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Innate immune escape by Dengue and West Nile viruses

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Abstract

Dengue (DENV) and West Nile (WNV) viruses are mosquito-transmitted flaviviruses that cause significant morbidity and mortality worldwide. Disease severity and pathogenesis of DENV and WNV infections in humans depend on many factors, including pre-existing immunity, strain virulence, host genetics and virus–host interactions. Among the flavivirus–host interactions, viral evasion of type I interferon (IFN)-mediated innate immunity has a critical role in modulating pathogenesis. DENV and WNV have evolved effective strategies to evade immune surveillance pathways that lead to IFN induction and to block signaling downstream of the IFN- α/β receptor. Here, we discuss recent advances in our understanding of the molecular mechanisms by which DENV and WNV antagonize the type I IFN response in human cells.

Introduction

Dengue (DENV) and West Nile (WNV) viruses are arthropod-transmitted viruses of the genus *Flavivirus* (family *Flaviviridae*) that pose a significant global health concern. They are closely related to several other insect-transmitted viruses that cause disease worldwide including Zika (ZIKV), yellow fever (YFV), Japanese encephalitis (JEV), and tick-borne encephalitis viruses. DENV is transmitted to humans principally by two mosquito vectors, *Aedes aegypti* and *Aedes albopictus*, and causes a spectrum of disease ranging from dengue fever to severe dengue (previously called dengue hemorrhagic fever/ dengue shock syndrome [DHF/DSS]), which is potentially lethal. Severe dengue is characterized by extravasation of fluid into peritoneal and pleural spaces, bleeding in the skin and gastrointestinal tract, thrombocytopenia and mild to moderate liver injury [1]. Four serotypes of DENV (DENV 1–4) exist and infection by one serotype confers durable protection against disease only to that particular serotype. Poorly neutralizing or low affinity cross-

reactive antibodies generated during primary infection with one DENV serotype can facilitate severe disease during secondary infection with a heterologous serotype, a phenomenon attributed to ‘antibody-dependent enhancement of infection’ (ADE) [2]. DENV is the leading cause of mosquito-borne viral disease, with an estimated total of ~390 million infections globally each year, primarily in subtropical and tropical regions [3]. Although no specific therapies against DENV are available, this past year, the first tetravalent Dengue vaccine (Dengvaxia®) was approved for use in Brazil, Mexico, and the Philippines in individuals 9–45 years of age [4,5]. This tetravalent vaccine appears to reduce hospitalization in those with prior DENV immunity; however, concern has been raised as to whether it sensitizes naïve individuals to greater symptoms, disease, and hospitalization in the context of a subsequent naturally-acquired first DENV infection [6,7].

WNV is a neurotropic virus that circulates and amplifies in nature between several bird species and ornithophilic mosquitoes. Although WNV can be transmitted to humans by mosquitoes of the *Culex* species, humans are a dead-end host and do not participate in the enzootic cycle. WNV causes a self-limiting febrile illness in most individuals that occasionally can progress to severe neurological disease including encephalitis, meningitis, and acute flaccid paralysis, with a case mortality rate of up to 5–10%. Originally isolated in the West Nile region of Uganda in 1937, WNV is now endemic within areas of Asia, Europe, the Middle East, and also North America. Since its introduction into the United States in 1999, there have been more than 45 000 reported WNV cases and an estimated 3 million people infected in the US [8]. Currently there are no vaccines or specific antiviral agents licensed for use in humans to protect against WNV infection.

DENV and WNV are enveloped viruses that package a positive-sense single-stranded RNA genome encoding a single open reading frame. Translation results in a poly-protein that is cleaved by host and viral proteases and yields three structural (capsid [C], pre-membrane/membrane [prM/M] and envelope [E]) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins. Upon attachment and entry into target cells — predominantly myeloid cells for DENV and multiple cell types for WNV, including skin-resident dendritic cells, keratinocytes, and neurons — these viruses replicate in endoplasmic reticulum (ER)-derived membrane vesicles in the host cytoplasm. The cytoplasmic replication strategy makes these viruses vulnerable to several innate immune sensors (also termed pattern recognition receptors [PRRs]), which detect viral RNA, or in some cases, host-derived molecules generated after virus infection. Upon their activation, PRRs initiate antiviral defense programs in infected and uninfected neighboring cells to impede viral replication and dissemination. However, DENV and WNV have evolved to evade or actively suppress innate immune responses through a variety of mechanisms. Many of the DENV and WNV non-structural proteins, which also are essential for viral RNA synthesis and assembly, have important functions in viral evasion of innate immunity. Recent discoveries have provided insights into the molecular mechanisms by which DENV, WNV, and other globally relevant flaviviruses (*e.g.*, ZIKV) escape innate immune defenses.

Herein, we summarize recent findings on innate immune detection of DENV and WNV and discuss in detail the strategies by which these viruses antagonize type I IFN-mediated

immunity, with a focus on the RIG-I-MAVS, cGAS-STING, and IFN- α/β receptor (IFNAR) signaling pathways.

Innate immune sensing of DENV and WNV infection

Mammalian cells sense unique features of invading viral pathogens through a sophisticated innate immune surveillance network comprised of membrane-bound, cytoplasmic and nuclear PRRs (reviewed in [9,10]). Multiple PRRs work in concert to sense viral infections by recognizing viral nucleic acids, proteins and/or carbohydrates, commonly termed pathogen-associated molecular patterns (PAMPs). In addition, some PRRs sense host-derived ‘danger signals’ (also called damage-associated molecular patterns [DAMPs]) that are produced by the cell upon viral infection. At least four major classes of PRRs contribute to the efficient detection of WNV and DENV infection in human cells: the RIG-I-like receptors (RLRs), Toll-like receptors (TLR3 and 7), NOD-like receptors (NLRs), and the cGAS-STING-dependent sensing pathway (reviewed in detail in [11,12]). These sensors, many of which are upregulated upon WNV and DENV infection [13], activate signaling cascades that induce antiviral or proinflammatory genes, including cytokines such as type I (mainly IFN- α subtypes and IFN- β) and III (IFN- λ) IFNs, chemokines, and IFN-stimulated genes (ISGs). Proteins encoded by ISGs then interfere with specific steps in the viral lifecycle or regulate innate immune signaling, ultimately establishing an antiviral state. Furthermore, cytokines and chemokines produced upon PRR activation help shape the adaptive immune response as well as the infiltrating inflammatory cell response to infection.

RLRs detect cytoplasmic viral RNA species, such as viral replication products or RNA genomes, and are considered as key intracellular PRRs for the detection of DENV, WNV, and other flaviviruses. The RLR family members, RIG-I, MDA5, and LGP2 are expressed in most cell types and characterized by a DExD/H-box helicase domain and a C-terminal domain (CTD), which are both important for binding viral RNA. In addition, RIG-I and MDA5 have two N-terminal caspase activation and recruitment domains (CARDs), which are responsible for initiation of antiviral signaling *via* the adaptor protein MAVS, which is localized at the mitochondria, mitochondria-associated membranes (MAMs) and peroxisomes. In contrast, LGP2 lacks the CARD signaling module and has been suggested to function as a regulator of RIG-I and MDA5 signaling (reviewed in [9,14]).

RIG-I senses viral RNA species that are characterized by extensive secondary structures (*e.g.*, dsRNA loops or panhandle-like structures) with an adjacent 5′ tri- or di-phosphate moiety. RIG-I also recognizes poly-U/UC sequence motifs in the genome of the distantly related *Flaviviridae* family member, hepatitis C virus (HCV). MDA5 is thought to recognize long dsRNA or web-like RNA aggregates (reviewed in [15,16]); however, the physiological RNA ligands detected by MDA5 are largely unknown. In the context of WNV infection, RLRs are crucial for controlling pathogenesis in mice [17,18,19]. Furthermore, gene targeting of RIG-I and/or MDA5 in human and murine cells showed that both sensors contribute to efficient detection of DENV and WNV [18,20,21,22]. During WNV infection, RIG-I and MDA5 act in a temporally distinct manner: while RIG-I was activated early in infection (<12 h post-infection), MDA5 was activated later (>24 h post-infection) [18]. Whether RLRs act analogously during DENV infection remains to be determined.

Furthermore, the RNA species that are recognized by RIG-I and MDA5 during DENV and WNV infection are unknown. As the RNA genomes of DENV and WNV contain a 5' type I cap structure and thus are expected to not stimulate RLR activity, it is likely that RNA replication intermediates (*e.g.*, negative strand RNA), which contain a 5' triphosphate group and may form dsRNA structures, or non-coding subgenomic flavivirus RNAs (sfRNA; reviewed in [23]) are sensed by RIG-I and MDA5.

Recently it has been shown that the cGAS-STING pathway, which is known to sense DNA viruses (reviewed in [24]), also restricts DENV and WNV infection. Gene silencing of the ER-resident adaptor protein STING (also called MITA, MPYS, or ERIS) enhanced DENV replication through reduction of proinflammatory cytokine induction [25,26]. Furthermore, the cytosolic DNA sensor cGAS (cyclic GMP-AMP synthase) potently inhibited the replication of positive-sense RNA viruses including DENV and WNV in a RIG-I-independent manner, and has been suggested to be important for basal expression of ISGs [27^{**},28]. Consistent with this hypothesis, mice lacking cGAS or STING sustained higher levels of WNV infection and lethality compared to control animals [27^{**},29]. Negative-stranded RNA viruses, however, were not restricted by cGAS or STING, suggesting that the cGAS-STING-mediated signaling pathway may be activated specifically during positive-sense RNA virus infection. Intriguingly, it was reported recently that not only microbial cytoplasmic DNA, but also host cell-derived DNA (*e.g.*, mitochondrial DNA), can stimulate cGAS-STING signaling [30,31,32]. Furthermore, viral-induced membrane remodeling was shown to activate STING [33]. It will be important to define how cGAS-STING-dependent signaling is activated during DENV and WNV infection, and whether flavivirus infection causes mitochondrial damage that liberates self-DNA ligands for recognition by cGAS.

To induce antiviral resistance, RLR-MAVS- and cGAS-STING-induced signaling activates several TRAF proteins and IKK family kinases, namely IKK ϵ , TBK1 and the IKK α / β / γ complex. These serine/threonine (Ser/Thr) kinases activate the transcription factors IFN-regulatory factor 3, 5, and 7 (IRF3/5/7) [34^{**}] and NF- κ B, which along with AP-1 induce the transcription of IFNs and other proinflammatory cytokines (reviewed in [35]). Upon secretion, type I and III IFNs bind to their respective receptors (IFNAR1/2 and IFN- λ receptor 1 (IFNLR1)/IL10R- β) and induce the expression of hundreds of ISGs, several of which (*e.g.*, IFITM1-3, IFIT2, Ifi2712a, viperin (RSAD2), and ISG20) have been demonstrated to have anti-DENV and/or anti-WNV activity [28,36,37,38,39].

In addition to the RLR-MAVS and cGAS-STING sensing pathways, TLR3 and 7 as well as NLRP3 are important for host immunity to DENV and WNV infection. For the latter families of PRRs, we refer to two recent reviews that detail their role in restricting DENV and WNV [11,12].

Antagonism of type I IFN induction by DENV and WNV

Although the type I IFN response is critical for controlling DENV and WNV infection [40,41,42,43,44], both viruses nonetheless block type I IFN gene expression either by escaping recognition by PRRs, or by actively inhibiting PRR-mediated IFN- α / β induction. Here, we discuss the molecular strategies by which DENV and WNV antagonize the RLR-

MAVS and cGAS-STING signaling pathways. These evasion strategies fall into three major categories: first, sequestration or modification of viral RNA; second, direct inhibition of PRRs or their adaptor proteins; and third, antagonism of key signaling proteins downstream of PRRs (Figure 1).

Sequestration or modification of viral RNA

A major strategy that DENV and WNV use to prevent RLRs from accessing viral RNA in the cytoplasm is the formation of replication compartments confined by cellular membranes. Specifically, DENV and WNV replicate in ER-convoluted membrane vesicles, also called viroplasm-like structures (VLS) [45,46,47,48], which have been proposed to function as a physical barrier to conceal dsRNA from cytoplasmic RLR sensors and delay IFN induction. A recent study suggested that in contrast to DENV and WNV, JEV does not efficiently conceal its dsRNA and therefore strongly induces IFNs and other cytokines [49]. However, more investigation is needed to directly address the relevance and contribution of DENV- and WNV-induced VLSs to viral escape from RLRs and other innate immune receptors.

As RLRs and other intracellular RNA sensors recognize specific features in the viral RNA that are absent in most mature cellular RNAs, many viruses modify their viral RNA to escape innate immune detection. Some viruses, including many flaviviruses (*e.g.*, WNV, DENV, JEV, ZIKV, and YFV), encode 2'-*O*-methyltransferases as part of their NS5 proteins to modify the 5' cap structure of their viral mRNAs, thereby mimicking eukaryotic type 1 cap structures (m7GpppNm-RNA). Flaviviral 2'-*O*-methyltransferase activity evades viral RNA detection by IFIT proteins [50,51], which can block viral protein synthesis by binding preferentially to non-2'-*O*-methylated type 0 cap structures (m7GpppN-RNA) and competing for eIF4e binding [52,53]. A recombinant WNV encoding an NS5 E218A mutant protein that is defective in 2'-*O*-methyltransferase activity was more sensitive to the antiviral action of IFIT family members [50,54]. The mutant WNV did not trigger increased type I IFN gene expression; however, this may be due to the efficient inhibition of IFN induction by other WNV-encoded proteins (as described below). Similarly, ablation of the 2'-*O*-methyltransferase activity from JEV and DENV led to growth attenuation of the mutant viruses in the context of cellular innate immune responses [55,56].

Direct inhibition of PRRs or their adaptor proteins

Apart from viral RNA binding, K63-linked polyubiquitination of RIG-I mediated by the E3 ubiquitin ligase TRIM25 is required for RIG-I activation [57]. This unconventional polyubiquitin-linkage type does not induce RIG-I degradation, but instead promotes RIG-I tetramerization, RIG-I-MAVS binding, and ultimately, an effective antiviral response [57,58,59]. The protein stability of TRIM25 is regulated by a balance of degradative K48-linked ubiquitination and stabilizing deubiquitination, the latter catalyzed by the deubiquitinating enzyme USP15 [60]. A recent study showed that sfRNA, a non-coding RNA derived from the 3' UTR of viral genomic RNA that contributes to pathogenicity and evasion of the type I IFN response [61,62], can inhibit TRIM25-mediated RIG-I activation [63*]. The sfRNA of an epidemic DENV strain bound to TRIM25 in a sequence-specific manner, and this interaction prevented TRIM25 deubiquitination and stabilization by USP15, thereby blunting the RIG-I-mediated IFN response [63*]. Further investigation is

warranted, as the precise mechanism for how sfRNA binding to TRIM25 modulates its protein stability and/or function and whether this strategy is common to other flaviviruses remain unknown.

RIG-I must translocate to signaling-competent organelles where MAVS is localized — the mitochondria and peroxisomes — to initiate downstream signaling. The mitochondrial-targeting chaperone protein, 14-3-3 ϵ , interacts with RNA-bound and TRIM25-activated RIG-I to promote the translocation of RIG-I from the cytoplasm to the outer membrane of mitochondria and MAMs [64]. Gene targeting studies showed that 14-3-3 ϵ was required for an effective RIG-I-mediated antiviral response [64]. It was recently established that 14-3-3 ϵ is a binding partner of the DENV NS3 protein [65**]. The DENV protease, composed of NS3 and its cofactor NS2B (commonly called NS2B/3), did not cleave 14-3-3 ϵ (neither RIG-I or TRIM25), but competed with RIG-I for 14-3-3 ϵ binding, thereby inhibiting the translocation of RIG-I to mitochondria. The strong 14-3-3 ϵ -binding affinity of DENV NS3 was attributed to a highly conserved four-amino-acid motif 64-RxEP-67, in which the central glutamic acid residue (E66) mimics the phosphorylated Ser/ Thr residue of cellular 14-3-3-binding motifs. Mutation of the NS3-14-3-3 ϵ interaction in the context of a recombinant DENV (64-RIEP-67 $\rightarrow$ 64-KIKP-67 mutation in the DENV2 strain 16681) resulted in greater cytokine induction in hepatocytes and primary human monocytes and dendritic cells [65**]. The NS3 proteins of WNV strains have a similar phosphomimetic 14-3-3 ϵ -binding motif, 64-RLDP-67, to block RIG-I-induced signaling in a cleavage-independent manner. Thus, in contrast to the NS3/ 4A protease of HCV, which cleaves MAVS to prevent RIG-I antiviral signaling [66,67], the NS2B/3 of DENV and WNV block RIG-I translocation to mitochondrial MAVS in a proteolysis-independent manner. Elimination of a critical RIG-I-escape mechanism from DENV resulted in an attenuated yet highly immunogenic virus (DENV_{RIEP $\rightarrow$ KIKP}). Future studies likely will address the physiological relevance of 14-3-3 ϵ antagonism by DENV and WNV NS3 for evasion of innate and adaptive immunity in relevant *in vivo* models.

In contrast to RIG-I/14-3-3 ϵ antagonism by DENV NS3, which is not dependent on its proteolytic activity, the DENV NS2B/3 protease cleaves STING, thereby preventing type I IFN induction mediated by cGAS [25,26]. As the cleavage site for DENV NS2B/3 (LRRQ⁹⁶G) is present in human STING, but not in the murine ortholog MPYS, it has been proposed that STING functions as a species-specific restriction factor for DENV.

Mimicry of host innate immune molecules is an established strategy of many viral pathogens to inhibit or subvert immune responses. Intriguingly, the crystal structure of the DENV and WNV NS1 protein revealed that the α/β subdomain of the NS1 ‘wing’ has a resemblance to the superfamily 2 helicase domain of RIG-I and MDA5 [68**]; however, whether RLR mimicry by NS1 is used to antagonize host immunity still needs to be addressed. Against a direct effect on RLR-mediated antiviral immunity, NS1 folds and assembles into its dimeric and hexameric forms within the lumen of the ER, and thus should not be accessible to cytoplasmic innate immune defense elements.

Antagonism of key signaling proteins downstream of PRRs

Several non-structural proteins of DENV and WNV dampen IFN α / β induction by targeting important signaling molecules downstream of several PRRs; however, the precise mechanisms and physiological role of these viral escape strategies still need to be demonstrated.

The NS2B/3 protease of DENV was shown to bind to IKK ϵ and block its kinase activity, thereby inhibiting IRF3 phosphorylation at position S386 and its nuclear translocation [69]. Ectopic expression of NS2A and NS4B from DENV1, 2 and 4, as well as NS4B of WNV (New York 1999 strain), inhibited the autophosphorylation-dependent activation of TBK1 [70]. The NS4A protein from DENV1, but not other DENV serotypes, also inhibited TBK1, suggesting that DENV1 contains additional IFN-regulating virulence determinants. As NS2A and NS4B did not co-immunoprecipitate with TBK1 [70], it remains to be elucidated how these viral proteins block TBK1 activation.

The NS2A protein of WNV also reportedly blunts type I IFN gene expression. Analysis of adaptive mutations of the replicon RNA of the Kunjin strain, a less pathogenic WNV lineage I isolate, showed that WNV NS2A suppressed IFN- β promoter activation, and adaptive mutation of alanine at position 30 to proline (A30P) abrogated type I IFN antagonism [90]. A recombinant WNV encoding a mutant A30P NS2A protein exhibited growth attenuation and was less neuroinvasive in mice than the parental strain [91].

Apart from non-structural proteins of DENV and WNV, which are the main IFN-antagonistic proteins, few structural proteins have been reported to suppress IFN α / β induction. For example, the E protein of WNV was shown to block dsRNA-induced type I IFN induction by targeting the kinase RIP1 (receptor-interacting protein 1), which is a positive regulator of RIG-I (and also TLR) signaling. RIP1 inhibition required a high mannose glycosylation profile on the E protein, which is only found on mosquito cell-derived, but not mammalian cell-produced, WNV [71].

Inhibition of IFNAR signaling by DENV and WNV

IFN- α and IFN- β proteins bind to the heterodimeric receptor IFNAR1/2 on infected and surrounding uninfected cells, inducing the activation of Janus kinase 1 (JAK1) or tyrosine kinase 2 (TYK2), which ultimately phosphorylate and activate the transcription factors STAT1 and 2. STAT1 and 2 subsequently bind to IRF9 and the STAT1-STAT2-IRF9 ternary complex translocates into the nucleus to induce the gene expression of many ISG-encoded antiviral effector proteins. DENV and WNV strains have evolved mechanisms to interfere with the IFNAR signaling pathway to sustain higher levels of infection. These viruses use two major strategies to block IFNAR signal transduction: direct inhibition of JAK1 or TYK2 activity, and targeting of the key transcription factors STAT1 and 2 (Figure 2).

The NS4B proteins of DENV and WNV have been shown to block the IFNAR-dependent signaling cascade [72,73,74]. WNV NS4B inhibits the phosphorylation-mediated activation of both JAK1 and TYK2, thereby blocking STAT activation [72,75]. WNV has evolved a second mechanism to block IFNAR-dependent signal transduction by upregulating the

expression of suppressors of cytokine signaling (SOCS) 1 and 3 [76], which dampen JAK1 activity. Mechanistically, WNV and also other enveloped viruses induce SOCS1/3 gene expression by binding to and activating TAM (Tyro3/Axl/Mer) receptors on dendritic cells, thereby blunting the antiviral IFN response [77]. Furthermore, disruption of cholesterol-rich lipid raft domains at the plasma membrane and WNV-induced IFNAR1 degradation also dampen IFN signaling [78,79].

Another important evasion strategy employed by WNV and DENV is direct antagonism of STAT1 and/or STAT2, and both viruses have dedicated multiple non-structural proteins to block STAT1/2 activation. The NS5 proteins of both WNV and DENV potently block STAT2 activation [80,81,82,83]. The NS5 protein of DENV recruits the host factor UBR4, which induces the proteasomal degradation of STAT2 [81,84]. Similar to the NS3-STING interaction, DENV NS5 interacts with human, but not mouse STAT2 [85], indicating that STAT2 also contributes to the species-specific restriction of DENV infection. Finally, a recent study showed that introduction of the capsid and E proteins (and also the non-structural proteins NS1–5) from a pathogenic WNV strain (Texas 2002) into an attenuated strain (Madagascar 1978) led to more potent IFN-antagonistic activity and pathogenesis, suggesting that these structural proteins of WNV also contribute to efficient STAT1/2 inhibition [86]. However, the molecular mechanism(s) by which structural proteins contribute to IFNAR-signal inhibition remain to be defined.

Conclusions

Several innate immune escape strategies of DENV and WNV target key signaling molecules in the type I IFN response, which underscores the importance of bypassing this early immune response for effective viral replication. For many of these immune evasion tactics, however, their relevance and relative contribution to viral pathogenesis need to be established. Another key question is whether this knowledge can be translated into the design of vaccines and antiviral agents. The rational design of live-attenuated viral strains may be possible by systematically eliminating specific immune evasion mechanisms. A caveat of this approach is the potential risk for reversion, but this may be overcome by disabling several immune evasion mechanisms in combination (such as viral targeting of STAT2 and 14-3-3 ϵ), which may improve the safety profile of potential vaccine strains at the expense of over-attenuation. Another active avenue of research is pharmacological targeting of the enzymatic activity of flaviviral proteins, such as NS2B/3 and NS5, or their enzymatic-independent interactions with host immune molecules for the development of antiviral therapies.

Finally, an in-depth understanding of the evasion strategies used by DENV and WNV also will enhance our knowledge of the innate immune restriction mechanisms of the recently emerging ZIKV. Indeed, recent studies in *Ifnar* or *Irf3/5/7* knockout mice demonstrated the importance of the type I IFN response in controlling ZIKV replication and pathogenesis in mice [87,88]; immunocompetent wild type mice were relatively resistant to infection whereas mice with compromised IFN immunity developed disease including *in utero* infection and congenital abnormalities. Given that ZIKV causes disease in ostensibly immunocompetent humans, it likely has evolved means to block type I IFN-mediated host

immunity. As such, some of the IFN-suppressive strategies utilized DENV and WNV may be conserved in ZIKV. In support of this, a recent study demonstrated that the NS5 protein of ZIKV, analogous to DENV NS5, induces the proteasomal degradation of STAT2, thereby blocking IFNAR signal transduction [89*]. Identifying the molecular details of how ZIKV suppresses the human innate immune system may reveal targets for the development of antiviral agents and vaccines to combat ZIKV infections.

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Figure 1. Antagonism of RLR-MAVS- and cGAS-STING-mediated IFN induction by DENV and WNV

Upon viral infection, RIG-I and MDA5 recognize cytoplasmic viral RNA species: short dsRNA containing a 5' tri- or di-phosphate moiety by RIG-I and long dsRNA structures by MDA5. The RNA ligands recognized by RIG-I and MDA5 during DENV and WNV infection remain unknown. Upon RNA binding, RIG-I and MDA5 multimerize and translocate from the cytoplasm to the outer membrane of mitochondria to bind to the adaptor protein MAVS. For RIG-I multimerization and translocation, K63-linked polyubiquitination of RIG-I induced by the E3 ligase TRIM25 and binding to the mitochondrial-targeting chaperone protein 14-3-3e are critical. The cGAS-STING pathway also restricts DENV and WNV infection. Upon binding to dsDNA-viral DNA in the case of DNA virus infection, or likely host-derived DNA (*e.g.*, mitochondrial DNA) associated with DENV or WNV infection—cGAS produces the second messenger cGAMP (cyclic GMP-AMP), which binds to and activates STING, a critical adaptor protein localized at the ER.

Following their activation, MAVS and STING multimerize and assemble a large 'signalosome' complex, comprised of RIP1, TRAF family members, and other signaling proteins (not illustrated), ultimately inducing the activation of TBK1, IKKε, and the

IKK α / β / γ complex. TBK1/IKK ϵ phosphorylate the transcription factors IRF3, IRF5, and IRF7, whereas the IKK α / β / γ complex activates NF- κ B. IRF3/5/7 and NF- κ B together with AP1 (not illustrated) induce the transcriptional activation of type I and III IFNs, and other proinflammatory cytokines and chemokines to establish an antiviral state. DENV and WNV inhibit the RIG-I-MAVS and cGAS-STING signaling pathways through several mechanisms. The NS3 protein of DENV and WNV competes with RIG-I for 14-3-3 ϵ binding, thereby preventing RIG-I translocation from the cytosol to mitochondrion-localized MAVS. NS3 encodes a phosphomimetic motif that resembles a conventional phosphorylation motif found in cellular 14-3-3-binding partners to interact with 14-3-3 ϵ . Furthermore, the sfRNA of an epidemic DENV strain binds to TRIM25, blocking its deubiquitination and stabilization by USP15 (not illustrated). To dampen cGAS-STING signaling, the DENV NS2B/3 protease cleaves STING. Furthermore, WNV and DENV block common signaling molecules downstream of innate sensors. The WNV E protein inhibits activation of RIP1, whereas DENV NS2B/3 interferes with IKK ϵ activity. The NS2A, NS4A, and NS4B proteins of DENV, and the NS4B protein of WNV, block the autophosphorylation-dependent activation of TBK1. Finally, DENV and WNV use the 2'-*O*-methyltransferase (MTase) activity of their NS5 proteins to generate type 1 cap structures in their mRNAs, thereby preventing translational inhibition by IFIT family proteins. Solid arrows indicate direct effects or well-established signaling events. Dashed arrows indicate signaling events that are either indirect or that have not yet been completely established. CARD, caspase activation and recruitment domain; CTD, C-terminal domain; DENV, dengue virus; K63-Ub, Lys63-linked ubiquitination; MTase, 2'-*O*-methyltransferase; P, phosphorylation; WNV, West Nile virus; sfRNA, subgenomic flavivirus RNA.

Figure 2. Inhibition of IFNAR signaling by DENV and WNV

IFN- α and IFN- β bind to and activate the heterodimeric IFN- α/β receptor (IFNAR1/2), which leads to the recruitment and autophosphorylation-dependent activation of the kinases JAK1 and TYK2. JAK1 and TYK2 phosphorylate the transcription factors STAT1 and STAT2, which then heterodimerize and bind to IRF9. The STAT1-STAT2-IRF9 ternary complex translocates into the nucleus to induce the expression of hundreds of IFN-stimulated genes (ISGs). ISGs encode for innate signaling molecules, or antiviral effector proteins that block specific steps in the DENV and WNV lifecycle. DENV and WNV have evolved multiple mechanisms to inhibit IFNAR-mediated signal transduction. The NS4B protein of WNV blocks the autophosphorylation and activation of JAK1 and TYK2. WNV and likely other flaviviruses (*e.g.*, DENV) utilize a second strategy to block JAK1 activation: they upregulate SOCS1 and SOCS3 gene expression through binding to and activation of TAM receptors. Multiple non-structural proteins of DENV and WNV prevent the activation of STAT1 and/or STAT2. For example, the NS2A, NS4A and NS4B proteins of DENV block STAT1 phosphorylation, whereas the NS5 protein of WNV inhibits activation of STAT2. Furthermore, the NS5 protein of DENV recruits the host factor UBR4 to induce the proteasomal degradation of STAT2. Solid arrows indicate direct signaling effects. Dashed arrows indicate signaling events that are either indirect or that have

not yet been entirely established. DENV, dengue virus; TAM receptor; Tyro3/Axl/Mer receptor; WNV, West Nile virus; P, phosphorylation.

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