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## Innate immunity to dengue virus infection and subversion of antiviral responses

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### Abstract

Dengue is a major public health issue in tropical and subtropical regions worldwide. The four serotypes of dengue virus (DENV1-4) are spread primarily by *Aedes aegypti* and *Ae. albopictus* mosquitoes, whose geographic range continues to expand. Humans are the only host for epidemic strains of DENV, and the virus has developed sophisticated mechanisms to evade human innate immune responses. The host cell's first line of defense begins with an intracellular signaling cascade resulting in production of interferon (IFN)- $\alpha/\beta$ , which promotes intracellular antiviral responses and helps initiate the adaptive response during the course of DENV infection. In response, DENV has developed numerous ways to subvert these intracellular antiviral responses and directly inhibit cellular signaling cascades. Specifically, DENV manipulates the unfolded protein response and autophagy to counter cellular stress and delay apoptosis. The DENV non-structural protein NS4B and subgenomic sRNA interfere with the RNAi pathway by inhibiting the RNase Dicer. During heterotypic secondary DENV infection, subneutralizing antibodies can enable viral uptake through Fc $\gamma$  receptors and down-regulate signaling cascades initiated via the pattern recognition receptors TLR3 and MDA5/RIG-I, thus reducing the antiviral state of the cell. The DENV NS2B/3 protein cleaves human STING/MITA, interfering with induction of IFN- $\alpha/\beta$ . Finally, DENV NS2A, NS4A, and NS4B complex together to block STAT1 phosphorylation, while NS5 binds and promotes degradation of human STAT2, thus preventing formation of the STAT1/STAT2 heterodimer and its transcriptional induction of ISGs. Here we discuss the host innate immune response to DENV and the mechanisms of immune evasion DENV has developed to manipulate cellular antiviral responses.

### Introduction

Four dengue virus serotypes (DENV-1,-2, -3, -4) cause dengue fever (DF) as well as more severe disease manifestations, traditionally referred to as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)<sup>1</sup>. DF is an acute febrile illness with headache, retro-

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orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations, and/or leukopenia. The hallmark features of DHF consist of thrombocytopenia, hemorrhagic manifestations, and signs of plasma leakage, which can lead to hypotensive shock (DSS) and, without appropriate treatment, death. The disease was recently reclassified into dengue, with and without warning signs, and severe dengue<sup>2</sup>. Bhatt *et al.*<sup>3</sup> recently estimated that up to 96 million dengue cases and a total of 390 million DENV infections occur each year worldwide<sup>3</sup>, leading to approximately 500,000 hospitalizations and 25,000 deaths, primarily among children<sup>4</sup>. Dengue occurs throughout tropical and subtropical regions around the world, with disease burden most well documented in Southeast Asia and Latin America<sup>3</sup>. DENV is transmitted by *Aedes aegypti* and *Ae. albopictus* mosquitoes, which continue to expand geographically, facilitated by increased global trade and travel, unplanned urbanization, poor waste and water management, as well as human behavior and ecology<sup>5</sup>. No commercial vaccine or specific antiviral treatment exists for dengue, though these are areas of substantial research and development efforts. Dengue is a human disease with no known animal reservoirs, and the virus has evolved successfully to evade human immune responses, especially innate antiviral immunity. This review focuses on mechanisms of the innate intracellular antiviral response and DENV evasion within infected cells.

### The dengue virus life cycle

DENV is a positive-strand RNA enveloped flavivirus whose 10.7 kb genome contains a 5' type I m<sup>7</sup>G cap structure and encodes a polyprotein that is post-translationally cleaved by host and viral proteases into three structural proteins (C, capsid; pr/M, membrane; E, envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). In humans, DENV primarily infects immune cells of the myeloid lineage, including monocytes, macrophages and dendritic cells as well as hepatocytes, as shown *in situ* in human autopsy tissues by immunohistochemistry<sup>6,7,8,9,10</sup>, in peripheral blood mononuclear cells (PBMC) during the acute phase of infection by flow cytometry<sup>11</sup>, and *ex vivo* in skin explants<sup>12</sup>. Though several reports exist of DENV infection of endothelial cells *in vivo* and *in vitro*, the role of viral infection of endothelial cells in dengue disease progression remains controversial<sup>7,8,9,10,13,14,15</sup>. Finally, only one autopsy report from a patient with fatal DHF showed DENV protein in myocytes, indicating that myocytes are likely not a primary target of DENV infection<sup>16</sup>.

Initial viral infection is mediated by clathrin-dependent receptor-mediated endocytosis<sup>17,18</sup>, and in a secondary infection, virions complexed with antibodies from a prior DENV infection(s) can enter cells via Fc $\gamma$  receptors (Fc $\gamma$ R) (Figure 1). Once inside the cell, the endosomal vesicle becomes acidified, and the virus undergoes conformational changes that enable fusion with the endosomal membrane, releasing the single-stranded RNA (ssRNA) into the cytosol<sup>19</sup>. The DENV ssRNA is then translated and replicated in the endoplasmic reticulum (ER), which undergoes hypertrophy after flavivirus infection<sup>20</sup>. Recent electron microscopy and electron tomography studies have shown that DENV RNA replication occurs in virus-induced membrane vesicles in the ER, with budding of DENV particles on ER membranes directly apposed to vesicle pores<sup>21</sup>. The newly replicated positive-strand viral RNA is packaged with capsid protein and assembled into an enveloped virion that is covered with 180 E and prM/M proteins, with the E proteins arranged in antiparallel

dimers<sup>22</sup>. Vesicles containing the newly-formed virions pass through the Golgi, where the host protease furin cleaves prM to generate mature and partially mature virions that are secreted from the host cell (Figure 1). The non-structural viral proteins participate in the replication complex<sup>23</sup> and have also been shown to play a role in disrupting cellular antiviral pathways to enhance viral replication, as discussed below.

### The dengue virion

DENV replicates in two distinct host environments, human and mosquito. During replication, cleavage of prM/M on the virion surface is required for a mature virion to be generated. Mature and immature virions differ in size and structure. Immature virions prior to prM/M cleavage are ~600Å in size and have a spiky appearance, whereas cleavage of prM allows the E protein to change conformation and form a smoother layer over the viral phospholipid membrane. During viral processing, prM has been shown to remain associated with mature virus at acidic pH, preventing membrane fusion<sup>24</sup>. Recent studies have shown that the maturation state of flaviviruses influences both the infectable cell type(s) and the interaction of the virion with particular antibodies, thus impacting the results of neutralization assays<sup>25</sup> and potentially affecting the triggering of innate immune responses. However, the relative percentage of immature or mature DENV virions in humans and the role of maturation state in disease are currently unknown. Previous work has shown that during virus propagation *in vitro*, both mature and immature virions are produced<sup>26,27</sup>, although the relative amounts vary substantially by cell type<sup>28,29</sup>. Recent reports have shown that not only maturation state, but temperature as well plays a role in the structure of the virion. At temperatures above 33°C, the mature virion lacking prM is irreversibly altered from a “smooth marble-like” structure to a bumpy form that is 10% larger, which has been shown to be a stable prefusion intermediate<sup>30,31</sup>. Another important conceptual advance is that DENV virions are not static but rather dynamic, breathing structures, thus enabling antibodies with “cryptic” epitopes to bind and also exposing the membrane underneath the layer of viral E and M proteins<sup>32,33,34</sup>. As a result, both the temperature and time of incubation of antibodies and virus can dramatically alter the measurement of neutralizing antibody titers<sup>33</sup>.

### Host receptors for dengue virus

DENV is transmitted to the human host during the mosquito's blood meal and infects human cells via several distinct receptors (Figure 1). Direct interaction between the glycosylated residues in Domain II of the DENV E protein and the carbohydrate recognition domain (CARD) of dendritic-cell-specific ICAM3-grabbing non-integrin (DC-SIGN) facilitates infection of dendritic cells<sup>27,35,36</sup>. However, as DENV infection of DCs does not require DC-SIGN internalization signals, DC-SIGN is considered an attachment factor<sup>37</sup>. Interestingly, polymorphisms in the human DC-SIGN gene CD209 have been suggested to correlate with an increased risk of dengue and disease severity<sup>38,39</sup>. DENV also utilizes the DC-SIGN homolog L-SIGN to infect liver endothelial cells<sup>40</sup>. Of note, virus produced *in vitro* in mosquito cells was found to utilize DC-SIGN, whereas virus propagated in human dendritic cells utilized L-SIGN to infect target cells<sup>36,40</sup>. In addition to DC-SIGN and L-SIGN, the mannose receptor expressed on human macrophages was found to bind the carbohydrate moieties on the DENV envelope protein<sup>41</sup>.

DENV has been shown to bind to a number of cell surface molecules. DENV is able to complex with heat shock protein (HSP) 90 and HSP70 on the surface of mammalian cells<sup>42,43</sup> and p74 on the surface of mosquito cells<sup>44</sup>, among others. Following heat shock treatment, host cells were found to have increased HSP expression, viral uptake and virus output<sup>43,44</sup>. In cells lacking selectin-type receptors, recent studies have shown that DENV utilizes the transmembrane receptors TIM and TAM, two receptors involved in phosphatidylserine-dependent removal of cells undergoing apoptosis<sup>45</sup>. TIM binds DENV directly, whereas TAM interacts indirectly with DENV via two bridge proteins, Gas6 and ProS<sup>45</sup>. Finally, during secondary DENV infection with a heterotypic serotype, the adaptive immune response can act to enhance viral infection via antibody-dependent enhancement (ADE) of Fc $\gamma$ RI- and Fc $\gamma$ RII-bearing cells<sup>46,47</sup>. Cross-reactive antibodies from a previous infection with a different serotype bind to the infecting DENV serotype, forming an immune complex that is recognized by Fc $\gamma$ Rs, which then mediate uptake into the target cells of myeloid lineage<sup>48</sup>.

### The host innate immune response against dengue virus

Pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), particularly TLR-3, TLR-7, and TLR-8, and intracellular sensors (e.g., the DExE/H box RNA helicases) are some of the first lines of defense in the innate immune recognition of double-stranded RNA (dsRNA), ssRNA or modified RNA. The primary TLR involved in dengue viral recognition, TLR-3, recognizes DENV RNA after endosomal acidification (Figure 2A) and has been shown to induce strong IL-8 and interferon (IFN)- $\alpha/\beta$  responses *in vitro*<sup>49</sup>. Recognition of DENV RNA by TLR-3 results in phosphorylation of TIR domain-containing-adaptor-inducing interferon  $\beta$  (TRIF). TRIF interacts with both TNF receptor associated factor (TRAF) 3 and 6. TRAF3 interacts with TANK binding kinase (TBK-1) and Ik kinase 1 (Ikk1), resulting in the phosphorylation of IRF-3. TRAF6 signals through association with TAK1, activating AP-1 and initiating Ikk1/2 dephosphorylation of IkkB, leading to NF $\kappa$ B activation. Nuclear translocation of IRF3, AP-1 and NF $\kappa$ B induces production of IFN- $\alpha/\beta$ , interferon stimulating genes (ISGs) and chemokines<sup>50</sup>. Experiments in non-human primates infected with DENV demonstrated that the administration of TLR-3 and TLR7/8 agonists resulted in significantly decreased viral replication and increased production of pro-inflammatory chemokines as well as increased anti-DENV serum antibody titers, indicating a protective role for TLRs during DENV infection<sup>51</sup>.

In conjunction with TLRs, cytoplasmic helicases sense RNA in the cytoplasm. Both retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), which recognize dsRNA<sup>52</sup>, are induced during DENV infection and are involved in IFN- $\beta$  induction<sup>52,53</sup> (Figure 2B). Once bound to the dsRNA, RIG-I oligomerizes in an ATP-dependent fashion<sup>54</sup>. One important role of these cytoplasmic dsRNA sensors is to distinguish between viral and host cell RNA<sup>55</sup>. In particular, MDA5 is able to distinguish host RNA from viral RNA based on a ribose 2'-O methylation found on host mRNA.<sup>56</sup> RIG-I and MDA5 signal through the mitochondrial antiviral signaling (MAVS) protein on mitochondria. MAVS interacts with the ER protein stimulator of interferon genes (STING, also known as MITA). Once activated, MAVS oligomerizes and attracts multiple ubiquitin E3 ligases such as TRAF3 and TRAF6 to activate the signaling cascade<sup>57</sup>. TRAF3

activation results in translocation of both IRF3 and IRF7 to the nucleus to induce transcription of IFN- $\alpha/\beta$ , as described above<sup>50</sup>. In addition, activation of TRAF6 results in NF $\kappa$ B translocation to the nucleus and production of IFN- $\alpha/\beta$ . Both RIG-I and MDA5 were found to synergize with TLR3 to limit DENV replication *in vitro*<sup>53</sup>.

IFN- $\alpha/\beta$  secreted by virus-infected cells triggers warning signals to adjacent cells that an infection is occurring as well as an autocrine induction of cellular antiviral responses. IFN- $\alpha/\beta$  is a powerful inhibitor of DENV infection. IFN- $\alpha/\beta$  signals through the IFN- $\alpha/\beta$  receptor (IFNAR) and activates the JAK/STAT pathway via phosphorylation of the adaptor molecules TYK2 and JAK1<sup>58</sup>. The activation of these adaptor molecules results in the phosphorylation and dimerization of various signal transducer and activator of transcription (STAT) molecules, including STAT1, STAT2, STAT3, and STAT5. An important signaling complex formed by STAT1 and STAT2 along with IRF9 in response to IFNAR activation is the interferon stimulating gene factor 3 (ISGF3) complex that translocates to the nucleus and binds to IFN-stimulated response elements located in the promoter region of IFN-stimulated genes. ISGF3 complexes bind to these key elements and induce the production of numerous antiviral proteins and pro-inflammatory cytokines<sup>59</sup>. IFNAR signaling also induces alternative signaling cascades, including the mitogen-activated protein (MAP) kinase p38 cascade and the phosphatidylinositol 3 (PI3) kinase cascade<sup>59</sup> that result in production of proinflammatory cytokines and chemokines.

In addition to IFN- $\alpha/\beta$  production, induction of NF $\kappa$ B translocation to the nucleus results in production of pro-interleukin 1 and activation of the inflammasome, which results in production of mature interleukin 1 and 18. Both cytokines are important for antiviral responses and result in recruitment of immune cells to the site of infection<sup>60</sup>. During infection, DENV interacts with the macrophage surface marker C-type lectin domain family 5, member A (CLEC5A) and induces inflammasome activation in human macrophages<sup>61</sup>. In addition, blockage of the CLEC5A/DENV interaction *in vivo* reduced mortality by 50% in a murine lethal challenge model<sup>62</sup>.

Small RNAs such as micro RNAs (miRNA) are utilized by the host to control cellular gene expression and in response to viral infections (Figure 2C). miRNAs are processed by and interact with proteins in the RNAi pathway, such as Dicer, Drosha, Ago1 and Ago2. Interestingly, knockdown of these proteins during DENV infection of mammalian cells resulted in increased viral replication, indicating the RNAi pathway may play an important role in the cellular anti-DENV response<sup>63</sup>. miRNAs not only regulate TLR and IL-1 signaling, but are also induced in response to these signals during viral infection, thus providing control of host innate immune responses. Approximately 22 nucleotides in length, miRNAs inhibit translation of target RNAs. Base-pair matching between miRNA and RNA does not need to be exact; thus, one miRNA can interact with many different RNAs.

## Dengue virus subversion of the cellular antiviral response

DENV has evolved to evade the host's innate immune response in two distinct ways: sequestration and active subversion of innate immune responses. DENV infection of a host cell causes extensive rearrangements of cellular membranes, yet the virus must control the

innate immune mechanisms such as stress that trigger cell death. DENV manipulates the cell to maintain host metabolism and protein production while sequestering itself in vesicles that are not degraded by host lysosomes. This complex process involves a delicate balance of activating cellular pathways for ER expansion and inducing lipid metabolism while preventing ER stress-induced death. DENV uses the cell's own pathways of survival such as autophagy to facilitate viral replication. Finally, DENV non-structural proteins act directly on components of the innate immune response's signaling cascade, inhibiting the RNAi pathway and IFN- $\alpha/\beta$  induction and signaling.

### ER stress/Unfolded protein response (UPR)

DENV utilizes several mechanisms to hijack the host cell machinery to facilitate replication. DENV replication occurs in the ER of host cells, and early during infection (e.g., 12 hours post-infection), the ER undergoes rearrangement and expansion<sup>64</sup> (Figure 2D). This ER expansion requires *de novo* synthesis of viral proteins but does not require host transcription and is independent of the unfolded protein response (UPR) and the sterol-regulatory-element-binding-protein-2 (SREBP-2) pathway, as shown using a series of mouse embryo fibroblasts deficient in key genes in the UPR and SREBP pathways<sup>64</sup>.

While early rearrangement of the ER has been shown to be independent of the UPR response, DENV infection induces the UPR and can manipulate the UPR to cope with ER stress (Figure 3A)<sup>65</sup>. In particular, DENV NS2B/3 was shown to induce X-box binding protein 1 (XBP1), leading to an expansion of the ER for viral replication<sup>66</sup>. This also results in reduction of the UPR response and decreased DENV-induced cytopathic effects, indicating that a reduced UPR may prevent or delay host cell death during viral replication<sup>66</sup>. It has also been reported that enhancing eIF2 $\alpha$  phosphorylation using Salubrinal, a small molecule inhibitor of eIF2 $\alpha$  phosphatases, increased translational inhibition, leading to reduced DENV production<sup>67</sup>. Overall, these data indicate that virus-mediated induction of the UPR is beneficial for viral production but must be controlled to prevent cell death.

### Autophagy and lipid metabolism

Autophagy is lysosomal degradation of cytoplasmic contents that is part of the normal cellular recycling of macromolecules as well as a cellular host response to starvation or stress<sup>68</sup>. Autophagy has been implicated as an innate immune response to trigger the death of pathogen-infected cells. A critical aspect of the autophagic pathway is the formation of cytosolic double-membrane vesicles, autophagosomes, that engulf portions of cytoplasmic material and facilitate fusion with lysosomes<sup>69</sup>. However, many positive-strand RNA viruses have evolved to benefit from the autophagic pathway by subverting autophagosome formation and using the machinery to enhance viral replication<sup>70</sup>.

DENV-2 was first found to activate autophagic machinery (Figure 2E), resulting in increased autophagosome formation along with enhanced viral replication<sup>71</sup> (Figure 3B). Further *in vitro* work found that both DENV-2 and -3 can inhibit autophagosome fusion with lysosomes<sup>72</sup> and that the autophagosomal membrane-bound microtubule-associated protein light chain 3 (LC3) co-localized with DENV dsRNA and NS1 in infected HepG2



cells, indicating that replicating virus can be found in autophagic vacuoles<sup>73</sup>. More recent work tested an inhibitor of autophagy, spautin-1, *in vitro* and in the AG129 mouse model, concluding that autophagic capabilities are required for infectious viral particle production<sup>74</sup>. Thus, DENV manipulates the autophagy pathway to increase viral replication while avoiding lysosomal degradation.

Lipid droplets are cellular storage vesicles containing triglycerides and cholesterol. As a part of normal lipid metabolism, cells degrade lipid droplets as needed to obtain fatty acids for metabolism or vesicle formation<sup>75</sup>. Lipid metabolism is linked to autophagy, as during autophagy lipid droplets are degraded and engulfed into autophagosomes. Therefore, the regulation of lipid metabolism by DENV was suggested to result from the induction of autophagy that can increase the degradation of lipid droplets and produce more fatty acid material for viral replication<sup>75</sup>. In addition, several studies have implicated a direct role for DENV in controlling lipid metabolism for enhanced viral replication. Using an siRNA library, the DENV NS3 protein was found to recruit fatty acid synthase (FASN) and increase fatty acid biosynthesis<sup>76</sup>. In another study, the N-terminus of DENV capsid (C) protein was shown to contain a lipid droplet-binding motif<sup>77</sup>. The ability to target the DENV C protein to lipid droplets may provide a scaffolding for infectious viral particle formation<sup>78</sup>. Heaton *et al.*<sup>79</sup> reported that DENV NS3 modulates host cellular fatty acid synthesis by recruiting fatty acid synthase to sites of DENV replication. The interaction between the NS3 and FASN is consistent with other studies in which DENV has been shown to modulate lipid metabolism by reabsorption of lipid droplets from host cells<sup>64,76</sup>. Thus, through careful manipulation of cellular processes, such as ER expansion, autophagy and lipid metabolism, DENV is able to sequester itself from the host's antiviral responses.

### sfRNA

RNA interference is an important host defense against viral infections. Recent studies have shown that DENV is able to interfere with RNAi pathways via two distinct mechanisms, involving NS4B and subgenomic flavivirus RNA (sfRNA) (Figure 3C). DENV infection is able to suppress RNAi pathways by expression of the nonstructural protein NS4B, which directly interferes with Dicer's ability to process small RNAs *in vitro*<sup>80</sup>. Utilizing a GFP reporter that is silenced by miRNA, Kakumani *et al.*<sup>80</sup> showed that NS4B was sufficient to increase GFP expression in mammalian, mosquito, and plant cell lines, thus providing evidence that NS4B is able to inhibit RNAi. These findings were confirmed by the use of NS4B mutants that were unable to modulate the RNAi pathway. In addition, DENV infection results in production of sfRNA from the 3' untranslated region of the viral genomic RNA. sfRNA is generated by stable secondary structures at the 5' end of the 3'UTR blocking the host 5'-3' exonuclease XRN1<sup>81</sup>. Flavivirus sfRNA has been shown to inhibit cleavage of dsRNA by Dicer by binding to and saturating the RNase. In a dose-dependent manner, increasing concentrations of sfRNA inhibited Dicer cleavage of long dsRNA into siRNAs<sup>82</sup>. In addition, the sfRNA directly interacts with XRN1, increasing overall messenger RNA stability within the host cell<sup>83,84</sup>, which could also benefit the viral RNA.

### Interference with innate signaling responses during ADE

During a secondary DENV infection, the virus can infect Fc $\gamma$ R-bearing cells via ADE. A series of *in vitro* experiments have shown that when human monocytic cell lines are infected by DENV under conditions of ADE, as compared to high-dose infection in the absence of antibody, the innate antiviral state of the cell is compromised. Specifically, uptake of DENV in complex with anti-DENV antibodies through Fc $\gamma$ RI and Fc $\gamma$ RIIa on THP-1 monocytic cells yielded enhanced infection as a result of down-regulation of both TLR gene expression and negative regulators of the NF $\kappa$ B pathway, as well as disruption of RIG-I and MDA5 signaling cascades, leading to decreased innate immune responses and increased viral replication<sup>85,86</sup> (Figure 3D). In addition, viral uptake through ADE enhanced anti-inflammatory cytokine production such as IL-10 from dendritic cells<sup>86,87</sup>. This phenomenon has been termed “intrinsic ADE”<sup>88</sup> and is still somewhat controversial. For instance, Kou *et al.*<sup>86</sup> showed that viral replication was increased during ADE in primary human monocytes in the absence of increased IL-10 production, indicating that mechanisms of antibody-dependent enhancement of infection are not yet fully understood.

### Interference with IFN- $\alpha/\beta$ induction

DENV non-structural proteins abrogate IFN- $\alpha/\beta$  production and signaling within infected cells via several distinct pathways. First, the 2'-O methylation of the viral mRNA cap structure by NS5 evades MDA5 recognition and host restriction via Ifit1-dependent and -independent mechanisms, blunting the IFN response against flaviviruses<sup>89,90,91</sup>. Second, DENV interferes with downstream signaling from RIGI and MDA5 by directly targeting human MITA/STING in this pathway to down-regulate the antiviral responses triggered upon viral infection. Recent reports have shown that expression of the DENV NS2B/3 protease directly cleaves MITA/STING, thereby disrupting IFN production<sup>92,93,94</sup> (Figure 3E). DENV NS2B/3 only recognizes human STING and not the murine homologue MPYS<sup>92,93</sup>. When human STING was engineered to contain the mouse sequence in the NS2B/3 cleavage site, IFN production was restored in DENV-infected cells<sup>93</sup>. Thus, NS2B/3 inhibits IFN- $\alpha/\beta$  induction in a species-specific manner.

### Interference with IFN- $\alpha/\beta$ signaling

Cells infected with DENV have been shown to be defective in responding to external treatment with IFN- $\alpha/\beta$ , indicating that DENV infection is able to prevent IFN- $\alpha/\beta$  signaling<sup>95,96,97</sup>. Signaling through the IFN- $\alpha/\beta$  receptor is mediated via the STAT1/2 signaling pathway and is an important component of the host cellular innate response to viral infection (Figure 4). DENV has evolved several mechanisms to interfere with this signaling pathway via inhibition of STAT1 phosphorylation as well as degradation of STAT2. DENV NS2A, NS4A and NS4B associate with cellular membranes and when expressed together have been shown to inhibit STAT1 phosphorylation and nuclear translocation in host cells<sup>95</sup> (Figure 4A). Expression of these three DENV non-structural proteins enhanced replication of  $\alpha/\beta$ -sensitive viruses in the presence of exogenous IFN- $\alpha/\beta$ <sup>95</sup>. In the related West Nile Virus (WNV), the sRNA has been implicated in viral immune evasion of the IFN- $\alpha/\beta$  pathway. WNV deficient in sRNA production was less virulent *in vitro* and *in vivo* when compared to wild-type virus. Interestingly, this phenotype



was rescued when the mutant virus was grown under Type I IFN-deficient conditions<sup>82</sup>, suggesting that sfRNA may play a role in modulating IFN- $\alpha/\beta$  responses during flaviviral infection.

Furthermore, DENV inhibits IFN- $\alpha/\beta$  signaling by triggering the degradation of STAT2. Initial studies observed reduced levels of STAT2 in cells expressing DENV replicons<sup>98</sup>. More recently, it has been shown that DENV NS5 mediates STAT2 degradation via the proteasome (Figure 4B). Mature NS5 was found to bind STAT2 but not to mediate protein degradation, as proteolytic cleavage of STAT2 required NS5 to be in the polyprotein form (Figure 4B). NS5 binds directly to the coiled-coil region in the first half of the human STAT2 protein<sup>99</sup> acting as a bridge between UBR-4 and STAT2, thus directing STAT2 toward the ubiquitination and proteasomal degradation pathway<sup>99,100</sup>. Interestingly, DENV is only able to degrade human STAT2, but not mouse STAT2<sup>101</sup>. Thus, these DENV non-structural proteins enable specific evasion of human IFN- $\alpha/\beta$  receptor signal transduction.

Finally, the ability of DENV to perturb the host's innate immune response may impact the adaptive immune response and modulate disease outcome. Though it is difficult to determine the direct effect of the reduced innate responses, dengue patients from Vietnam and Nicaragua with more severe disease (DSS) had decreased expression levels of IFN- $\alpha/\beta$ -stimulated genes<sup>102,103,104</sup>. DENV infection of monocytes, macrophages and dendritic cells may also affect the quality and quantity of T cells, as T cells from patients infected with DENV exhibited reduced proliferation *in vitro*<sup>105</sup>.

## Conclusions

DENV is an RNA virus that triggers innate antiviral immune mechanisms within infected cells that should protect the cell from viral replication and warn surrounding cells of the pathogen's presence. In response, DENV subverts the innate antiviral responses by sequestering itself and manipulating cellular machinery and signaling pathways. DENV controls cellular processes in order to delay cell death, inhibit RNAi pathways, and provide a niche hidden from intracellular innate RNA sensors. In addition, non-structural DENV proteins inhibit the induction and signaling cascade of IFN- $\alpha/\beta$ , thus preventing cellular pathways that lead to the expression of interferon response genes and antiviral mechanisms. Recent and ongoing research continues to uncover a growing number of unique mechanisms used by DENV to outsmart the host innate immune response.

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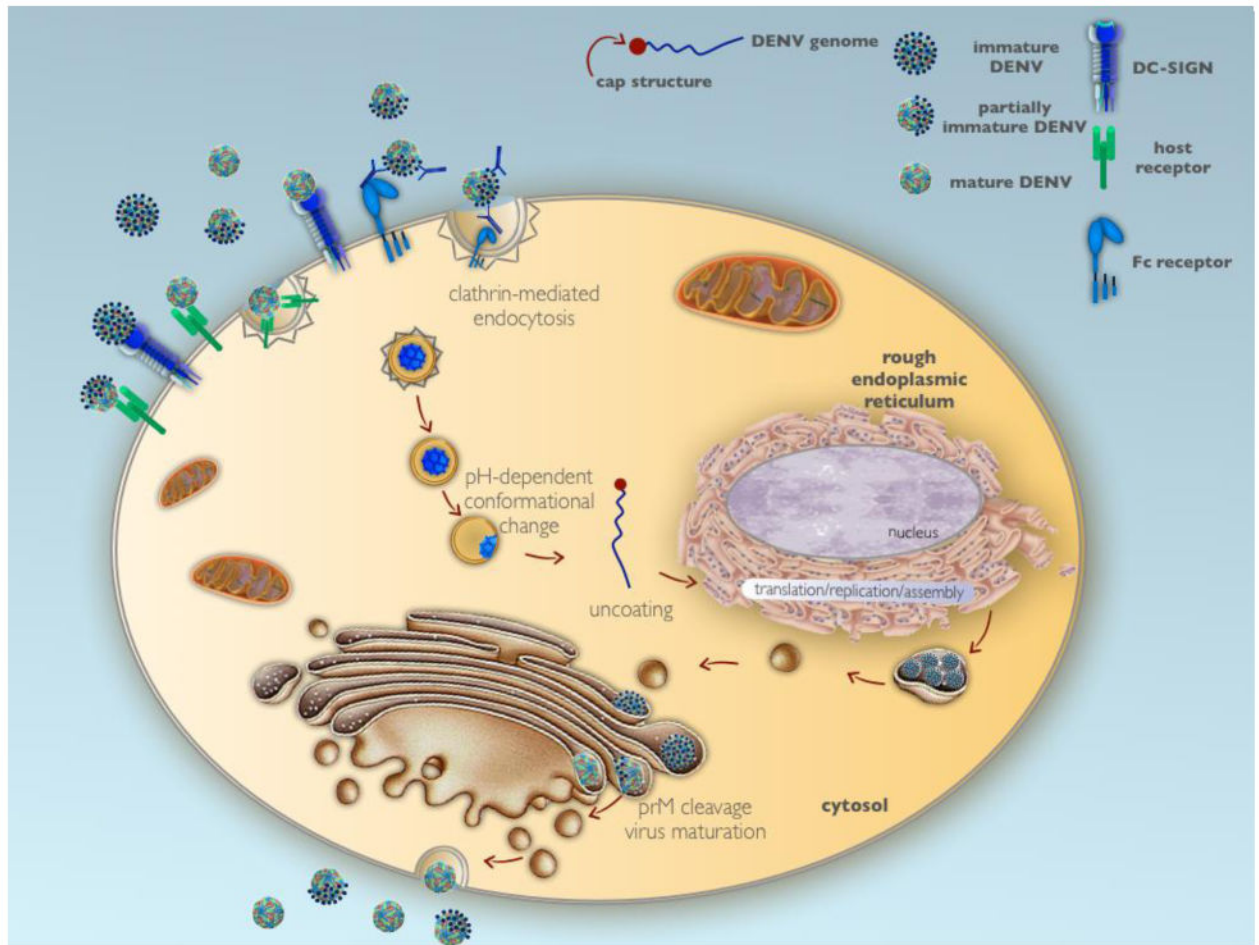
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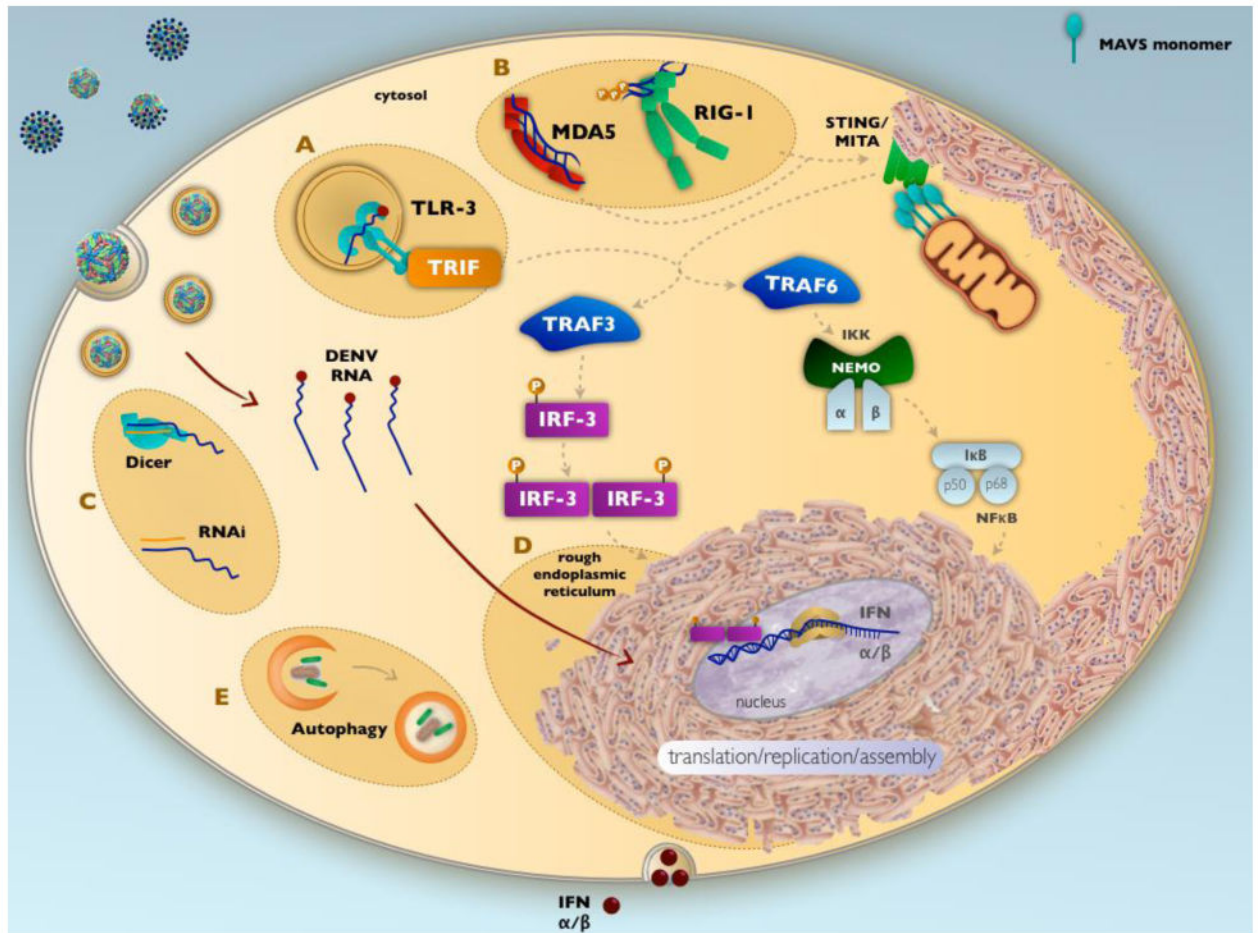
### Highlights

1. Dengue virus infection triggers numerous intracellular innate immune responses
2. DENV avoids intracellular detection via sequestration of viral replication in the ER
3. DENV manipulates cellular signaling cascades to inhibit innate antiviral responses



### Figure 1. Dengue virus life cycle

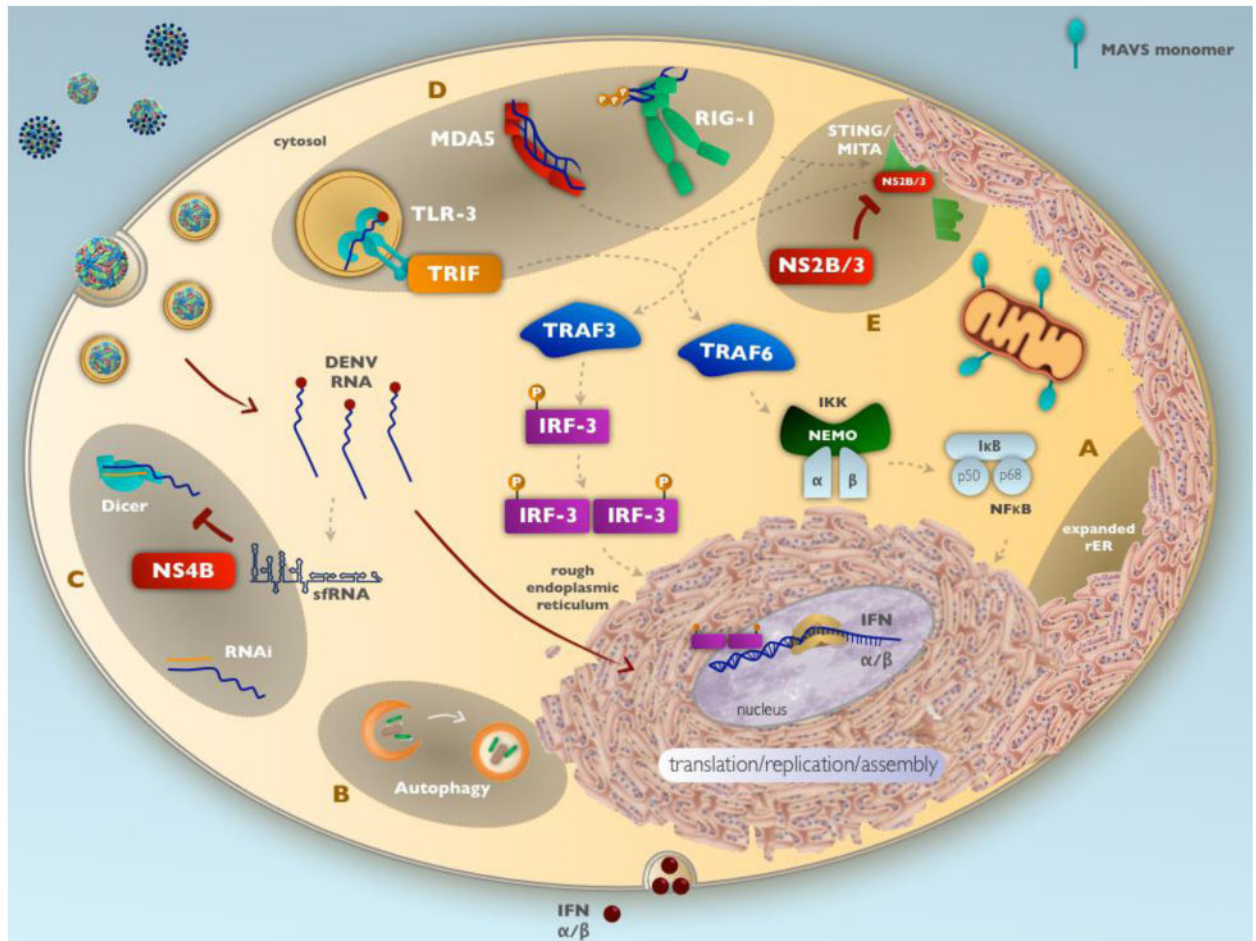
Dengue virus infects host cells via receptor-mediated endocytosis, and following vesicle acidification and conformational changes in the viral prM/M and E proteins, the viral RNA is uncoated and released into the cytosol. The viral RNA migrates to the endoplasmic reticulum where its translation, replication and assembly occur. Packaged virions are transported to the Golgi, where they undergo proteolytic cleavage that results in virus maturation and are then exported from the cell using the host secretory system.



**Figure 2. Host innate immune response to dengue virus infection**

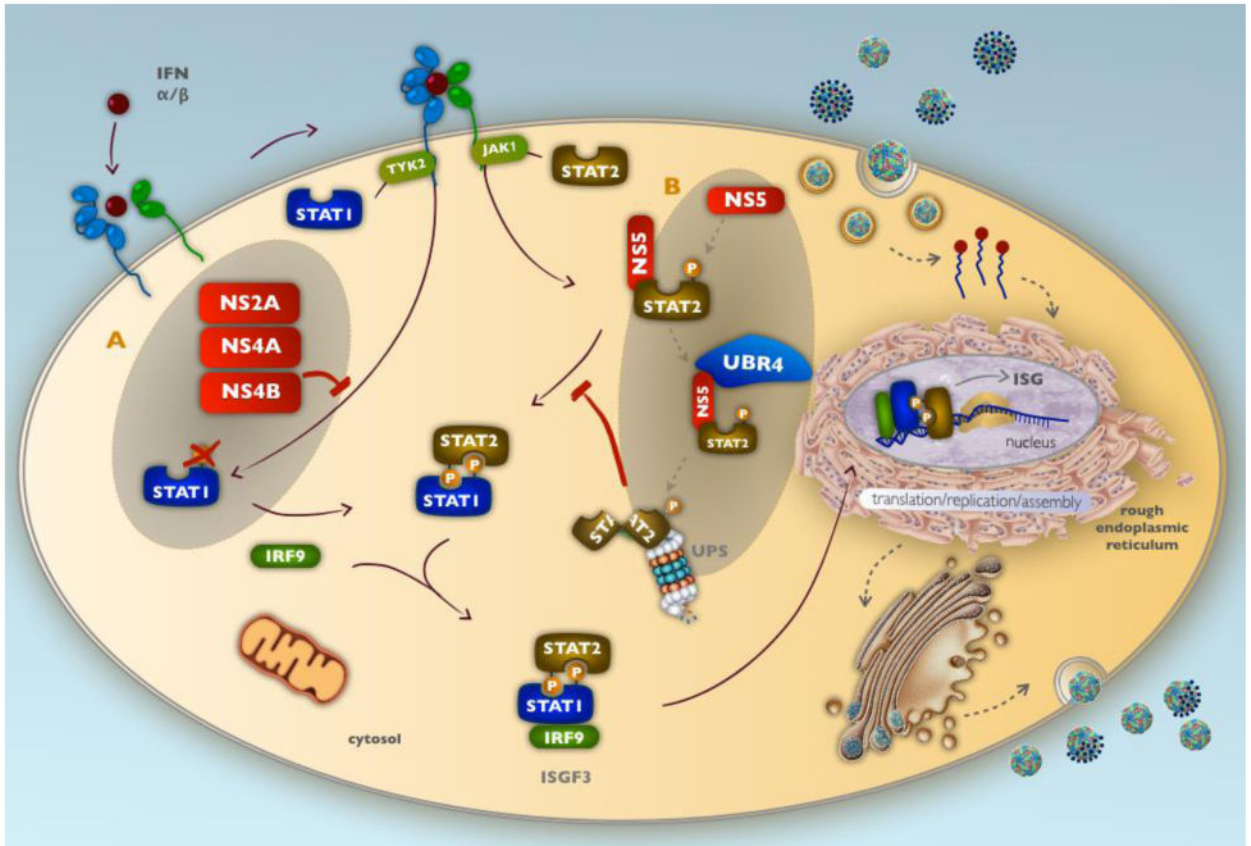
DENV RNA is recognized by pattern recognition receptors TLR-3 (A) and MDA5 and RIG-I (B), resulting in a signaling cascade that induces IFN- $\alpha/\beta$  production. Viral infection induces the host RNAi pathway (C) to control viral RNA replication; hypertrophy of the ER (D), where viral translation and replication occurs, and autophagy (E) to balance cellular stress and prevent apoptosis.





### Figure 3. Dengue virus subversion of host innate immune responses

DENV manipulates host cellular processes and innate immune responses within infected cells by A) manipulating the unfolded protein response (UPR) to counter cellular stress and delay apoptosis. B) To offset ER stress and access fatty acid metabolism networks, DENV also initiates autophagy, which has shown to be necessary for production of infectious virions. C) The DENV non-structural protein NS4B and subgenomic sfRNA interfere with the RNAi pathway by inhibiting the RNase Dicer. D) During heterotypic secondary DENV infection, subneutralizing antibodies can enable viral uptake through FcγR and down-regulate signaling cascades initiated via the pattern recognition receptors TLR3 and MDA5/RIG-I, thus reducing the antiviral state of the cell. E) The DENV NS2B/3 protein cleaves human STING/MITA, interfering with the signaling pathway leading to induction of IFN $\alpha/\beta$ .



**Figure 4. Dengue virus interference with interferon- $\alpha/\beta$  signaling**

DENV non-structural proteins impede IFN- $\alpha/\beta$  signaling in virally infected cells. A) NS2A, NS4A, NS4B complex together to block STAT1 phosphorylation, nuclear translocation, and transcriptional activation of ISGs. B) DENV NS5 binds and degrades human STAT2, targeting it toward the proteasomal degradation pathway, thus preventing formation of the STAT1/STAT2 heterodimer and its transcriptional induction of ISGs.