



Cytoplasm and Beyond: Dynamic Innate Immune Sensing of Influenza A Virus by RIG-I

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ABSTRACT Innate immune sensing of influenza A virus (IAV) requires retinoic acid-inducible gene I (RIG-I), a fundamental cytoplasmic RNA sensor. How RIG-I's cytoplasmic localization reconciles with the nuclear replication nature of IAV is poorly understood. Recent findings provide advanced insights into the spatiotemporal RIG-I sensing of IAV and highlight the contribution of various RNA ligands to RIG-I activation. Understanding a compartment-specific RIG-I-sensing paradigm would facilitate the identification of the full spectrum of physiological RIG-I ligands produced during IAV infection.

KEYWORDS influenza A virus, RIG-I, defective interfering RNA, innate immunity, panhandle

The compartmentalization of eukaryotic cells provides not only physical and functional boundaries for distinct biological processes but also specialized and coordinated immune surveillance mechanisms to detect and counteract viral infections. Depending on the site of viral replication and cellular localization of viral nucleic acids, different classes of pattern recognition receptors are involved in innate immune sensing, leading to the induction of antiviral responses such as type I interferon (IFN) production. The retinoic acid-inducible gene I (RIG-I) is the founding member of the RIG-I-like receptor family and is one of the key RNA sensors in the cytoplasm. Since its rediscovery as an essential regulator of innate immune signaling in response to poly(I:C) transfection and viral infections (1), RIG-I has been shown to sense viral RNAs derived from a panel of virus families. Viruses bearing a single-stranded RNA genome of negative or positive polarity, a double-stranded RNA, and even a DNA genome can all produce certain RNA species that are recognized by RIG-I during infection (2–7). The use of chemically synthesized RNA with well-defined 5' modification and strandedness further demonstrates that optimal RIG-I activation requires 5'-di- or -triphosphate and a juxtaposed short double-stranded RNA (dsRNA) stretch containing a blunt end (8–11). Nonetheless, the detailed structural attributes of many authentic viral RNAs activating RIG-I are not fully elucidated, nor is the information regarding the spatiotemporal RIG-I detection and the relative contribution of various RNA ligands to RIG-I activation during infection. Here, we review the recent advances in these aspects with the focus on RIG-I sensing of influenza A virus (IAV), a unique nuclear-replicating RNA virus. We highlight the findings that elucidate the characteristics of RIG-I ligands produced from IAV infection and how these ligands are integrated into a dynamic RIG-I-sensing paradigm.

RIG-I activation by the single-stranded viral genome. IAV bears a negative-sense RNA genome comprising eight single-stranded RNA (ssRNA) segments. With an exception in plasmacytoid dendritic cells, the innate immune sensing of IAV is strictly dependent on RIG-I (12, 13). Interestingly, infections with many negative-strand viruses, such as IAV, do not generate detectable amounts of dsRNA (14, 15), whereas transfection with virion-derived genomic RNA (vRNA) or total RNA from virally infected cells induces IFN production mediated by RIG-I (3, 10, 15–18). This led to an intriguing

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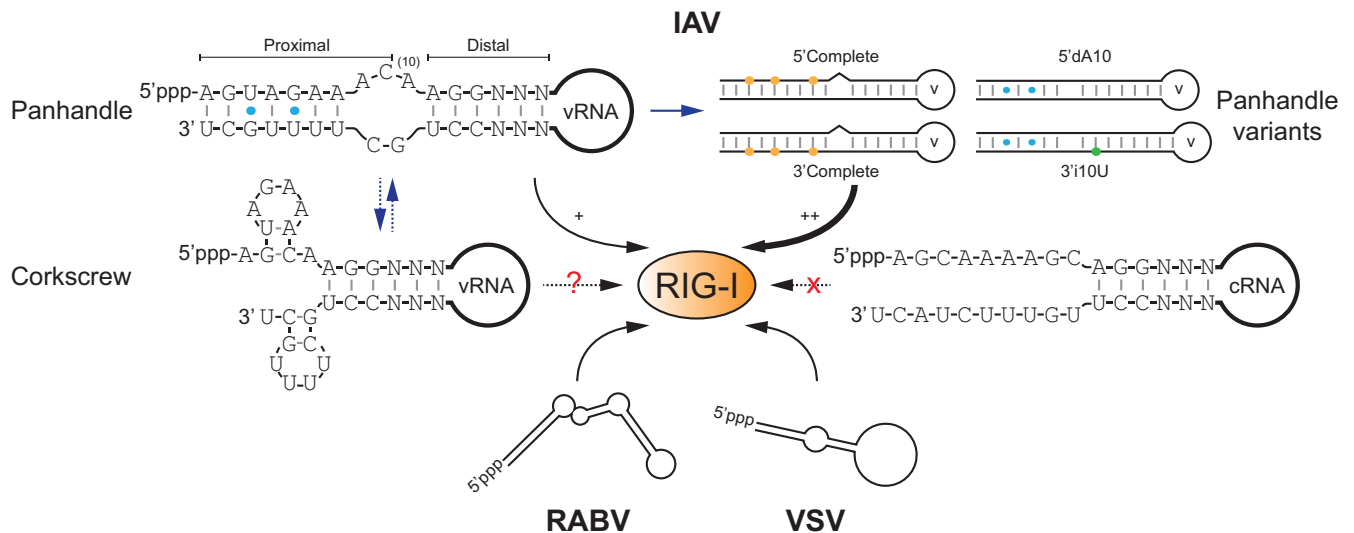


FIG 1 RIG-I activation by genomic panhandle structures of IAV and other negative-strand RNA viruses. The IAV genomic promoter region adopts alternate RNA structures, such as the panhandle and corkscrew configurations. The panhandle structure can be divided into a proximal stem (5' nucleotide positions 1 to 9 and 3' nucleotide positions 1 to 9) and a distal stem (5' nucleotide positions 11 to 16 and 3' nucleotide positions 10 to 15) linked through an unpaired adenosine (5' nucleotide position 10). The panhandle proximal stem contains two G:U wobble pairs and an A:C mismatch that together constitute a bulge element. The wild-type IAV panhandle directly binds to and activates RIG-I, while panhandle variants with the destabilizing elements eliminated (5' and 3' complete constructs, 5'dA10, and 3'i10U) activate RIG-I more efficiently than does the wild type. Whether the IAV promoter region in the corkscrew configuration is able to activate RIG-I remains unknown. The IAV antigenomic promoter region contains two A:C mismatches in close proximity to the 5' triphosphate which largely disrupt the double-strandedness of the panhandle proximal stem and has been shown to lack RIG-I-activating ability. Synthetic panhandle RNAs stimulating that of rabies virus (RABV) and vesicular stomatitis virus (VSV) have also been shown to potentially activate RIG-I.

question as to how these negative-strand viruses meet the dsRNA requirement for RIG-I activation. Bioinformatics analysis reveals that the panhandle structure, a short dsRNA duplex formed from the partial complementarity between viral genome extremities, is the only conserved RNA secondary structure among negative-strand viruses (19). Such a panhandle structure also serves as the viral promoter region for viruses containing a segmented genome (20). Accordingly, it had been proposed that the panhandle structure of the negative-strand virus genomic RNA fulfills the dsRNA requirement for RIG-I. Corroborating the association of IAV genomic RNA and defective interfering RNA with RIG-I (16, 18, 21), the IAV panhandle structure is directly involved in RIG-I activation and IFN induction (22). Despite being relatively shorter than that of other viruses, the *in vitro*-transcribed IAV panhandle RNA binds to RIG-I and induces IFN production (22). Synthetic panhandle RNAs simulating those of rabies virus and vesicular stomatitis virus also robustly induced RIG-I-mediated IFN responses (9, 23). In a detailed compositional analysis of the authentic IAV genomic panhandle structure in relation to RIG-I activation, mutations that eliminated the wobble base pairs, mismatch, or unpaired nucleotide enhanced its immunostimulatory activity, indicating an inherent mechanism adopted by the wild-type genomic panhandle in mitigating RIG-I activation (22). A similar mechanism of RIG-I evasion was demonstrated for IAV antigenomic panhandle (i.e., complementary RNA [cRNA]), which contains two mismatches in proximity to the 5'-triphosphate that largely disrupt the double-strandedness of the panhandle proximal stem (24, 25) (Fig. 1). Examination of the effect of panhandle-stabilizing mutations on IAV promoter activity further sheds lights on how the IAV panhandle promoter region coordinates viral RNA synthesis with the induction of host innate immune responses. The IAV genomic panhandle adopts a nucleotide composition to achieve an optimal balance of viral transcription/replication and suboptimal RIG-I activation, both of which are beneficial to virus survival (22).

The IAV genomic and antigenomic RNA, as with those of other segmented negative-strand viruses, are encapsidated in the form of circular ribonucleoprotein complexes (RNPs). The panhandle promoter region associates with the viral polymerase complex and has been shown to adopt alternative RNA configurations, such as the "corkscrew"

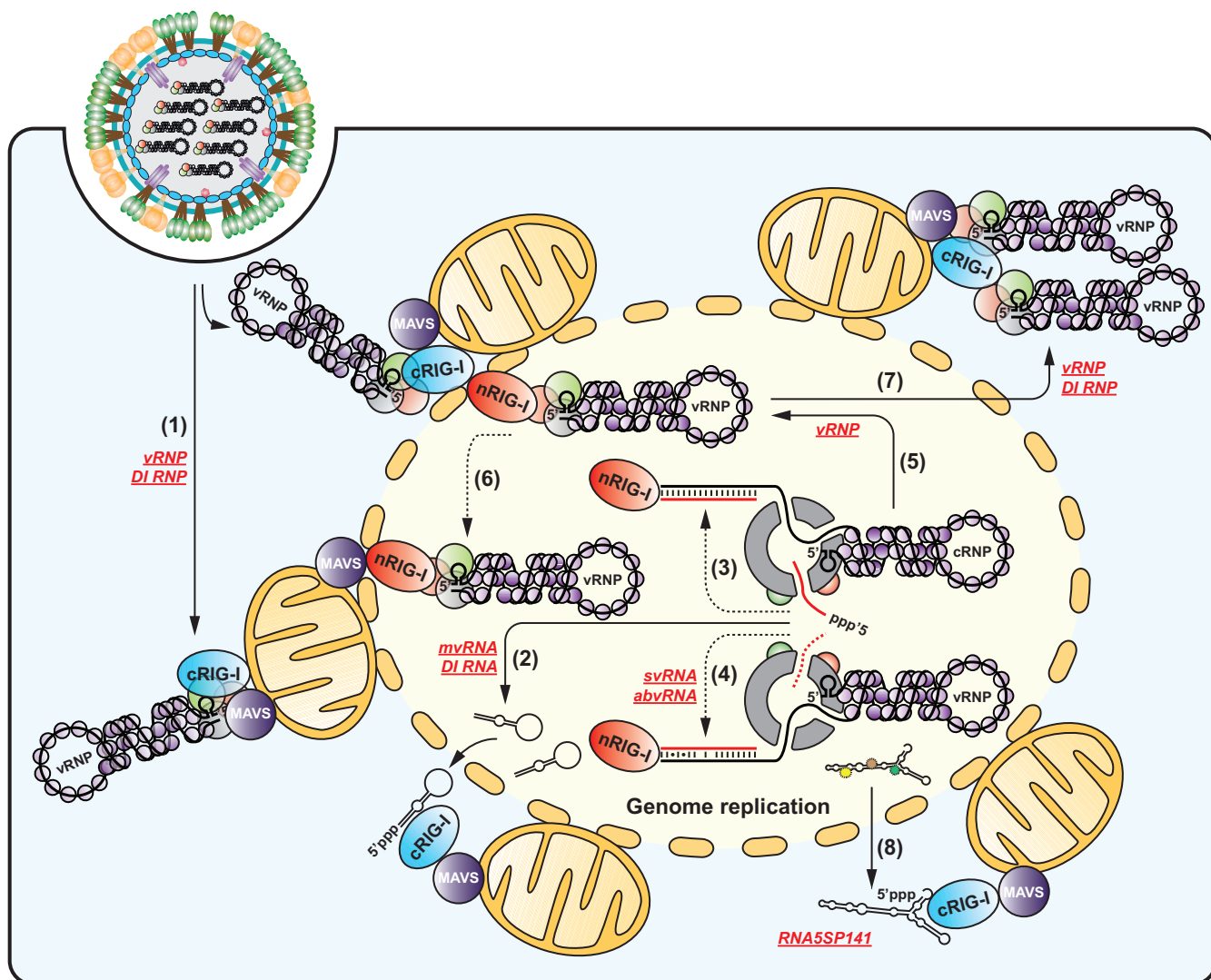


FIG 2 Spatiotemporal RIG-I activation by the IAV panhandle RNA signatures during infection. (1) Incoming vRNPs and DI RNAs (presumably encapsidated in DI RNPs) directly activate cytoplasmic RIG-I (cRIG-I); (2) during genome replication, mini viral RNAs (mvRNA) and DI RNAs (naked or RNPs) synthesized by an erroneous viral polymerase are exported into the cytoplasm whereby activating cRIG-I; (3 and 4) small viral RNAs (svRNA) and aberrant viral RNAs (abvRNA) anneal to the full-length template cRNA (3) or vRNA (4) to form fully or partially complementary (panhandle-like) RNA duplexes activating nRIG-I; (5) nRIG-I recognizes the panhandle structure within progeny vRNPs and forms oligomers with activated cRIG-I that cross the nuclear membrane, thereby activating MAVS; (6) at the late stage of infection, activated nRIG-I directly engages MAVS localized on perinuclear mitochondria owing to the increased nuclear membrane permeability; (7) exported vRNPs and DI RNPs activate cRIG-I prior to genome packaging; (8) nuclear-to-cytoplasmic relocation of 5S rRNA pseudogene 141 (*RNA5SP141*) activates cRIG-I upon IAV-induced shutoff of RNA binding protein synthesis.

model (26–28). The crystal structure of promoter RNA-bound IAV polymerase complex also reveals the formation of an intrastrand hook structure in the 5' arm of the promoter (29). Despite these alternative RNA structures, RIG-I has been shown to colocalize with incoming viral RNPs (vRNPs) (30), interact with viral polymerase subunits (31), and associate with the IAV promoter region in the context of vRNP (32). The involvement of the panhandle configuration in RIG-I activation was indirectly demonstrated by the sensitivity of purified vRNPs to dsRNA-specific RNase III in inducing RIG-I conformational change, an effect that was likewise observed for bunyavirus nucleocapsids (32–34). However, conflicting results exist regarding the effect of RNase III on the immunostimulatory activity of total RNA from IAV-infected or RNP reconstituted cells (15, 18). Interestingly, the interaction of RIG-I with incoming vRNPs appears to contribute little to IFN induction (Fig. 2, step 1). RIG-I confers a signaling-independent mechanism of host restriction specifically on IAV vRNPs of avian origin, due in part to

their lower stability which may more likely expose the panhandle structure (32). A similar function of RIG-I has also been observed during hepatitis B virus infection, where RIG-I competed with the viral polymerase in binding to pregenomic RNA, thereby suppressing viral reverse transcription (35). While RIG-I could efficiently displace dsRNA-bound IAV NS1 or vaccinia virus E3L protein (36), direct biochemical evidence for RIG-I displacement of IAV polymerase prebound to the panhandle RNA is still lacking.

RIG-I activation during IAV infection within and beyond the cytoplasm. Despite highly depending on RIG-I for IFN induction, IAV differs from most RNA viruses in a way that its RNA synthesis takes place in the cell nucleus. Knowledge of how this nuclear replication nature is reconciled with the cytoplasmic localization of RIG-I in IFN induction has only been recently advanced. Both belonging to the *Orthomyxoviridae* family, IAV and influenza B virus (IBV) exhibit opposite kinetics of RIG-I-dependent IFN induction. IBV induces rapid IFN response upon endocytic release of vRNPs, presumably via RIG-I sensing of the IBV panhandle structure, which is slightly longer than that of IAV (37–39). In contrast, IFN induction by IAV shows delayed onset which requires viral RNA synthesis (18, 38, 40, 41). While conventional views emphasize the requirement of viral RNA nuclear export in activating RIG-I in the cytoplasm (41–43), the recent identification of nuclear-resident RIG-I has shifted the RIG-I sensing paradigm of IAV toward the nuclear compartment (44). Corroborating the previously known interaction between RIG-I and vRNP components (30–32), nuclear RIG-I interacts with vRNPs in the nucleus and efficiently senses vRNP reconstitution and IAV infection (44). The temporal involvement of nuclear and cytoplasmic RIG-I in vRNP association fits in the time frame of IAV life cycle, where nuclear RIG-I constantly associates with vRNPs and cytoplasmic RIG-I preferentially binds vRNPs at the late stage of infection (Fig. 2, steps 5 and 7). The nuclear RIG-I also exhibits compartment-specific signaling activity by its inability to sense Sendai virus, a cytoplasmic-replicating virus; yet, it induces maximal IFN responses to IAV along with its cytoplasmic counterpart (44). Although nuclear RIG-I relies on the tripartite motif protein 25-mitochondrial antiviral-signaling protein (TRIM25-MAVS) axis for downstream signaling transduction, how the activated nuclear RIG-I engages cytoplasmic MAVS is currently unknown. Since no MAVS reorganization was observed upon IAV infection, it was proposed that an increase in the nuclear envelope permeability, which is likely mediated by IAV-induced nuclear pore enlargement (45), may facilitate the formation of a sensing milieu for nuclear RIG-I at the perinuclear region (Fig. 2, step 6). Moreover, the mechanistic underpinnings of the cooperation between the two cellular pools of RIG-I remain unclear. A series of structural and biochemical studies have shown that MAVS activation requires RIG-I oligomerization, which could be achieved by filament formation on an individual RNA ligand or oligomerization of multiple RNA-bound monomeric RIG-I in a polyubiquitin-dependent manner (46–49). The short length of an individual IAV genomic panhandle structure does not support RIG-I filament formation (22, 50). It is thus tempting to speculate that oligomerization of monomeric RIG-I bound to separate panhandle RNA constitutes the major mechanism of MAVS activation during IAV infection. Accordingly, the activated nuclear and cytoplasmic RIG-I may bridge across the nuclear envelope to achieve oligomerization (Fig. 2, step 5) and therefore cooperate to efficiently activate MAVS. Future studies are warranted to evaluate these hypotheses.

Physiological RIG-I ligands beyond full-length viral genomes? Another possibility for the cooperation between the two cellular pools of RIG-I is that they may recognize distinct but overlapping classes of viral agonists. Over the last decade, the understanding of the physiological RIG-I ligands produced from IAV infection has been limited. The relative contributions of various ligands to RIG-I activation are also poorly elucidated. In addition to the full-length viral genomic RNA (18), viral defective interfering (DI) RNA also contributes to RIG-I activation (21). Historically discovered as an artifact from IAV passages at high multiplicity of infection, DI RNA has recently been identified from clinical human nasopharyngeal specimens and correlates with antiviral responses against IAV infection *in vivo* (51–53). Structurally, IAV DI RNAs are primarily

full-length viral genomes with monogenic internal deletions and are theoretically encapsidated into vRNP-like structures that are packaged into virions (54). They retain the 5' and 3' genomic noncoding regions and thus the same panhandle structure activating RIG-I. While several lines of evidence argue against IFN induction by incoming vRNPs (41, 55, 56), chemical inhibition of ongoing viral replication reveals the direct contribution of incoming DI RNA as contained in the IAV stocks to RIG-I activation and IFN induction (Fig. 2, step 1) (56). The mechanism for the preferential RIG-I sensing of incoming DI RNA over vRNP is currently unknown but likely lies in the level of exposure of the panhandle structure and/or its cellular distribution. Moreover, there is currently a lack of approaches to differentiating the contribution of incoming and *de novo*-synthesized DI RNA to RIG-I activation. Serial passages of the wild-type virus in the absence of IFN-mediated immune pressure have been shown to select for mutants accumulating great amounts of DI RNA that are packaged into viral stocks (57); however, it remains unclear whether the strong immunostimulatory activity of these mutants upon infection is a result of the incoming or *de novo* synthesized DI RNA, or a combination of both.

Recent studies further provide novel insights into the contribution of viral RNA in aberrant forms to RIG-I activation. Mini viral RNA (mvRNA), a panhandle-forming DI-like RNA <80 nucleotides (nt) in length, has been identified to constitute a class of physiological RIG-I ligand during IAV infection *in vivo* (58). Notably, mvRNA was produced to higher levels by the deadly 1918 H1N1 pandemic virus and highly pathogenic avian H5N1 viruses, which likely contributed to their high virulence. The generation of mvRNA was mechanistically attributable to an erroneous viral polymerase activity as a result of low PB1 catalytic fidelity or avian PB2 signatures. Moreover, an imbalanced polymerase-to-NP ratio also led to mvRNA production and increased IFN induction, an effect echoing previous reports (41, 44, 58). As the key RNA chain elongation factor, viral NP regulates the synthesis of full-length viral genomes and abortive replication products (59). Using an NP-free condition that abolished full-length viral genome synthesis, it was recently demonstrated that the viral polymerase produces aberrant viral RNAs activating nuclear RIG-I (56). Two distinct aberrant RNAs representing abortive replication products of negative polarity were revealed whose accumulation was sensitive to expression of the viral nuclear export protein (NEP). Interestingly, NEP-mediated depletion of these aberrant RNAs did not affect IFN induction but concomitantly primed the synthesis of small viral RNA (svRNA), a class of single-stranded aberrant RNA of 22 to 27 nt in length that was previously shown to lack immunostimulatory activity in isolation (60, 61). While the sequence information of the abortive replication products remains uncharacterized, the compensatory role of svRNA in resuming RIG-I activation indicates the formation of dsRNA structures with complementary sequences to meet RIG-I ligand features. Given that both svRNA and abortive replication products possess the 5' end of vRNA, it was proposed that either RNA could form intermolecular RNA duplexes with the 3' end of full-length vRNA (partial complementarity, panhandle-like) or the 3' end of full-length cRNA (full complementarity) (Fig. 2, steps 3 and 4). Partially supporting the potential formation of these RNA duplexes, the 3' end of vRNA (and possibly for cRNA) adopts no apparent secondary structure in a polymerase-bound promoter configuration (29). NP depletion may also abolish the recruitment of RNA helicase UAP56, which would otherwise prevent dsRNA formation between the positive- and negative-sense viral RNA (62, 63). Both being generated under NP-deficient conditions, it also remains obscure whether the abortive replication products are equivalent to mvRNA. The immunostimulatory activity of mvRNA, like DI RNA, is resistant to RNA extraction, whereas that of abortive replication products is not, indicating a distinct origin (56, 58). A difference in the cellular distribution of the two classes of aberrant RNA may also exist that contributes to differential activation of cytoplasmic versus nuclear RIG-I (Fig. 2, step 2).

Concluding remarks and open questions. It is widely acknowledged that the IAV panhandle structure constitutes the key attribute of RIG-I ligands produced from IAV

infection; however, the relative contribution of viral RNA species bearing such structure to RIG-I activation and IFN induction is still poorly understood. The emerging role of endogenous cellular RNA, such as 5S rRNA pseudogene transcripts (64), in RIG-I activation further complicates the picture (Fig. 2, step 8). With the identification of nuclear-resident RIG-I, deep sequencing of RNA species preferentially associated with the two cellular pools of RIG-I may further expand our view of the full spectrum of physiological RIG-I ligands produced from IAV infection. Approaches to selectively modulating the synthesis of one or more known viral ligands are of the utmost importance to better understand their relative contributions to RIG-I activation. Apart from the IFN response (57), cellular constraints that attenuate or potentiate IAV to produce DI RNA or other aberrant forms of viral RNA are also underexplored. Cell type-specific difference may play a critical role in conferring these constraints. Some innate immune cells, such as macrophages, occasionally lead to abortive viral replication depending on the virus strain, yet they are the major IFN-producing cell types (65, 66). Single-cell analyses further reveal stochastic IFN events among IAV-infected cell population, which partially correlates with viral genetic diversity, such as mutations and defective viral gene expression including but not limited to NS1 (55, 67, 68). The definite physiological RIG-I ligands produced by NS1 deletion viruses which stimulate strong IFN responses *in vitro* and *in vivo* (21, 69), and the mechanism by which *de novo*-synthesized DI RNA and mVNA override NS1-mediated RIG-I inhibition are also unclear (57, 58). Only with these questions answered would the dynamic RIG-I-sensing paradigm of IAV be completely revealed.

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