



How Dengue Virus Circumvents Innate Immunity

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In the battle between a virus and its host, innate immunity serves as the first line of defense protecting the host against pathogens. The antiviral actions start with the recognition of pathogen-associated molecular patterns derived from the virus, then ultimately turning on particular transcription factors to generate antiviral interferons (IFNs) or proinflammatory cytokines via fine-tuned signaling cascades. With dengue virus (DENV) infection, its viral RNA is recognized by the host RNA sensors, mainly retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) and toll-like receptors. DENV infection also activates the cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS–STING)-mediated DNA-sensing pathway despite the absence of a DNA stage in the DENV lifecycle. In the last decade, DENV has been considered a weak IFN-inducing pathogen with the evidence that DENV has evolved multiple strategies antagonizing the host IFN system. DENV passively escapes from innate immunity surveillance and also actively subverts the innate immune system at multiple steps. DENV targets both RNA-triggered RLR–mitochondrial antiviral signaling protein (RLR–MAVS) and DNA-triggered cGAS–STING signaling to reduce IFN production in infected cells. It also blocks IFN action by inhibiting IFN regulatory factor- and signal transducer and activator of transcription-mediated signaling. This review explores the current understanding of how DENV escapes the control of the innate immune system by modifying viral RNA and viral protein and by post-translational modification of cellular factors. The roles of the DNA-sensing pathway in DENV infection, and how mitochondrial dynamics participates in innate immunity are also discussed.

Keywords: dengue virus, interferon, RLR–MAVS, cGAS–STING, mitochondrial dynamics

Dengue virus (DENV) hijacks the host's cellular machinery and accesses cell resources in multiple ways to accomplish its lifecycle. Cellular immune signaling then turns on various cascades to fight back when the host cell senses this invading pathogen. Therefore, DENV confronts a series of challenges at each step of its lifecycle from virus entry to the release of mature virion. To counteract, DENV not only passively hides to escape the immune surveillance but also directly targets immune mediators to block the antiviral signaling transduction (1–3).

In this review, we discuss how the host cell activates innate immunity in response to DENV infection and the strategies DENV uses to evade the innate immune system. We illustrate the main theme of this article in **Figure 1** and summarize the DENV antagonism (**Table 1**) described in the text.

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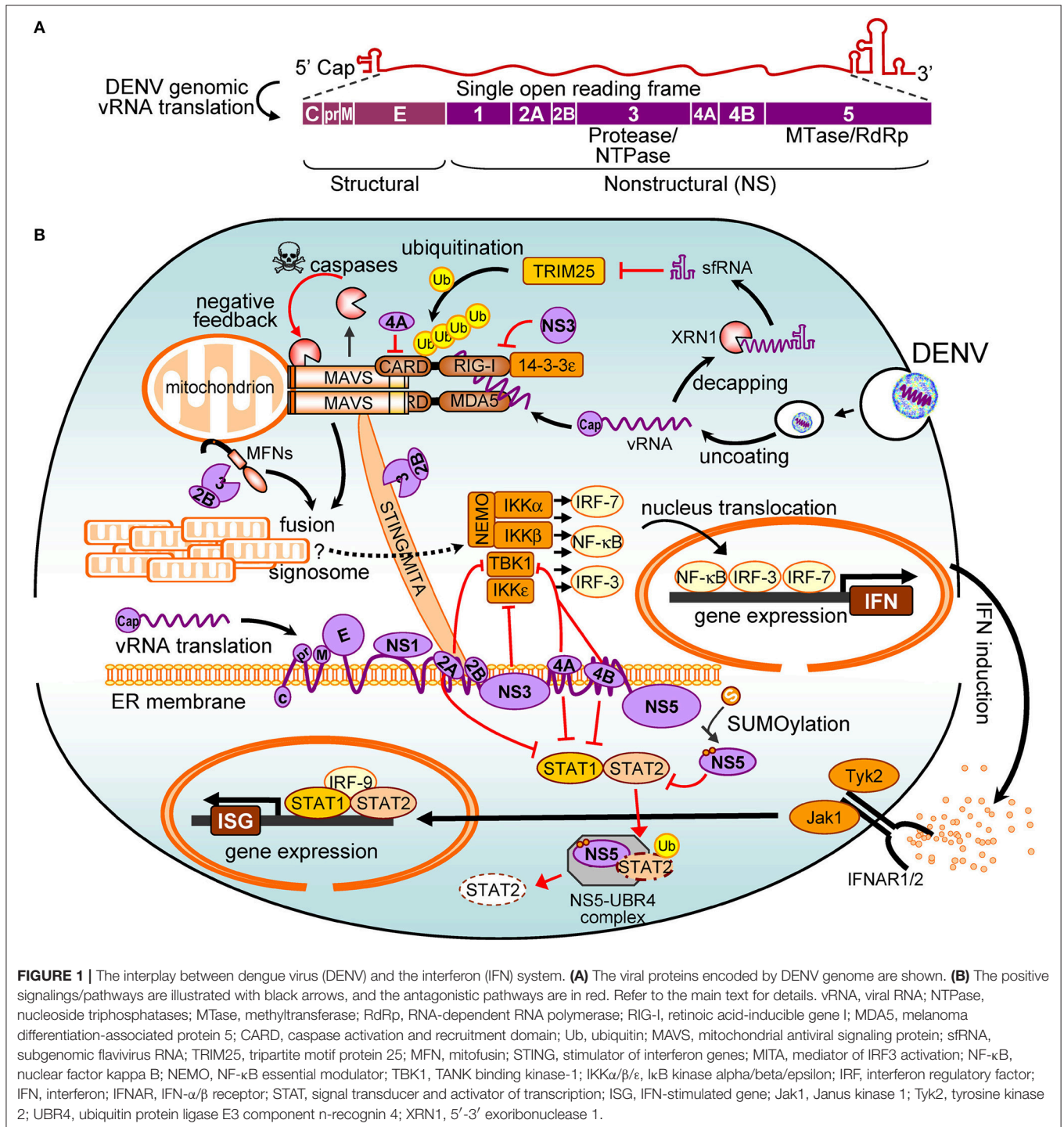


FIGURE 1 | The interplay between dengue virus (DENV) and the interferon (IFN) system. **(A)** The viral proteins encoded by DENV genome are shown. **(B)** The positive signalings/pathways are illustrated with black arrows, and the antagonistic pathways are in red. Refer to the main text for details. vRNA, viral RNA; NTPase, nucleoside triphosphatases; MTase, methyltransferase; RdRp, RNA-dependent RNA polymerase; RIG-I, retinoic acid-inducible gene I; MDA5, melanoma differentiation-associated protein 5; CARD, caspase activation and recruitment domain; Ub, ubiquitin; MAVS, mitochondrial antiviral signaling protein; sRNA, subgenomic flavivirus RNA; TRIM25, tripartite motif protein 25; MFN, mitofusin; STING, stimulator of interferon genes; MITA, mediator of IRF3 activation; NF- κ B, nuclear factor kappa B; NEMO, NF- κ B essential modulator; TBK1, TANK binding kinase-1; IKK α / β / ϵ , I κ B kinase alpha/beta/epsilon; IRF, interferon regulatory factor; IFN, interferon; IFNAR, IFN- α / β receptor; STAT, signal transducer and activator of transcription; ISG, IFN-stimulated gene; Jak1, Janus kinase 1; Tyk2, tyrosine kinase 2; UBR4, ubiquitin protein ligase E3 component n-recogin 4; XRN1, 5'-3' exoribonuclease 1.

BRIEF MOLECULAR VIROLOGY OF DENV

DENV belongs to the genus *Flavivirus* of *Flaviviridae* and is the leading cause of mosquito-borne viral diseases. The DENV virion harbors a messenger-sense, single-stranded RNA (ssRNA) genome that contains a 5' cap but lacks a 3' poly-A tail. The DENV invasion starts with cell-surface attachment and receptor

binding. After internalization, the nucleocapsid is uncoated, and the virus genome then releases to the cytoplasm. The DENV RNA genome is similar to cellular mRNA, translating a polyprotein precursor in a cap-dependent manner. Viral and cellular proteases then process the polyprotein into three structural proteins (capsid [C], precursor membrane [prM], and envelope [E]) and seven non-structural (NS) proteins

TABLE 1 | Summary of dengue virus (DENV) factors antagonizing the interferon (IFN) system.

| DENV factors | Target pathway | Actions | References |
|--------------|------------------------|-------------------------------------------------------------------------------------------------------------------------|------------|
| sfRNA | RNA-sensing | Binds to TRIM25 to inhibit viral RNA recognition by RIG-I | (4, 5) |
| NS2A | IFN induction | Antagonizes the phosphorylation of TBK1 and RIG-I-induced IRF3 | (6) |
| | IFN signaling | Inhibits IFN-triggered antiviral actions | (7) |
| NS2B | DNA-sensing | Targets cGAS for degradation | (8) |
| NS2B3 | DNA-sensing | Cleaves STING through protease-dependent manner | (9, 10) |
| | IFN induction | Interacts with IKK ϵ to mask part of its kinase domain to prevent the phosphorylation of IRF3 | (11) |
| | Mitochondrial dynamics | Cleaves MFN1 and MFN2 to modulate the MFN-mediated host antiviral defense | (12) |
| NS3 | RNA-sensing | Competes with RIG-I for 14-3-3 ϵ binding to block RIG-I activation | (13) |
| NS4A | RNA-sensing | Translocates to mitochondrion-associated endoplasmic reticulum membranes to prevent the binding between RIG-I and MAVS. | (14) |
| | IFN induction | Blocks TBK1 activation | (6) |
| | IFN signaling | Inhibits of IFN-triggered gene expressions | (7) |
| NS4B | IFN induction | Antagonizes the phosphorylation of TBK1 and RIG-I-induced IRF3 | (6) |
| | IFN signaling | Inhibits STAT1 phosphorylation and transcriptional activation | (7) |
| NS5 | RNA-sensing | Catalyzes DENV genomic RNA 2'-O methylation mimicking cellular mRNA | (15) |
| | IFN signaling | Binds and degrades STAT2 | (16–18) |

sfRNA, subgenomic flaviviral RNA; TRIM25, tripartite motif protein 25; RIG-I, retinoic acid-inducible gene-1; TBK1, TANK binding kinase-1; IRF, interferon regulatory factor; STAT, signal transducer and activator of transcription; cGAS, cyclic GMP-AMP synthase; STING, stimulator of interferon genes; IKK ϵ , I κ B kinase epsilon; MFN, mitofusin; MAVS, mitochondrial antiviral signaling protein.

(NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). After that, viral RNA is replicated by the viral RNA-dependent RNA polymerase NS5 in the replication complex. Structural proteins are assembled with the DENV RNA genome in the endoplasmic reticulum (ER) and then transmitted to the Golgi apparatus. Ultimately, the mature and infectious virions are secreted into the extracellular space and await the next round of infection (19, 20).

DENV has evolved many strategies to minimize its exposure *in vitro* because the virus is membrane-enveloped and is liable to dysfunction *in vitro*. Thus, DENV uses the mosquito, the natural syringe, as the vector to preserve, replicate, and transmit itself. Natural feeding of human blood containing DENV viral RNA more than 5 log₁₀-copies/ml seems sufficient to transmit all serotypes of DENV from human to the primary mosquito vector *Aedes aegypti* (21). Therefore, the period of human DENV infectiousness to the *A. aegypti* mosquitoes may vary between viral serotypes but concentrates on the days when a patient develops illness/fever (22). Despite the presence of a protein D7 capable of inhibiting DENV in mosquito saliva (23), the bites with mosquito saliva increase DENV dissemination into the mammalian host (24, 25).

DENV takes advantage of the mammalian host machinery for replication, but the immune system can detect and attack this invading pathogen. In the last decade, DENV has been considered a weak interferon (IFN)-inducing pathogen (26, 27), with the knowledge that DENV has evolved multiple strategies to antagonize the host IFN system (1–3, 28). Understanding how DENV escapes the control of innate immunity may shed some light on the complicated pathogenesis of DENV infection.

THE CONCEPT OF IFN SYSTEM IN INNATE IMMUNITY

Innate immunity specifies particular pattern recognition receptors (PRRs) to distinguish pathogen-associated molecular patterns (PAMPs) of invading pathogens, including both RNA and DNA viruses. The aberrant nucleic acid species in the cytoplasm, such as double-stranded RNA (dsRNA) in the endosome, cytoplasmic DNA and 5'-triphosphorylated RNA, are the unique viral PAMPs that activate corresponding PRRs (29, 30). Once activated, the sensor hands over the signal to its adaptor proteins, which then recruit kinases to phosphorylate transcription factors and ultimately turn on the production of antiviral IFNs and proinflammatory cytokines. The secreted type-I/III IFNs bind to their receptors IFNAR1/2, which activates Janus kinase (Jak)–Signal transducer and activator of transcription (STAT)-mediated signaling and leads to generation of antiviral proteins encoded in IFN-stimulated genes (ISGs) (31, 32). Various antiviral proteins interfere with steps of the viral lifecycle. For example, ribonuclease L (RNase L) is encoded by an ISG that degrades viral RNA to inhibit DENV replication (33). To counteract the host antiviral actions, DENV evolves strategies targeting various steps of the whole defense system, from sensing of the foreign DNA/RNA to the induction, signaling, and manipulation of IFN system. We categorized these various strategies by the stages of IFN system and discussed them below.

THE RNA SENSING PATHWAY

Camouflage is the first strategy to keep DENV away from the alarm bell of innate immunity. Similar to cellular mRNAs,

DENV genomic RNA is capped at the 5'-end. Cellular mRNA is posttranscriptionally capped at the 5'-end comprising a N⁷-methylguanosine and one or two 2'-O methyl nucleotides (34, 35). Thus, viral RNA lacking 2'-O methylation will be recognized as non-self RNA that elicits innate immunity (35–38). DENV NS5 contains methyltransferase activity that catalyzes both N⁷ and 2'-O methylations sequentially (39–41). The DENV lacking 2'-O-methyltransferase activity elicits a significant early innate immune response in host cells and thus replicates with a lower viral load than the wild-type (15). Therefore, DENV hides and stays under the radar in host cells.

DENV enters host cells by receptor-mediated endocytosis, and its RNA is released to the cytosol for translation and replication. The localization of DENV-derived dsRNA is important for recognition by PRRs. By electron tomography analysis, the cytosolic DENV dsRNA was detected in DENV-induced vesicles derived from ER membrane (42). These vesicles quarantine DENV dsRNA from the cytosolic RNA sensors in a digitonin-resistant membrane structure until 72 h postinfection (43). However, these viral RNA species, when leaked to the cytosol, also become targets for several PRRs, including melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene-I (RIG-I) in the cytoplasm and toll-like receptor (TLR)-3 in the endosome. These PRRs are essential for host defense surveillance, which synergistically recognizes DENV RNA and then initiates IFN induction (44–46). MDA5 and RIG-I are similar RNA helicases expressed in most cell types. Both contain two caspase activation and recruitment domains (CARDs) at the N-terminus for antiviral signaling initiation. After viral RNA binds to the C-terminal helicase domain, the CARD domain of RIG-I/MDA5 then interacts with the CARD domain of their downstream adaptor, mitochondrial antiviral signaling protein (MAVS) (a.k.a. IPS-1/VISA/Cardif) (47–50). This CARD–CARD interaction clusters MAVS for a signaling cascade, which is required for inducing IFN to establish an antiviral state (49). Actually, RIG-I and MDA5 recognize different RNA structures even though they share a high degree of functional and structural homology. MDA5 mainly recognizes long dsRNA or web-like RNA aggregates, whereas RIG-I preferentially senses short dsRNA and single-stranded uncapped RNA with a tri- or di-phosphate at the 5'-end (51–53). These species/forms of RNA differ from self-RNA in the cytoplasm and can be detected in DENV-infected cells (44, 54). Even though RIG-I or MDA5 alone is sufficient to potentiate DENV-induced IFN-induction signaling, RIG-I and MDA5 together trigger a higher level of IFN induction. Therefore, overexpressing RIG-I or MDA5 can suppress DENV replication; silencing of RIG-I and MDA5 contributes to DENV RNA replication and virus production (44). Because RIG-I and MDA5 share the same adaptor MAVS, lack of MAVS impairs IFN induction in DENV-infected cells (54–56).

The protein level of both RIG-I and MDA5 can be further enhanced by IFN (57), so activation of the RIG-I-MAVS pathway forms a positive feedback loop against DENV infection. In the context of RIG-I activation, the ubiquitin ligase tripartite motif protein 25 (TRIM25) binds to and adds lysine-63 (K63)-linked polyubiquitin at the CARD domain of RIG-I (58–60). The

mitochondrial-targeting chaperone protein 14-3-3 ϵ stabilizes the interaction between TRIM25 and RIG-I, thus facilitating K63-linked ubiquitination of RIG-I, which results in MAVS activation (61).

Because ubiquitination and translocation of RIG-I are both required for MAVS activation, DENV evolves strategies to antagonize this step and thus prevents RIG-I-mediated IFN responses. In DENV-infected cells, the uncapped DENV genomic RNA can be digested from the 5'- to 3'-end by the cellular exoribonuclease 1 (XRN1) leaving the incomplete degradation product subgenomic flaviviral RNA (sfRNA) (62). The DENV sfRNA binds to TRIM25, whose binding capacity depends on the sfRNA sequence, thus dampening ubiquitination-mediated RIG-I activation (4, 5). Moreover, DENV NS3 protease contains a 14-3-3 ϵ protein-binding motif RxEP; the binding of these proteins prevents the activated RIG-I from moving from cytosol to mitochondria. Thus, infection of a recombinant DENV, with the RxEP motif replaced by KIKP, triggered a high IFN response that inhibited DENV replication (13). Also, DENV NS4A colocalizes and interacts with MAVS in mitochondrion-associated ER membranes where RIG-I relays the signal to MAVS. The TM3 domain of DENV NS4A is responsible for binding MAVS and thus prevents the association of RIG-I and MAVS (14). Therefore, DENV can disrupt the RIG-I-MAVS interaction directly to suppress IFN production.

In addition to the RIG-I-MAVS pathway, TLR3 and TLR7 are important for recognizing DENV RNA in the endosome. TLR7 senses ssRNA with G- and U-rich sequences (63), whereas TLR3 recognizes dsRNA derived from DENV replication (64). Although, both TLRs are involved in producing a type I IFN response during DENV infection, TLR3 is more effective than TLR7 in IFN induction and DENV inhibition (64). TLR7 also mediates a virus-specific humoral immune response for DENV clearance: administration of combined TLR3 and TLR7 agonists could decrease DENV replication and increase the anti-DENV humoral response in macaques (65). Even though, the direct modulation of TLRs by DENV infection remains to be seen, DENV has been shown to block TLR-mediated antiviral signaling by targeting downstream immune mediators, I κ B kinase epsilon (IKK ϵ) and TANK-binding kinase-1 (TBK1) (6, 11).

THE DNA SENSING PATHWAY

Despite the absence of a DNA stage in the DENV lifecycle, DENV infection still activates the DNA-sensing pathway. The cellular DNA should be located in the nucleus or mitochondria. Presence of a DNA molecule in the cytoplasm is thus expected to trigger innate immune responses, such as inflammation and IFN production (66). Cyclic GMP-AMP synthase (cGAS) is a cytosolic DNA sensor that synthesizes cGAMP, a non-canonical cyclic dinucleotide, in response to DNA stimuli (67, 68). cGAMP is a second messenger that binds and activates the adaptor protein encoded in the gene *tmem173*, namely, stimulator of IFN genes (STING) [a.k.a. mediator of IRF3 activation (MITA), or ER IFN stimulator (ERIS)] (69–71). Human STING is a transmembrane protein located on ER membrane and shares 81% similarity (68%)

identity) with its murine ortholog MPYS (72). After stimulation, STING is dimerized and then translocated to a perinuclear site where it forms a punctate structure and interacts with TBK1 for activating IFN regulatory factors (IRFs) (70, 71). In addition to activating IRFs and producing IFN, STING activation also triggers NF- κ B signaling that leads to the production of pro-inflammatory cytokines (73, 74).

Because DENV is an RNA virus without a DNA stage in its lifecycle, the roles of the DNA-sensing pathway in DENV infection were ignored until DENV protease NS2B3 was found to cleave human STING but not its murine ortholog MPYS (9, 10). Thus, murine MPYS is more competent than human STING in suppressing DENV replication. Even though STING is not essential for IFN production stimulated by a dsRNA analog (73), STING is involved in both DNA and RNA pathogen-sensing pathways. STING can interact with RIG-I and MAVS to enhance the antiviral response (69, 70), which may suggest a crosstalk between viral RNA- and DNA-sensing pathways (75). Therefore, the possibility that DENV may target the DNA-sensing pathway to subvert innate immunity seems logical.

Stimulation of double-stranded DNA but not dsRNA analog enhances the interaction between DENV protease and STING, which then contributes to DENV protease-mediated STING cleavage (10). Therefore, the presence of DNA in cytosol upon DENV infection may contribute to DENV pathogenesis. The release of both genomic and mitochondrial DNA (mtDNA) has been proposed to activate the STING signaling pathway in DENV-infected cells (10). Indeed, aberrant DNA signal appears in cytosol and co-localizes with cGAS upon DENV infection, with the DNA signal resulting from the release of mtDNA rather than genomic DNA (8). Moreover, DENV NS2B mediates cGAS degradation dependent on autophagy-lysosome pathway to avoid IFN production (8). Even though the requirement of mtDNA in the DENV-activated cGAS-STING pathway remains unclear, the roles of mtDNA in DENV pathogenesis are of interest.

THE IFN INDUCTION PATHWAY

After RNA/DNA recognition, both RIG-I-MAVS and cGAS-STING pathways recruit and activate the IKK ϵ /TBK1 and IKK α / β / γ complexes (3). These kinases activate transcription factors, such as NF- κ B and IRFs, to turn on IFN mRNA expression (29). Regardless of the multiple strategies used to antagonize RNA/DNA recognition, DENV also subverts this IFN induction step to minimize the antiviral response in infected cells (27, 76).

Although DENV protease activity is required to cleave and block STING signaling, the protease structure itself is able to inhibit IKK ϵ kinase activity. By interacting with the N-terminus of IKK ϵ , NS2B3 masks part of the kinase domain of IKK ϵ to prevent the S386-phosphorylation of IRF3 (11). Despite the presence of two NS2B3-putative cleavage sites within IKK ϵ , neither catalytic nor inactivated NS2B3 protease affects the protein level of IKK ϵ (11). Therefore, DENV protease is able to counteract IFN induction via both catalysis-dependent and

-independent mechanisms, with the wild-type DENV protease more competent than the protease-dead mutant. Moreover, DENV NS2A and NS4B regulate innate immune responses by inhibiting TBK1/IKK ϵ -directed downstream signaling instead of targeting MAVS or STING directly (6). Thus, both NS2A and NS4B antagonize IRF3 phosphorylation resulting from the activation of RIG-I, MDA5, MAVS, TBK1, or IKK ϵ . Only NS4A of DENV1 but not those of DENV2 or DENV4 blocks TBK1 activation (6), which suggests that DENV1 contains an additional regulatory mechanism against innate immunity.

THE IFN SIGNALING PATHWAY

DENV uses various strategies as described above to prevent the production of IFN by infected cells. Nevertheless, the secreted IFN actively binds to the heterodimeric IFN receptor, IFNAR1/2, which ultimately turns on the expression of many antiviral proteins against DENV infection. After IFN binding, the IFNAR-associated tyrosine kinases Jak1 and tyrosine kinase 2 (Tyk2) undergo autophosphorylation, which then activates downstream transcription factors, mainly STAT1 and STAT2, by phosphorylation. The phosphorylated STATs form a heterotrimeric complex with IRF9, called IFN-stimulated gene factor 3 (ISGF3), which translocates to the nucleus and awakens ISGs to fight against the virus (32, 77, 78). Meanwhile, STAT1 is also modified by the K48-linked conjugation of ubiquitins (79), which tags STAT1 for degradation and shuts off an antiviral response. Accordingly, removing these ubiquitins by the deubiquitinating enzyme USP13 increases the stability of STAT1 proteins and potentiates a stronger IFN-mediated antiviral response against DENV infection (80).

Several viral proteins of DENV are involved in suppressing the IFN-induced signaling. In the presence of IFN, the DENV NS2A, NS4A, and NS4B proteins were found to enhance the replication of an IFN-sensitive recombinant reporter Newcastle disease virus, whereas only DENV NS4A and NS4B significantly reduced the expression of a reporter gene driven by IFN-sensitive response element (7). The NS4B was found to inhibit IFN-induced STAT1 phosphorylation and nuclear translocation, which impairs the transcriptional activity of ISGF3 to turn on antiviral genes (7). Moreover, DENV NS5 can bind to and inhibit the transcription factor STAT2 activated by IFN treatment (16, 17). DENV NS5 recruits the host factor UBR4 to suppress human but not murine STAT2 via the proteasomal degradation pathway (18). Because conjugation of small ubiquitin-like modifier (SUMO) stabilizes DENV NS5 protein to maintain its biological functions, SUMOylation is required for NS5-mediated antagonism of IFN signaling (81).

MANIPULATION OF IFN SYSTEM BY MITOCHONDRIAL DYNAMICS

The roles of mitochondria in innate immunity were largely unknown until strong antiviral activity was detected by overexpressing the mitochondrial protein MAVS (47–50).

Mitochondria move along the cytoskeleton and continuously undergo fusion and fission, which results in the diverse morphology of each mitochondrion (82, 83). MAVS forms prion-like aggregates upon activation (84), which also leads mitochondria to become aggregated in cells overexpressing MAVS (55). Therefore, manipulation of mitochondrial dynamics may regulate antiviral activity in response to virus infection. Indeed, overexpression of the mitochondrial fusion mediator mitofusin 1 (MFN1) rather than MFN2 resulted in a higher-order aggregation of mitochondria that facilitated IFN-induction signaling (12). In contrast, MAVS-mediated IFN-induction signaling was dampened in cells harboring highly fragmented mitochondrial morphology, either by overexpressing a dominant-negative MFN1 (12, 85) or by administration of a chemical disrupting mitochondrial membrane potential (MMP) (86). To manipulate mitochondria toward fragmentation, the virus may suppress fusion or enhance fission. Even though DENV infection triggers MMP disruption (55), which may result in fragmentation of mitochondria (87), the DENV protease NS2B3 alone is sufficient to cleave both MFNs and manipulate mitochondrial morphology (12). Cleavage of both MFN1 and MFN2 suppresses MFN-mediated mitochondrial fusion processes and interferes in MAVS-mediated signalings, such as IFN and cell death induction (12). Hence, mitochondria may serve as platforms transmitting the IFN-induction signal, so that aggregated mitochondria help form a more operative signosome by tethering related molecules with each other. A seemingly conflicting report showed that DENV NS4B induces mitochondria elongation and thus restricts the RIG-dependent IFN response (88). This notion is also consistent with the scenario that disrupted mitochondrial fusion or misassembled signosome leads to disturbed IFN-induction signaling in DENV-infected cells.

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CONCLUSIONS

With DENV infection, disease symptoms range from asymptomatic, classical dengue fever to life-threatening dengue hemorrhagic fever and severe dengue shock syndrome. The diverse disease symptoms result from a complicated interaction between DENV and the host. Innate immunity helps the host fight against infection by eliminating DENV and regulating follow-up immune responses. The non-canonical functions of DENV proteins and DENV-derived sRNA in antagonizing the IFN system further damage infected cells in the battle between DENV and its host. DENV may defeat the host immunity at first line of defense. The seesaw of DENV-inducing and -antagonizing innate immunity in the initial state of infection may contribute to the DENV pathogenesis at some later time. Recent evidence shows that both viral and cellular factors are involved in the host responses upon DENV infection. Therefore, we highlight critical regulatory mechanisms of innate immunity by showing how DENV manipulates it. Notwithstanding unfinished puzzles, antiviral applications derived from all these studies are anticipated.

AUTHOR CONTRIBUTIONS

Y-TK and C-YY conceived and wrote the draft. Y-TK, ML, and C-YY wrote and proofread the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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