IKKβ complex binding [59]. TRAF3 K63-linked ubiquitination also creates a platform for NEMO-TBK1/IKKε complex recruitment and activation [60]. Notably, a recent study unveiled a novel K63-K48 branched ubiquitination of TRAF6 contributing to IL-1β signaling, which led us to consider whether branched or mixed ubiquitination are also relevant for antiviral immunity [61]. Several ubiquitin-modifying enzymes, including A20, CYLD and OTUB2, are shown to remove the K63-linked ubiquitination of TRAF6, and a set of deubiquitinating enzymes including DUBA, OTUB1 and UCHL1 abolish TRAF3 K63-ubiquitination, which moderates TRAF6 and TRAF3 activation respectively [62–67] (Fig. 3).

In addition, NEMO, the cognate binding partner of the IKKα/IKKβ complex and TBK1/IKKε kinase complex, has been shown to undergo K27-, K29- and linear ubiquitination, and these PTMs regulate NF-κB and IRF3 activation [68–70] (Fig. 3). TBK1 is targeted for K63-linked ubiquitination by RNF128 and Nrdp1, which facilitate TBK1 activation [57, 71] (Fig. 3). By contrast, Triad3A and DTX4 catalyze K48-linked ubiquitination of TRAF3 and TBK1, respectively, leading to target protein degradation and moderate IFN-β production [72–75] (Fig. 3). IRF3 is reported

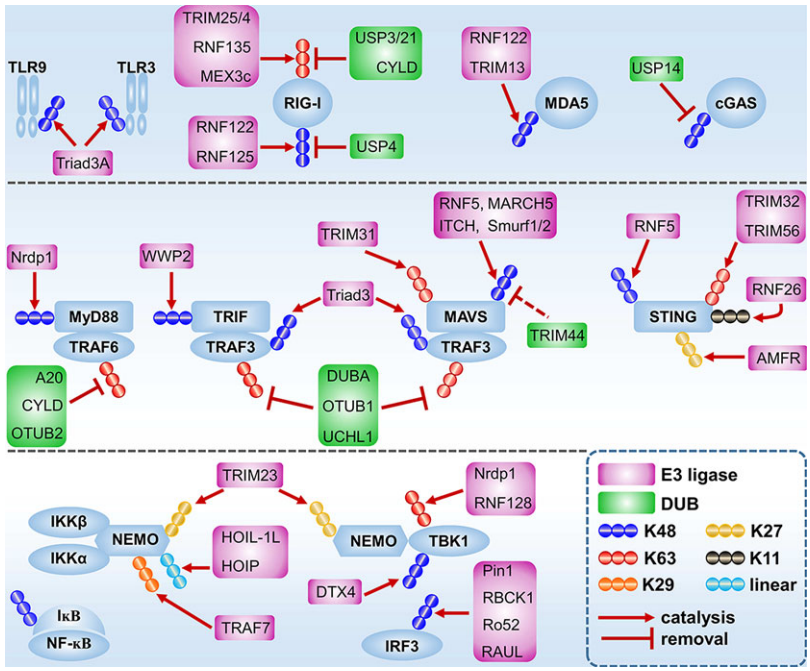

Figure 3. Regulation of antiviral signaling pathways by ubiquitination. TLR9 and TLR3 are K48-linked ubiquitinated by Triad3A and subsequently degraded by proteasome. RIG-I is K48-linked ubiquitinated by RNF122 and RNF125 and then undergoes proteolytic degradation. USP4 remove the K48-linked ubiquitination of RIG-I and promotes RIG-I signaling. TRIM25, TRIM4, RNF135 and MEX3c catalyzed RIG-I K63-linked ubiquitin chain and promote RIG-I activation. USP3, USP21 and CYLD inhibit RIG-I K63-linked ubiquitination and activation. MDA5 is K48-linked ubiquitinated by RNF122 and TRIM13 and then degraded by proteasome. USP14 promotes cGAS signaling by removing K48-linked ubiquitin chain of cGAS. MyD88 and TRIF are K48-linked ubiquitinated by Nrdp1 and WWP2 respectively, and then they are sequestered to proteolytic degradation. TRAF6 are inhibited by A20, CYLD and OTUB2 that specially remove TRAF6 K63-linked ubiquitination. TRAF3 are inhibited by DUBA, OTUB1 and UCHL1 that specilally remove TRAF3 K63-linked ubiquitination. TRAF3 are also targeted for proteolytic degradation by Triad3-mediated K48-linked ubiquitination. MAVS is activated by TRIM31-medited K63-linked ubiquitination. RNF5, MARCH5, ITCH and Smurf1/2 catalyze MAVS K48-linked ubiquitination and induce MAVS degradation by protease. TRIM44 removes ITCH-induced K48-linked ubiquitination of MAVS and thus promotes RIG-I signaling. TRIM32 and TRIM56 promote cGAS signaling by catalyzing K63-linked ubiquitination of STING. RNF5 catalyzes STING K48-linked ubiquitination and promotes proteolytic degradation of STING. RNF26 catalyzes STING K11-linked ubiquitination and inhibit STING degradation. AMFR catalyzes STING K27-linked ubiquitination and promotes STING activation. TRIM23 promotes NF-κB and IRF3 activation by adding K27-linked ubiquitinatin chain to NEMO. HOIL-1L/HOIP and TRAF7 promote NF-κB signaling by catalyzing NEMO linear and K29-linked ubiquitination respectively. Nrdp1 and RNF128 catalyzing TBK1 K63-linked ubiquitination and thus promoting TBK1 activation. DTX4 induces TBK1 proteolytic degradation by adding K48-linked ubiquitin chain to it. Pin1, RBCK1, Ro52 and RAUL catalyze IRF3 K48-linked ubiquitination and promote IRF3 proteolytic degradation.

to be K48-linked ubiquitinated by Pin1, RBCK1 and Ro52 as well as RAUL, which also catalyzes K48-linked ubiquitination of IRF7 [76–80] (Fig. 3).

Ubiquitination is a key regulatory mechanism for RLR pathways as well. For instance, TRIM25, an IFN-inducible E3 ligase belonging to the tripartite motif (TRIM) protein family, catalyzes RIG-I K63-linked ubiquitination at K172, which promotes RIG-I's interaction with MAVS and ultimately IFN-β production [81] (Fig. 3). Genetic and structural analyses show that TRIM25-mediated ubiquitination at K172 is crucial for RIG-I activation in mammalian cells in response to NDV, Sev, VSV and influenza infections [81–83]. Moreover, RIG-I is also K63-linked ubiquitinated by RNF135, TRIM4 and MEX3C, whose abrogation dampens virus-induced IFN production [84–87] (Fig. 3). Several reports suggested that it is unanchored K63-linked ubiquitin chain that binds with RIG-I and triggers downstream IRF3 phosphorylation [88, 89]. An unsolved question is which type of K63-linked ubiquitin chain, covalent or non-covalent, is essential for RIG-I activation in vivo.

As K63-linked ubiquitination is crucial for RIG-I activation, it is not surprising that this PTM is tightly controlled by deubiquitinating enzymes. For example, CYLD, USP21 and USP3 are shown to remove K63-linked ubiquitin chains of RIG-I, thereby moderating IRF3 phosphorylation and IFN-β production [90–92] (Fig. 3). Moreover, the LUBAC complex, which is composed of HOIL-1L and HOIP, counteracts TRIM25-mediated RIG-I activation by sequestering TRIM25 to K48-linked ubiquitination and degradation [93]. MAVS also undergoes K63-linked ubiquitination at K500, yet the E3 ligase has not been identified [94]. Interestingly, a recent study in Raw264.7, HEK293T and BMDM cells suggests that K500 of MAVS is also K48-linked ubiquitinated by MARCH5, a mitochondria-resident E3 ligase [95] (Fig. 3). Perhaps a spatial and/or temporal regulation of K500 ubiquitination on MAVS is in place to control this. Besides, TRIM31 is shown

to serve as a positive regulator of MAVS aggregation by promoting K63-linked ubiquitination of MAVS at K10, K311 and K461 in HEK293T and BMDM cells, thus driving IFN-β production [96] (Fig. 3). The K48-linked ubiquitination, often leading to protease degradation of target proteins, also plays an important role in the RLR pathway as the abundance of receptors and signaling proteins must be tightly controlled. Indeed, RIG-I, MDA5 and MAVS are K48-linked ubiquitinated and thus repressed by RNF125 while ubiquitin-specific protease 4 (USP4) specifically removes the K48-linked ubiquitin chain of RIG-I, thus stabilizing the RIG-I protein and facilitate RIG-I activation [97, 98] (Fig. 3). RIG-I is also reported to be K48-linked ubiquitinated by RNF122 and CHIP, while another E3 ligase, TRIM13, catalyzes MDA5 K48-linked ubiquitination and promotes its degradation [99–101] (Fig. 3). MAVS is targeted for K48-linked ubiquitination by multiple E3 ligase including Smurf1/2, RNF5 and ITCH, which induces MAVS degradation and RLR signal termination [102–104] (Fig. 3). Interestingly, TRIM44 dampens ITCH-induced K48-linked ubiquitination of MAVS, thus facilitating IFN production [105] (Fig. 3).

It has become evident that the cGAS-STING pathway is also regulated by ubiquitination. STING is targeted for K63-linked ubiquitination by TRIM32 and TRIM56 at K150, which potentiates STING dimerization and interaction with TBK1 [106] (Fig. 3). Interestingly, the STING K150 residue also undergoes K48-linked ubiquitination and K11-linked ubiquitination by RNF5 and RNF26, respectively [107, 108] (Fig. 3). RNF5-mediated K48-linked ubiquitination at K150 is dampened by RNF26-induced K11-linked ubiquitination. In contrast, RNF26 did not affect the K63-linked ubiquitination of STING at K150 [108]. Besides, AMFR, an endoplasmic reticulum (ER) located E3 ligase, catalyzes the K27-linked ubiquitination at K150 as well, which promotes TBK1 activation and IFN-β production in MEF and BMDM cells in response to HSV infection [109] (Fig. 3). The discrepancies of ubiquitination at K150 of STING might be caused by different experimental methods and/or a temporal regulation of homogeneous/mixed/branched ubiquitin chain during STING activation. More detailed studies are required to fully understand the dynamic interplay of these ubiquitin-related processes.

Autophagy is a central cell homeostatic process by which damaged organelles, protein aggregates and invading microbes are sequestered in autophagosomes and delivered to the lysosome for degradation, however, the interplay between antiviral immunity and autophagy is largely unknown. A recent study showed that cGAS is targeted for K48-linked ubiquitination and subsequent selective autophagic degradation [110]. Mechanistically, cGAS is constitutively K48-linked ubiquitinated and recognized by autophagy receptor p62, leading to selective autophagic degradation [110]. Upon viral infection, TRIM14 recruits USP14 to cleave the K48-linked ubiquitin chain of cGAS, which disrupts the cGAS-p62 interaction, thereby preventing cGAS autophagic degradation [110] (Fig. 3). An interesting question is whether other signal molecules in antiviral innate immunity are also regulated by ubiquitination-mediated selective autophagy. A detailed list of ubiquitination in antiviral signaling is included in Table 2.

Unconventional PTMs

In light of the development of mass spectrometry (MS) technology, and in the context of the extensively investigated antiviral signaling pathways, the importance of some unconventional PTMs in the innate response has been revealed. These unconventional PTMs include methylation, acetylation, SUMOylation and ADP-ribosylation.

Methylation refers to the transfer of a methyl group from the donor S-adenosylmethionine (SAM) to the lysine or arginine residues of target proteins. The histones are the first and the best described target proteins of methylation, and notably, several studies have emphasized the importance of non-histone methylation in regulating innate immunity [111]. For instance, the NF-κB subunit, p65, is hyper methylated and has distinct functions when methylated. In particular, the protein lysine methyltransferases (PKMT) SETD6 catalyzes monomethylation of p65 at K310 and dampens p65-driven inflammatory responses [112, 113]. Mechanistically, K310-methylated p65 recruits the ankryin repeat of the histone methyltransferase GLP to maintain a repressed chromatin state of p65 target genes, thus inhibiting the transcription of proinflammatory cytokines such as TNF and IL-1α [112, 113]. Moreover, the protein kinase C-ζ (PKC-ζ) phosphorylates p65 at Ser311, blocking the interaction of p65 with GLP and promoting proinflammatory gene expression [112]. In contrast, methylation of p65 at K218 and K221 by NSD1, as well as at K37 by SET9 potentiates transcription of p65-regulated genes [114, 115]. Notably, the NDS1-mediated methylation of p65 is clearly induced upon double-stranded RNA stimulation, yet the complete function of p65 methylation in viral infections is still unknown [114]. An interesting question is whether other signaling proteins are also methylated in antiviral immunity, since the role of methylation beyond histones is of comprehensive concern.

Acetylation is the inverse introduction of an acetyl group to protein lysine residues. Several studies have highlighted the importance of acetylation in antiviral responses. MAPK phosphatase 1 (MKP1), a dual-specificity phosphatase that inactivates p38 MAPK, is acetylated by p300, resulting in enhanced MKP1 activity and moderate proinflammatory cytokine expression [116]. RIG-I is shown to be acetylated at K909 in resting Huh7 and HEK293 cells, and HCV or VSV infections induce a quick deacetylation of RIG-I by histone deacetylase 6 (HDAC6) [117, 118]. This deacetylation is crucial for RIG-I activation and downstream IFN-β production in vivo, since HDAC6 knockout mice show increased susceptibility to HCV and VSV infection [117, 118] (Fig. 4). Notably, another histone deacetylase, HDAC9, deacetylates TBK1 and enhances its kinase activity [119] (Fig. 4). Moreover, the DNA methyltransferase Dnmt3a contributes to IFN-β production by maintaining high HDAC9 expression, which uncovers the crosstalk between epigenetics and post-translational modifications in antiviral immunity [119].

One of the features of immune homeostasis is the negative feedback regulation of signaling pathways by varying PTMs. Apart from IKKε-mediated RIG-I phosphorylation in preventing

Table 2. Ubiquitination of PRR signaling pathways

Signaling protein	Enzymes	Functions	Ref.
TLR3, TLR9	Triad3A	Catalyzing K48-linked ubiquitination and degradation	[56]
Myd88	Nrdp1	Inducing K48-linked ubiquitination and degradation	[57]
TRIF	WWP2	Mediating K48-linked ubiquitination and degradation	[58]
TRAF6	A20, CYLD and OTUB2	Removing the K63-linked ubiquitination of TRAF6 to inhibit TRAF6 activation	[62–65]
TRAF3	DUBA, OTUB1 and UCHL1	Depleting the K63-linked ubiquitination of TRAF3 to suppressTRAF3 activation	[65–67]
TRAF3	Triad3	Mediating K48-linked ubiquitination and degradation	[72]
NEMO	TRIM23	Catalyzing K27-linked ubiquitination and activation of NF-κB and IRF3	[68]
NEMO	HOIL-1L, HOIP	Inducing linear ubiquitination and NF-κB activation	[69]
NEMO	TRAF7	Catalyzing K29-linked ubiquitination to suppress NF-κB activation	[70]
TBK1	RNF128	Mediating K63-linked ubiquitination and activation	[71]
TBK1	DTX4	Catalyzing K48-linked ubiquitination and degradation	[74, 75]
IRF3	Pin1, RBCK1, Ro52 and RAUL	Catalyzing K48-linked ubiquitination and degradation	[76–80]
IRF7	RAUL	Inducing K48-linked ubiquitination and degradation	[80]
RIG-I	TRIM25, RNF135, TRIM4 and MEX3c	Catalyzing RIG-I K63-linked ubiquitination and RIG-I K63-linked ubiquitination is critical for RIG-I activation	[81–87]
RIG-I	CYLD, USP21, USP3,	Removing RIG-I K63-linked ubiquitination to inhibit RIG-I activation	[90–92]
RIG-I	RNF125, RNF122 CHIP	Inducing K48-linked ubiquitination and degradation of RIG-I	[97, 99, 100]
RIG-I	USP4	Removing RIG-I K48-linked ubiquitination to promote RIG-I activation	[98]
MDA5	RNF125 TRIM13	Inducing K48-linked ubiquitination and degradation of MDA5	[97, 101]
TRIM25	HOIL-1L, HOIP	Catalyzing K48-linked ubiquitination of TRIM25 to inhibit RIG-I activation	[93]
MAVS	TRIM31	Inducing K63-linked ubiquitination of MAVS and activation of MAVS	[96]
MAVS	MARCH5, Smurf1/2, RNF5 and ITCH	Catalyzing K48-linked ubiquitination of MAVS to inhibit MAVS	[95, 102–104]
MAVS	TRIM44	Removing ITCH-induced K48-linked ubiquitination and degradation of MAVS	[105]
cGAS	USP14	Removing K48-linked ubiquitination of cGAS to suppress autophagic degradation of cGAS	[110]
STING	TRIM32, TRIM56	Catalyzing K63-linked ubiquitination of STING to activate STING	[106]
STING	RNF5	Catalyzing K48-linked ubiquitination of STING and degradation	[107]
STING	RNF26	Catalyzing K11-linked ubiquitination and inhibiting K48-linked ubiquitination of STING	[108]
STING	AMFR, INSIG1	Catalyzing K27-linked ubiquitination of STING to activate STING	[109]

excessive RIG-I activation [46], ISG15, an IFN-stimulated ubiquitin-like protein, suppresses RIG-I signaling by conjugating to RIG-I [120, 121] (Fig. 4). The biological function of this process, termed ISGylation, is still poorly understood. In addition, during murine gamma herpesvirus 68 (γHV68) infection of MEF cells, RIG-I is also reported to be deamidated by PFAS, which converts glutamine or asparagine residues to glutamate or aspartate respectively [122]. Moreover, the viral homologues of PFAS, named vGAT, promote RIG-I deamidation and subsequent MAVS and IKKβ activation [122] (Fig. 4). A probable question is whether the deamidation of RIG-I is a general or γHV68 infection specific regulation process.

Interestingly, a recent study suggested that the aryl hydrocarbon receptor (AHR) pathway, known for mediating the toxic activity of many environmental xenobiotics, is unexpectedly linked to antiviral responses via ADP-ribosylation, a PTM referring the addition of one or more ADP-ribose moieties to proteins [123]. AHR signaling was shown to specifically induce the expression of TIPARP/PARP7 which in turn dampens TBK1 activity via catalyzing TBK1 ADP-ribosylation [123] (Fig. 4). The study highlighted the function of PTMs as an access point connecting different signaling pathways and physiological processes such as nutritional dysregulation stress and innate resistance against viral infections.

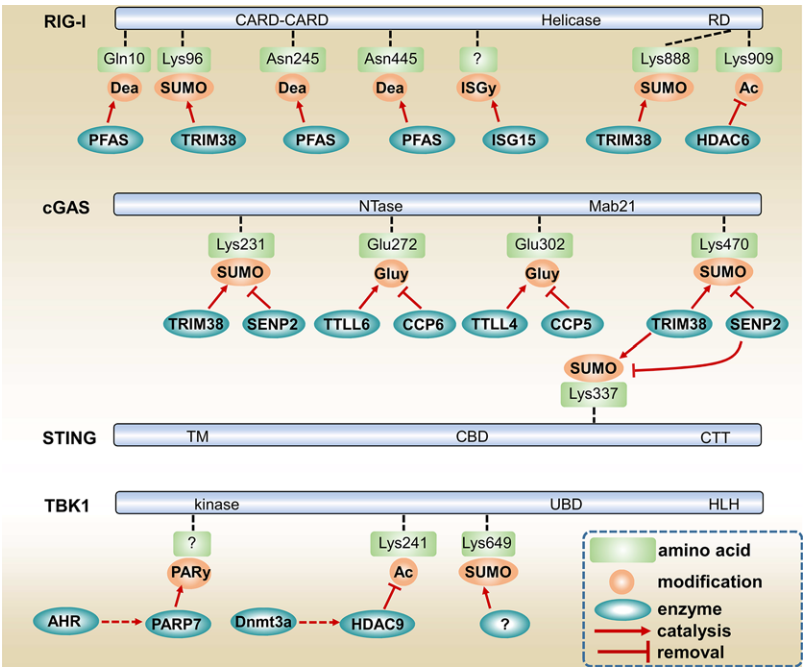

Figure 4. Non-canonical PTMs of RIG-I, cGAS, STING and TBK1. RIG-I is deamidated by PFAS at Q10, N245 and N445. RIG-I's deamidation is crucial for RIG-I signaling during γHV68 infection. TRIM38 promotes RIG-I activation by inducing RIG-I SUMOylation at K96 and K888. ISG15 limits RIG-I activation by catalyzing RIG-I ISGylation. HDAC6 is critical for RIG-I activation by removing RIG-I K909 acetylation. TRIM38 also promotes cGAS signaling by catalyzing cGAS K231 and K470 SUMOylation as well as STING K337 SUMOylation. SENP2 inhibits cGAS signaling by counteracting TRIM38-mediated SUMOylation of cGAS and STING. AHR signaling moderates antiviral response by promoting PARP7-mediated TBK1 ADP-ribosylation. Dnmt3a contributes to IFN-β production by promoting HDAC9 expression. HDAC9 removes TBK1 K241 acetylation to boost TBK1 kinase activity. Dea, deamidation. SUMO, SUMOylation. ISGy, ISGylation. Ac, acetylation. Gluy, glutamylation. PARy, ADP-ribosylation. CARD, caspase recruitment domain. RD, regulatory domain. Mab21, Mab-21 domain. TM, transmembrane domain. CBD, c-di-GMP binding domain. CTT, c-terminal tail. UBD, ubiquitin-binding domain. HLH, helix-loop-helix domain.

The cGAS pathway has been characterized for a few years and its regulatory mechanism is still not clear. Recently, a published work showed that cGAS activity is controlled by reversible glutamylation, a PTM which involves the addition of glutamate side chains to the γ-carboxyl groups of glutamic acid residues of the target proteins [124]. The tubulin tyrosine ligase–like (TTLL) 4 and 6 were shown to promote IFN-β production by catalyzing monoglutamylation and polyglutamylation of cGAS, respectively, while the carboxypeptidase CCP5 and CCP6 both remove the glutamate chain of cGAS in macrophages [124] (Fig. 4).

Processed by specific small ubiquitin related modifier (SUMO) enzymes E1, E2, and E3, SUMOylation is a PTM resembling ubiquitination as ubiquitination use ubiquitin to covalently attach to target protein while SUMOylation use SUMO [125]. A number of studies have highlighted the importance of SUMOylation in antiviral signaling. For example, SUMOylation of RIG-I and MDA5 by the E3 ligase PIAS2β and TRIM38 promotes type I IFN production and antiviral activities in the cell [126–128]. cGAS and STING are SUMOylated by the ubiquitin ligase TRIM38, which prevents their K48-linked ubiquitination and degradation, hence facilitating antiviral innate immune responses [129] (Fig. 4). In agreement, the deSUMOylating enzyme Sentrin/SUMO-specific protease (SENP) 2 alleviates antiviral responses by deSUMOylating cGAS, STING and IRF3 [129] (Fig. 4). Furthermore, TBK1 K694 SUMOylation contributes to its antiviral activities as K694 mutated TBK1 presents enforced interaction with TANK1, a known negative regulator of TBK1 [130] (Fig. 4). The E3 ligase responsible for TBK1 SUMOylation has not been identified yet, but at least it is known that the adenoviral protein Gam1 inhibits SUMOylation of TBK1 [130]. Notably, SUMOylation also represses antiviral responses via targeting the Ifnb1 gene promoter [131]. It seems that SUMOylation leads to both enhancing and suppressive functions in antiviral innate immunity, depending on the substrate specificity. Overall, however, an intrinsic question is why the cells extensively utilize the "long peptide chains" and "multi-enzymes" PTMs, such as ubiquitination, SUMOylation, ISGylation, polyglutamylation and ADP-ribosylation, to control antiviral signaling.

PTMs in virus related immune disorders and therapy

Due to the critical role of PTMs in antiviral responses, it is not surprising that virus have evolved strategies to actively interfere or hijack the PTMs of host proteins. Influenza A viruses (IAVs) are serious infectious RNA viruses that cause grievous economic and health consequences. The influenza non-structural protein 1 (NS1) is known to act as a virulence factor inhibiting host immune responses. The NS1 encoded by human, avian, swine and mouse-adapted influenza viruses can bind with human TRIM25 and inhibit TRIM25-catalyzed RIG-I ubiquitination [132]. However, the NS1 from mouse-adapted influenza viruses targets RNF135, but not TRIM25, to inhibit RIG-I K63-linked ubiquitination, which indicates a species divergence between human and mice influenza infections and immune responses [132]. The papillomaviruses induce expression of the cellular protein ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) in keratinocytes, which inhibits TRAF3 K63-linked ubiquitination and subsequent IRF3 phosphorylation, as well as IFN-β expression [67]. UCHL1 also promotes K48-linked ubiquitination of NEMO, leading to attenuated proinflammatory cytokines production [67]. In addition, a large number of viruses, such as severe acute respiratory syndrome (SARS) coronavirus, human coronavirus NL63, mouse hepatitis virus (MHV) A59, herpes simplex virus 1 (HSV-1), Kaposi's sarcoma-associated herpesvirus (KSHV), HBV, Epstein-Barr virus and some arteriviruses, have been shown to block IFN-β

production by encoding DUB motif-containing proteins [133, 134]. Given the distinct functions of ubiquitination in the same or different signaling proteins, further investigation is urgent to characterize how these virus DUB-containing proteins interact with target proteins and remove the "bad" type of ubiquitin chains on specific residues. Apart from ubiquitination, the Ebola Zaire virus dampens IRF7 activity and IFN-β transcription via VP35, which increases PIAS1-mediated SUMOylation of IRF7 and thus impairs the recruitment of IRF7 to type I IFN gene promoters [135].

Conclusion

The idea of PAMPs and PRRs was started two decades ago [136]. At that time, protein phosphorylation was already extensively explored and ubiquitination was just around the corner from receiving wide-spread interest [137]. Twenty years of study have not only highlighted the cornerstone role of the PRR pathway in infection immunity, but also reveals the importance of PTMs in regulating numerous cell activities. For the antiviral innate immune responses, several intriguing and crucial aspects still await further investigation.

Though much progress has been made in characterizing PTMs and enzymes, which regulate type I interferon production, the in vivo relevance of these enzymes and modifications to antiviral immunity still need to be addressed in future studies, as a considerable part of these functions and mechanisms are characterized in vitro or in non-immune cells. More detailed genetic and biochemical evidence is also needed for a full understanding of PTM role in antiviral responses. Moreover, while multiple kinases and E3 ligases contributing to IFN-β production are identified, not much is known about the phosphatases and deubiquitinating enzymes which functions as IFN-β production brake. Though insufficient interferon production may cause chronic infections, excessive interferon production often causes autoimmune and/or inflammatory diseases such as Aicardi–Goutières Syndrome, systemic lupus erythematosus, arthritis and influenza pneumonia. In this regard, we must address significant gaps in knowledge of PTMs and enzymes that are moderating antiviral responses, many of which could be new and favorable antiviral and autoimmune therapeutic targets.

While phosphorylation and ubiquitination are shown to regulate antiviral innate immunity, the exact roles of other PTMs, such as acetylation, methylation, ADP-ribosylation, SUMOylation, ISGylation and palmitoylation, are still poorly addressed. Even for phosphorylation and ubiquitination, a global map of regulation network and the detailed mechanisms of each signaling protein cannot clearly be drawn. Moreover, differences in observations generated from studies using different virus species, host species and host cell lineages make the questions even trickier. Meanwhile, whether additional novel PTMs might exist in antiviral signaling is unknown. In this regard, advanced technological platforms such as mass spectrometry, fluorescence imaging, nuclear magnetic resonance (NMR) and Raman spectroscopy are eagerly required for both in vitro and in vivo characterizing the dynamics and interplay of different PTMs.

Genome sequencing has unveiled the importance of antiviral signaling in various human diseases such as IRF7 in influenza infection, TRAF3 in herpes simplex encephalitis and MDA5 in systemic lupus erythematous [138–140]. Given the pivotal role of PTMs in modulating antiviral innate immune responses, we eagerly await the identification of disease-relevant mutations in numerous PTM enzymes and the possible diagnostic markers and therapeutic targets for virus-related immune disorders.

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Abbreviations: **AHR**: aryl hydrocarbon receptor · **BTK**: Bruton's tyrosine kinase · **cGAMP**: cyclic GMP-AMP · **HDAC6**: histone deacetylase 6 · **HSV**: herpes simplex virus · **IFNs**: interferons · **IKKε**: Inhibitor-κB kinase ε · **IRF**: IFN regulatory factor · **LGP2**: laboratory of genetics and physiology 2 · **MAVS**: mitochondrial antiviral-signaling · **MDA5**: melanoma differentiation-associated protein 5 · **MST4**: mammalian STE20-like protein kinase 4 · **MyD88**: myeloid differentiation factor-88 · **NS1**: nonstructural protein 1 · **PKCα**: protein kinase C α · **PRR**: pattern-recognition receptors · **PTMs**: post-translational modifications · **RIG-I**: retinoic acid-inducible gene I · **RLR**: RIG-I-like receptor · **Sev**: Sendai virus · **STING**: stimulator of interferon genes · **SUMO**: small ubiquitin related modifiers · **TBK1**: TANK-binding kinase-1 · **TLR**: toll-like receptor · **TRAF3**: TNF receptor-associated factor 3 · **TRIF**: TIR-domain-containing adaptor protein inducing interferon beta · **TRIM**: tripartite motif · **USP4**: ubiquitin-specific protease 4 · **VSV**: vesicular stomatitis virus

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