

# Dengue Virus NS Proteins Inhibit RIG-I/MAVS Signaling by Blocking TBK1/IRF3 Phosphorylation: Dengue Virus Serotype 1 NS4A Is a Unique Interferon-Regulating Virulence Determinant

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**ABSTRACT** Dengue virus (DENV) replication is inhibited by the prior addition of type I interferon or by RIG-I agonists that elicit RIG-I/MAVS/TBK1/IRF3-dependent protective responses. DENV infection of primary human endothelial cells (ECs) results in a rapid increase in viral titer, which suggests that DENV inhibits replication-restrictive RIG-I/interferon beta (IFN- $\beta$ ) induction pathways within ECs. Our findings demonstrate that DENV serotype 4 (DENV4) nonstructural (NS) proteins NS2A and NS4B inhibited RIG-I-, MDA5-, MAVS-, and TBK1/IKK $\epsilon$ -directed IFN- $\beta$  transcription (>80%) but failed to inhibit IFN- $\beta$  induction directed by STING or constitutively active IRF3-5D. Expression of NS2A and NS4B dose dependently inhibited the phosphorylation of TBK1 and IRF3, which suggests that they function at the level of TBK1 complex activation. NS2A and NS4B from DENV1/2/4, as well as the West Nile virus NS4B protein, commonly inhibited TBK1 phosphorylation and IFN- $\beta$  induction. A comparative analysis of NS4A proteins across DENVs demonstrated that DENV1, but not DENV2 or DENV4, NS4A proteins uniquely inhibited TBK1. These findings indicate that DENVs contain conserved (NS2A/NS4B) and DENV1-specific (NS4A) mechanisms for inhibiting RIG-I/TBK1-directed IFN responses. Collectively, our results define DENV NS proteins that restrict IRF3 and IFN responses and thereby facilitate DENV replication and virulence. Unique DENV1-specific NS4A regulation of IFN induction has the potential to be a virulence determinant that contributes to the increased severity of DENV1 infections and the immunodominance of DENV1 responses during tetravalent DENV1-4 vaccination.

**IMPORTANCE** Our findings demonstrate that NS2A and NS4B proteins from dengue virus serotypes 1, 2, and 4 are inhibitors of RIG-I/MDA5-directed interferon beta (IFN- $\beta$ ) induction and that they accomplish this by blocking TBK1 activation. We determined that IFN inhibition is functionally conserved across NS4B proteins from West Nile virus and DENV1, -2, and -4 viruses. In contrast, DENV1 uniquely encodes an extra IFN regulating protein, NS4A, that inhibits TBK1-directed IFN induction. DENV1 is associated with an increase in severe patient disease, and added IFN regulation by the DENV1 NS4A protein may contribute to increased DENV1 replication, immunodominance, and virulence. The regulation of IFN induction by nonstructural (NS) proteins suggests their potential roles in enhancing viral replication and spread and as potential protein targets for viral attenuation. DENV1-specific IFN regulation needs to be considered in vaccine strategies where enhanced DENV1 replication may interfere with DENV2-4 seroconversion within coadministered tetravalent DENV1-4 vaccines.

Received 2 April 2015 Accepted 7 April 2015 Published 12 May 2015

**Citation** Dalrymple NA, Cimica V, Mackow ER. 2015. Dengue virus NS proteins inhibit RIG-I/MAVS signaling by blocking TBK1/IRF3 phosphorylation: dengue virus serotype 1 NS4A is a unique interferon-regulating virulence determinant. *mBio* 6(3):e00553-15. doi:10.1128/mBio.00553-15.

**Editor** Michael J. Buchmeier, University of California, Irvine

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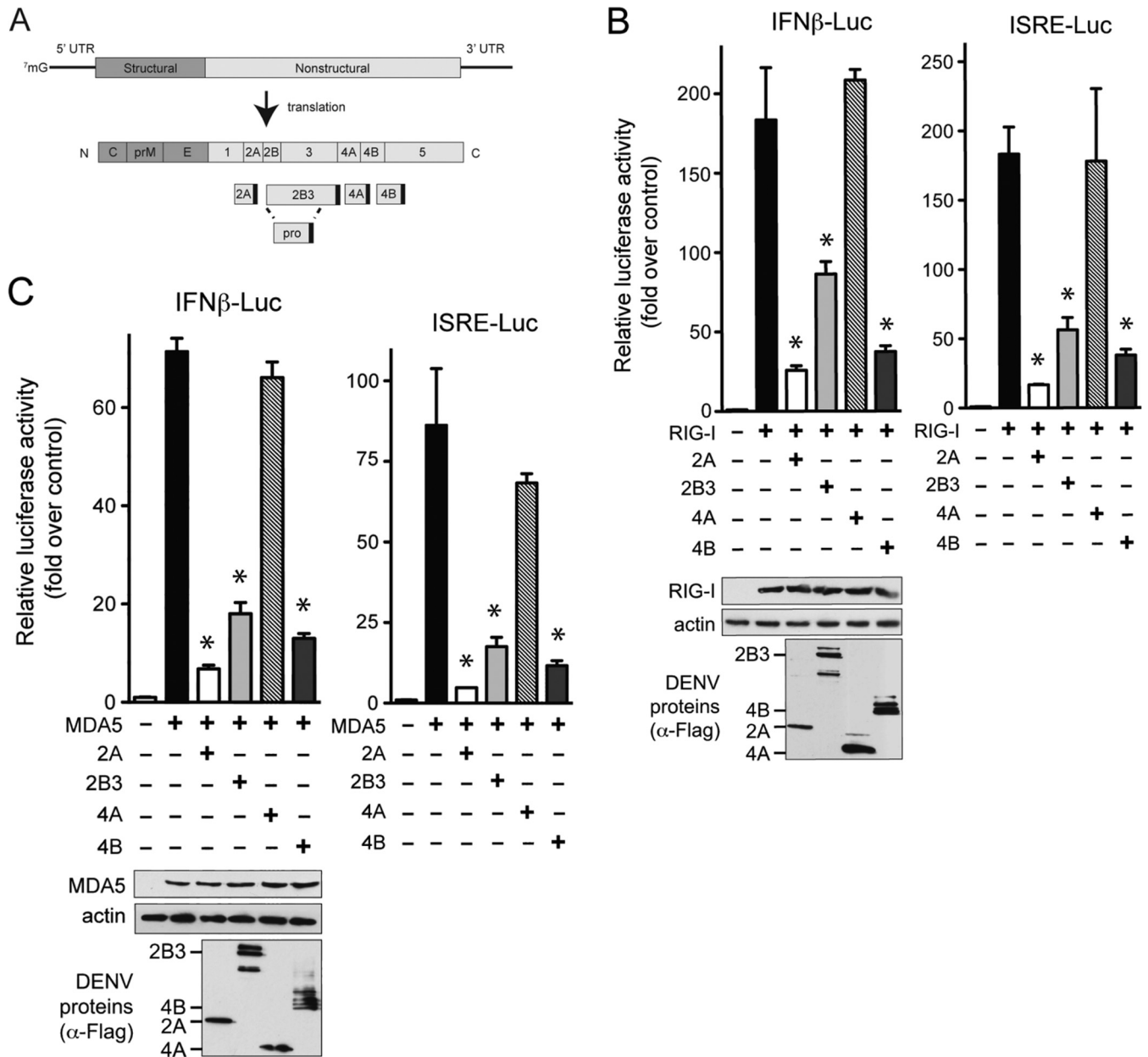
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This article is a direct contribution from a Fellow of the American Academy of Microbiology.

Dengue viruses (DENVs) are members of the *Flaviviridae* family and are transmitted to humans by *Aedes aegypti* mosquitoes (1). DENVs infect 50 to 100 million individuals each year primarily causing dengue fever (DF) (2). There are four discrete DENV serotypes (DENV1-4), and following infection by a second dengue serotype, ~1% of DENV infections result in more-severe disease: dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (2–7). There are currently no viable dengue virus therapeutics, and the mechanisms by which DENVs cause vascular leakage remain to be defined. Protection from DENV disease is focused on developing a tetravalent DENV1-4 vaccine that elicits protection against all four serotypes and prevents more severe disease resulting from exposure to a second DENV serotype (2,

7–13). In this context, individual DENV serotypes can be immunodominant when coadministered and cause antagonistic seroconversion responses that challenge the generation of serotypically balanced immunity to tetravalent vaccination (2, 8, 14).

DENVs have an 11-kb positive-stranded RNA genome that synthesizes a single cotranslationally cleaved polyprotein encoding three structural proteins (capsid, envelope, and prM) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Fig. 1A) (1, 15). Structural proteins distinguish viral serotypes and direct viral attachment and entry (1). Nonstructural proteins are essential for viral replication and largely conserved across DENV serotypes. DENVs infect immune and dendritic cells as well as human endothelial cells (ECs) (16–



**FIG 1** NS2A and NS4B antagonize RIG-I/MDA5-directed type I IFN induction. (A) Schematic of DENV polyprotein, indicating structural and nonstructural (NS) proteins produced after cleavage by host and viral proteases. Full-length (uncleaved) and pro (active) forms of the viral 2B3 protease are depicted. UTR, untranslated region. (B and C) HEK293T cells were cotransfected with identical amounts of total DNA, including IFN- $\beta$  promoter or ISRE-driven firefly luciferase (Luc) reporters, *Renilla* luciferase plasmid, and the indicated RIG-I-CARD or MDA5 expression vectors in the presence (+) or absence (-) of plasmids expressing Flag-tagged DENV4 NS2A, NS2B3, NS4A, NS4B, or empty control plasmid (11). Luciferase activity was measured 24 h posttransfection, normalized to *Renilla* luciferase activity, and reported as fold increase compared to controls lacking RIG-I-CARD or MDA5. Assays were performed in duplicate with similar results from at least three separate experiments. Expression of dengue virus nonstructural proteins and inducers was assessed by Western blot analysis using anti-Flag ( $\alpha$ -Flag) and anti-RIG-I or anti-MDA5.  $\beta$ -Actin serves as a loading control. Asterisks indicate statistical significance ( $P < 0.05$ ) as determined by Student's *t* test.

18), which are the ultimate targets of fluid barrier dysfunction in DHF and DSS disease (19). DENV4 infection of human ECs *in vitro* is productive, resulting in a rapid increase in viral titers 12 to 24 h postinfection (hpi) but with little additional virus production or viral spread at later time points (20, 21). Analysis of EC responses to DENV4 infection revealed the induction of interferon beta (IFN- $\beta$ ) and IFN-stimulated genes (ISGs) 24 and 48 hpi, and

viral spread was conferred by the addition of blocking IFN- $\beta$  antibodies to the medium (21). In contrast, IFN- $\beta$  and ISG responses are absent 12 hpi, suggesting that DENV inhibits the early induction of IFN responses in order to productively replicate in ECs (22). DENV infection of ECs may contribute to viremia and viral dissemination as well as provide targets for immune-enhanced vascular permeability.

In the cytoplasm, RNA virus replication generates 5' triphosphorylated RNA that is detected by constitutively expressed RIG-I (retinoic acid-inducible gene 1) sensors and triggers MAVS (mitochondrial antiviral signaling protein)/TRAF3 (tumor necrosis factor receptor-associated factor 3) activation of TANK-binding kinase 1 (TBK1) (22–25). TBK1 phosphorylates interferon regulatory factor 3 (IRF3) and activates NF- $\kappa$ B to transcriptionally induce type I IFN (23, 26–29). IRF3 binds IFN-stimulated response elements (ISREs) to induce additional antiviral genes (28, 30–32). Secreted interferon alpha/beta (IFN- $\alpha/\beta$ ) binds cellular IFN receptors (IFNAR) activating downstream JAK (Janus kinase)-STAT (signal transducer and activator of transcription factor) signaling pathways and inducing the transcription of antiviral ISGs.

RNA viruses regulate RIG-I-directed IFN induction in order to successfully replicate and spread. NS proteins from discrete flaviviruses have been shown to restrict type I IFN induction or cellular responses to IFN- $\alpha/\beta$  addition. The hepatitis C virus (HCV) NS2 protease as well as Kunjin virus and West Nile virus (WNV) NS2A proteins reportedly inhibit IFN induction induced by TBK1/IKK $\epsilon$  (I $\kappa$ B kinase  $\epsilon$ ) expression or Sendai virus (SeV) infection (33–35). The HCV NS3/4A protease and NS4B proteins regulate IFN induction by cleaving MAVS and STING, respectively (36–42).

DENV replication is also inhibited by the prior addition of IFN- $\alpha/\beta$ , and preventing early IFN induction is critical to DENV replication *in vitro* and *in vivo* (43–46). DENV infections are inhibited by expression of selected ISGs, including IFITM2/3, viperin, and ISG20 (47). To counteract this, DENV NS2A, NS4A, and NS4B proteins inhibit STAT1 activation, nuclear translocation, and ISG induction in response to IFN- $\beta$  addition (45, 48, 49).

Similar to responses in ECs, DENV also inhibits the induction of IFN- $\alpha/\beta$  in dendritic cells at early times postinfection but induces IFN by 36 to 48 hpi (50). Expression of DENV2 NS2B3 protein reduced IFN- $\beta$  transcription 30 to 50% following SeV or poly(I-C) induction (51). Similar to the HCV NS4B protein, DENV NS2B3 cleaves STING and restricts STING-directed IFN- $\beta$  induction (41, 42, 52, 53). A recent report indicates that RIG-I agonists induce pathway-dependent protective responses that specifically require RIG-I/MAVS/TBK1/IRF3 signaling effectors to inhibit DENV infection (54). RIG-I/MAVS/TBK1/IRF3 inhibition was largely independent of type I IFN (54), suggesting a fundamental role of TBK1/IRF3-directed responses in restricting DENV infection. Although DENV2 proteins reportedly fail to inhibit IFN responses induced by robust Sendai virus infections (49, 50), there is no indication of which DENV proteins regulate pathway-specific RIG-I/MAVS/TBK1-directed ISRE and IFN transcriptional responses.

In the present study, we investigate the ability of DENV NS2A, NS4A, NS2B3, and NS4B to regulate the ISRE and IFN- $\beta$  induction following RIG-I/TBK1 signaling pathway activation. We demonstrate that NS2A and NS4B, but not NS4A, regulate IFN transcriptional responses induced by RIG-I, MDA5 (melanoma differentiation-associated protein 5), MAVS, TBK1, or IKK $\epsilon$  but not by constitutively active IRF3-5D. NS2A and NS4B inhibited TBK1 and IRF3 phosphorylation, suggesting that regulation occurs at the level of TBK1 complex activation. The WNV NS4B similarly inhibited TBK1 phosphorylation, suggesting the conservation of NS4B function across flaviviruses. Importantly, we found that the NS4A protein from DENV1, but not DENV2/4,

uniquely inhibited TBK1-directed IFN- $\beta$  transcription. This suggests that NS4A is an additional DENV1-specific inhibitor of IFN signaling pathways. These findings functionally define new DENV NS proteins that antagonize RIG-I/TBK1 signaling pathways and suggest potential differences in IFN regulation by discrete DENV serotypes. Our results suggest the potential for NS2A and NS4B DENV proteins to synergistically foster robust early DENV replication within cells and for DENV1 to contain a novel IFN-regulating determinant that may enhance its replication and virulence.

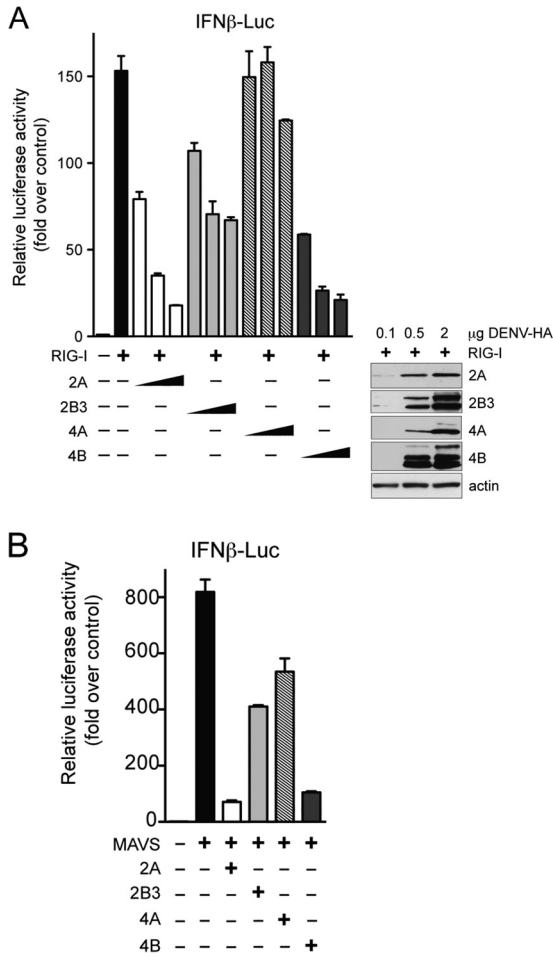
## RESULTS

**NS2A and NS4B regulate RIG-I/MDA5-directed pathway responses.** DENV infection of primary human endothelial cells results in the rapid production of  $1 \times 10^5$  virions 24 h postinfection (hpi), identical to replication in IFN-deficient VeroE6 cells (20, 21). IFN pretreatment of ECs inhibits DENV infection, and DENV spread is restricted by the late induction of IFN- $\beta$  and permitted by the addition of neutralizing anti-IFN- $\beta$  antibodies to endothelial cell supernatants (21). Recent studies indicate that 5' triphosphorylated RNA induces protective responses against DENV by activating signaling pathways dependent on RIG-I/MAVS/TBK1/IRF3 effectors (54). Collectively, these findings suggest that DENV restricts early IFN induction and IRF3-directed ISRE transcriptional responses to permit viral replication and spread.

Little is known about how DENV proteins regulate canonical RIG-I/MDA5/TBK1/IRF3-directed signaling responses. Here we define DENV4 NS proteins that inhibit pathway-specific RIG-I/MDA5 signaling inducers of ISRE and IFN- $\beta$  promoter transcriptional responses. NS2A, NS2B3, NS4A, and NS4B proteins were expressed in HEK293T cells with ISRE or IFN- $\beta$  promoter luciferase reporters in the presence or absence of plasmids expressing RIG-I-CARD (caspase activation and recruitment domain) or MDA5. We found that NS2A and NS4B dramatically inhibited transcription from IFN- $\beta$ - and ISRE-containing promoters (>80%) directed by RIG-I-CARD (Fig. 1B) and MDA5 (Fig. 1C). NS2B3, which acts on STING (52, 53), also partially reduced RIG-I-CARD- and MDA5-induced transcription. DENV4 NS4A had no effect on pathway activation by RIG-I or MDA5 (Fig. 1B and C), even when increasing amounts of protein were expressed (Fig. 2A). In contrast, NS2A and NS4B inhibited RIG-I-directed IFN- $\beta$  transcription in a dose-dependent manner (>85%) (Fig. 2A).

MAVS is a downstream effector of RIG-I and MDA5 activation, and MAVS activates TBK1 by recruiting TRAF3-TBK1 complexes to the mitochondrion (31, 55). We observed that NS2A and NS4B robustly inhibited MAVS-induced IFN- $\beta$  transcription 85 to 90% (Fig. 2B), while NS2B3 reduced only MAVS-directed IFN- $\beta$  induction ~50% (Fig. 2B). These novel observations indicate that NS2A and NS4B efficiently inhibit IFN- $\beta$  induction (>85%) and define DENV NS2A and NS4B proteins as inhibitors of RIG-I/MDA5/MAVS-directed signaling pathway responses.

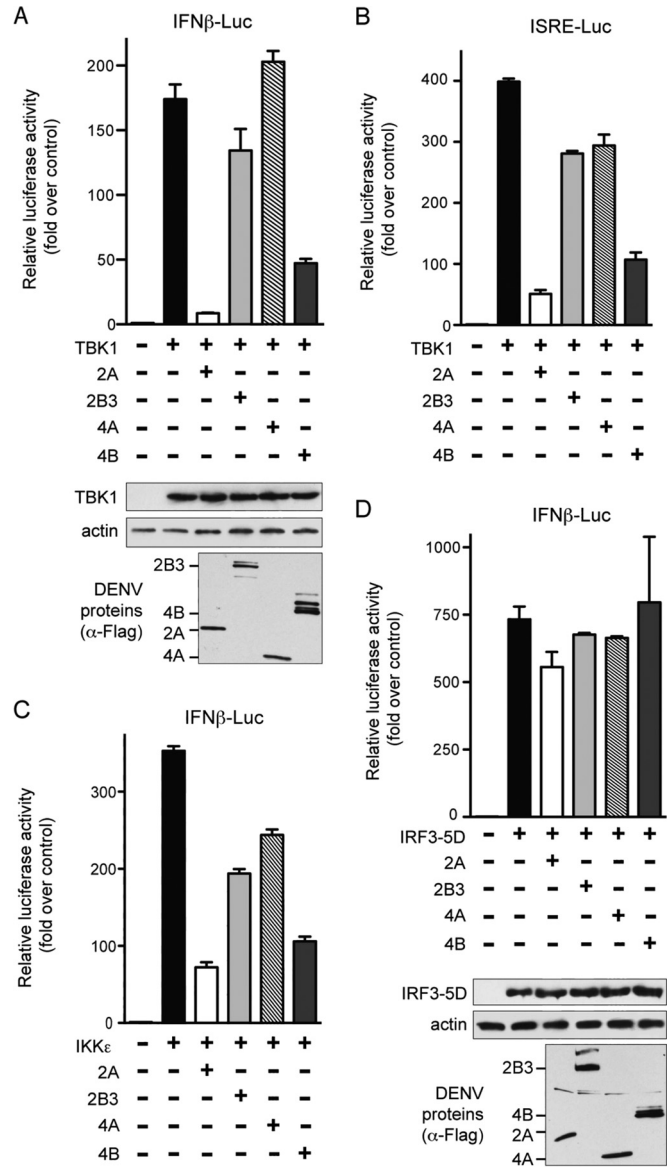
**NS2A and NS4B inhibit TBK1- and IKK $\epsilon$ -directed signaling.** MAVS activation of constitutively expressed TBK1, or its induced homologue IKK $\epsilon$ , directs IRF3/7 phosphorylation and transcription from ISRE and IFN- $\beta$  promoters (31, 32). Using TBK1 and IKK $\epsilon$  as downstream pathway activators, we observed that both NS2A and NS4B inhibited transcription from ISRE and IFN- $\beta$  promoters directed by TBK1 (Fig. 3A and B) and IKK $\epsilon$  (Fig. 3C).



**FIG 2** NS2A and NS4B dose dependently inhibit RIG-I- and MAVS-directed IFN induction. HEK293T cells were cotransfected as described in the legend to Fig. 1 with IFN-β promoter firefly luciferase reporter, *Renilla* luciferase plasmid, and RIG-I-CARD (A) or MAVS (B) expression vectors in the presence or absence of the indicated DENV NS expression plasmids (0.1, 0.5, or 2 μg [indicated by the black triangle] [A]) or control empty plasmid (2 μg) [B]. Luciferase activity was assessed and evaluated as described in the legend to Fig. 1, and expression of DENV4 NS proteins was determined by Western blot analysis using anti-HA with β-actin as a loading control (11).

In contrast, neither NS4A nor NS2B3 efficiently inhibited TBK1- or IKKε-directed transcriptional responses. Using the constitutively active IRF3 phosphomimetic, IRF3-5D, as a downstream inducer, we found no inhibition of IFN-β transcriptional responses by coexpressed NS2A, NS2B3, NS4A, or NS4B (Fig. 3D). These findings indicate that NS2A and NS4B inhibit pathway-specific signaling responses upstream of IRF3 activation at the level of TBK1/IKKε signaling complexes.

The DENV2 NS2B3 protease was previously shown to reduce type I interferon production ~50% by binding and cleaving STING (52, 53). To determine whether the DENV4 NS2B3 protein inhibits STING similar to DENV2 NS2B3, we assayed the ability of full-length NS2B3 and pro-NS2B3 (active, cleaved form) to inhibit STING-directed IFN-β induction. We also comparatively analyzed the ability of NS2A, NS4A, and NS4B proteins to induce STING cleavage. We found that only NS2B3 and active pro-NS2B3 significantly inhibited STING-induced IFN-β pro-

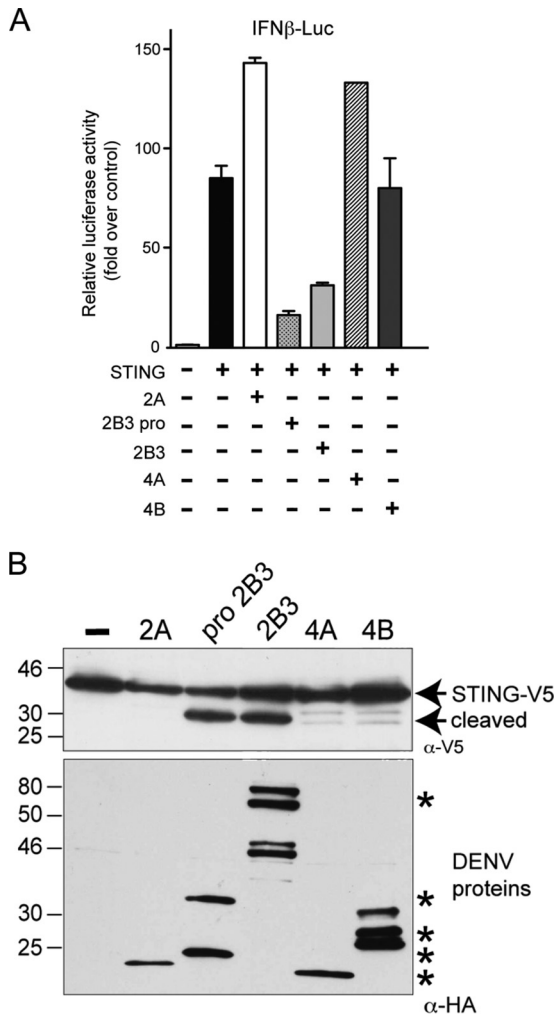


**FIG 3** NS2A and NS4B inhibit TBK1- and IKKε-directed IFN-β transcription. HEK293T cells were cotransfected as described in the legend to Fig. 1 in the presence or absence of TBK1 (A and B), IKKε (C), or IRF3-5D (D) and the indicated DENV4 NS expression plasmids. Luciferase activity was measured 24 h posttransfection and reported as described in the legend to Fig. 1. Assays were performed in duplicate with similar results from at least three separate experiments. Expression of DENV NS proteins and inducers was assessed by Western blot analysis using anti-Flag and anti-TBK1, anti-IKKε, or anti-IRF3 antibodies, respectively, with β-actin as a loading control.

moter transcription (>75%) (Fig. 4A). Figure 4B demonstrates that DENV4 NS2B3 cleaved STING similar to DENV2 NS2B3 (52, 53). This suggests a conserved NS2B3 innate immune regulatory mechanism that acts on STING, but not TBK1-directed IFN-β induction. Collectively, these findings demonstrate that NS2B3 selectively inhibits STING, while NS2A and NS4B proteins specifically inhibit TBK1/IKKε-directed IFN signaling pathways independent of STING.

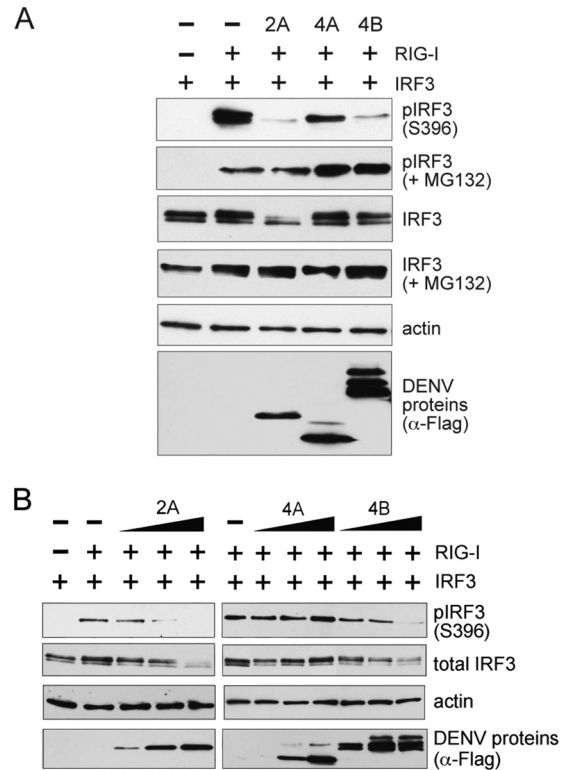
**NS2A and NS4B inhibit TBK1 autophosphorylation and IRF3 phosphorylation.** TBK1 activation results from its K<sup>63</sup>-





**FIG 4** NS2A and NS4B fail to inhibit the induction of IFN- $\beta$  by STING. (A) HEK293T cells were cotransfected as described in the legend to Fig. 1 with luciferase reporters and the STING-V5 expression plasmid as activator in the presence or absence of indicated plasmids expressing DENV4 NS2A, NS2B3pro (active, processed form), NS2B3 (full length), NS4A, or NS4B. Luciferase activity was measured 24 h posttransfection and reported as fold increase over the value for the empty plasmid control. Assays were performed in duplicate with similar results from at least three separate experiments. (B) HEK293T cells were transfected with plasmid expressing STING-V5 and either empty plasmid (-) or plasmid expressing the indicated DENV4 NS proteins. Cell lysates were analyzed 24 h posttransfection by Western blot analysis of STING-V5 expression using anti-V5 and DENV proteins using anti-HA. The positions of the full-length (36 kDa) and cleaved forms (32 kDa) of STING-V5 are indicated by the arrows to the right of the blots. Asterisks to the right of the Western blot denote expression of each DENV NS protein.

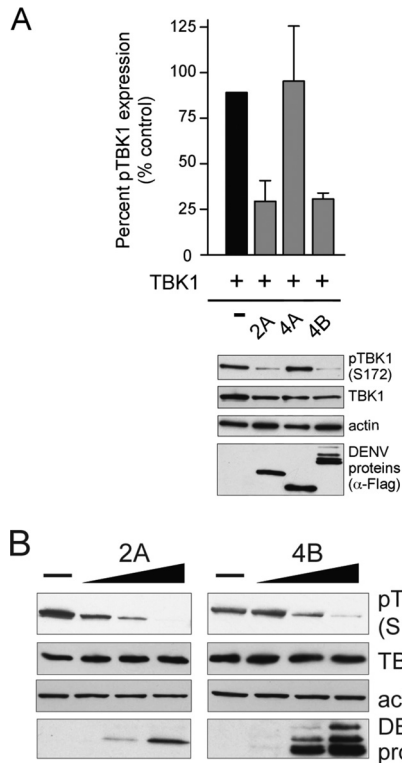
linked ubiquitination and recruitment to complexes that direct TBK1 autophosphorylation (Ser172) (24, 27). Activated TBK1 phosphorylates IRF3 and permits phosphorylated IRF3 (pIRF3)-directed transcription from ISRE-containing promoters (22). To determine whether NS2A and NS4B inhibit TBK1 and IRF3 phosphorylation, we coexpressed NS2A, NS4A, or NS4B proteins and analyzed TBK1 and IRF3 phosphorylation. We observed that NS2A and NS4B, but not NS4A, inhibited IRF3 phosphorylation (S396) (Fig. 5A) and that inhibition was dose dependent (Fig. 5B). Interestingly, both NS2A and NS4B also moderately decreased



**FIG 5** NS2A and NS4B block RIG-I-induced IRF3 phosphorylation. (A) HEK293T cells were cotransfected with IRF3-T7 expression plasmid, and as indicated, RIG-I-CARD expression plasmid, plasmids expressing DENV4 NS2A, NS4A, or NS4B, or empty vector. Cells were harvested 24 h posttransfection and analyzed by Western blotting for pIRF3 (anti-pIRF3 Ser396), total IRF3 (anti-IRF3),  $\beta$ -actin (anti- $\beta$ -actin), and DENV4 NS protein expression (anti-Flag). IRF3 levels were similarly analyzed in identical samples that were treated with MG132 (20  $\mu$ M) 5 h prior to harvest.  $\beta$ -Actin serves as a loading control. (B) Cells were cotransfected and analyzed as described in the legend to Fig. 6A with increasing amounts of indicated NS protein expression plasmid (0.5, 1, or 2  $\mu$ g).

total IRF3 levels, suggesting the potential for NS2A and NS4B to increase proteasome-mediated IRF3 degradation. Consistent with this, addition of the proteasomal inhibitor MG132 increased both total IRF3 and pIRF3 levels in the presence of NS2A and NS4B (Fig. 5A, +MG132). Similar to inhibition of IRF3 phosphorylation, expression of either NS2A or NS4B, but not NS4A, resulted in a dramatic decrease in phosphorylated TBK1 (phosphorylated S172 [pS172]) (Fig. 6A). Increasing NS2A or NS4B expression also resulted in a concomitant decrease in phospho-TBK1 levels; however, there was no effect on total TBK1 levels (Fig. 6B). These results suggest that NS2A and NS4B inhibit autophosphorylation of TBK1 and thereby prevent TBK1 activation and downstream IRF3 phosphorylation.

**The N-terminal domain of DENV4 NS4B directs RIG-I/TBK1 regulation.** We found that DENV4 NS4B regulates RIG-I and TBK1, but not STING, signaling responses. In order to define regulatory elements within NS4B, we evaluated IFN regulation by N- and C-terminal domains of NS4B (NS4B- $\Delta$ 118-260 and NS4B- $\Delta$ 1-117, respectively). We found that the NS4B N terminus (NS4B- $\Delta$ 118-260) uniquely inhibits RIG-I or TBK1 induction of IFN- $\beta$ , while the C-terminal domain had no effect on IFN- $\beta$  induction (Fig. 7A). These findings suggest that functional determi-

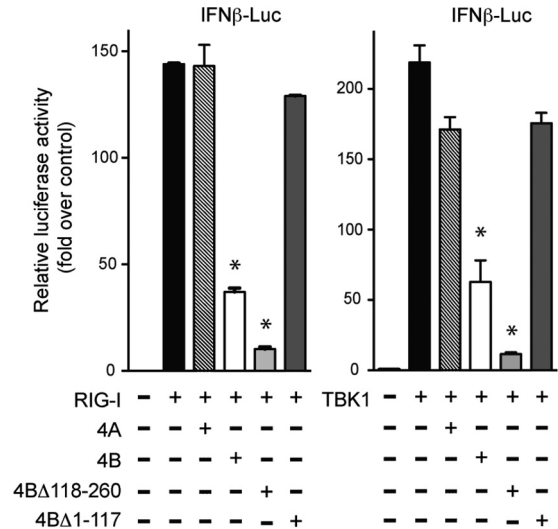


**FIG 6** NS2A and NS4B dose dependently inhibit TBK1 phosphorylation. (A) Plasmids expressing TBK1 and DENV4 NS2A, NS4A, and NS4B, as indicated, were cotransfected into HEK293T cells. Cells were harvested 24 h posttransfection and analyzed for pTBK1 (anti-pTBK1 Ser172), total TBK1 (anti-TBK1),  $\beta$ -actin (anti- $\beta$ -actin), and DENV4 NS protein (anti-Flag) expression by Western blot analysis. Phosphorylated TBK1 expression levels were measured from three replicate Western blot analyses and graphed as a percentage of control levels in the absence of DENV protein expression. (B) Increasing amounts of NS2A or NS4B plasmid (0.5, 1, or 2  $\mu$ g) were cotransfected with TBK1 expression plasmid and analyzed as described above for panel A.

nants within N-terminal NS4B domains regulate RIG-I/TBK1 signaling pathways.

**Conserved IFN inhibition by West Nile virus NS4B.** The NS4B protein from West Nile virus (WNV) has thus far only been implicated in regulating signaling pathways downstream of the IFN receptor (56), and WNV NS4B is only 40% identical to the DENV4 protein. To determine whether WNV NS4B regulates IFN induction similar to DENV proteins, we synthesized the WNV (NY99 strain) NS4B coding sequence (GenBank accession no. AF260967) and assayed the ability of the expressed WNV NS4B to regulate RIG-I-induced IFN- $\beta$  transcription. As shown in Fig. 8A and B, WNV NS4B blocked both RIG-I- and TBK1-induced IFN- $\beta$  induction >90%. Similar to DENV proteins, WNV NS4B also inhibited TBK1 autophosphorylation without altering total TBK1 levels (Fig. 8C). These findings demonstrate a conserved function of the WNV NS4B protein that inhibits IFN- $\beta$  induction by blocking TBK1 phosphorylation and activation.

**Dengue virus serotype-specific regulation of RIG-I signaling.** Although there is little understanding of serotype-specific differences in IFN regulation between DENVs, NS proteins are largely conserved across DENV serotypes. To initially examine differences in serotype (ST)-specific regulation of RIG-I/TBK1-directed IFN signaling pathways, we expressed NS2A, NS4A, and

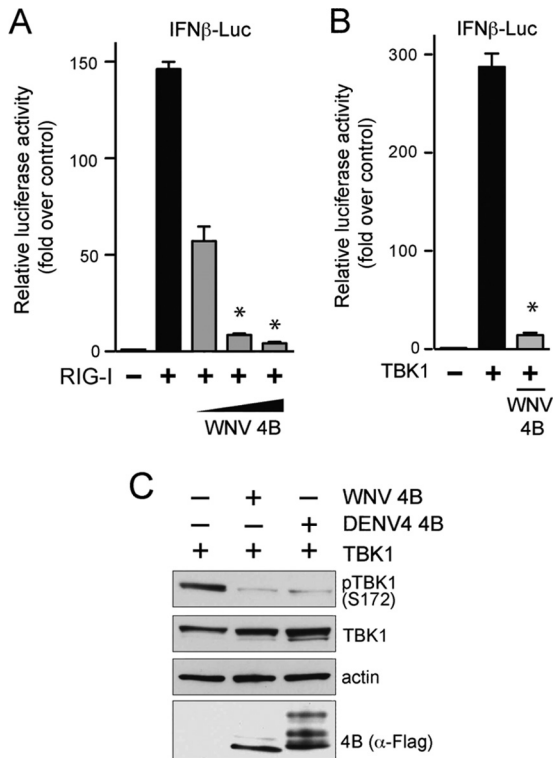


**FIG 7** The N-terminal domain of DENV NS4B inhibits RIG-I and TBK1 induction of IFN- $\beta$ . HEK293T cells were cotransfected as described in the legend to Fig. 1 with RIG-I-CARD or TBK1, IFN- $\beta$  promoter luciferase reporter, *Renilla* luciferase plasmid, and as indicated, plasmids expressing HA-tagged DENV4 wild-type NS4B, NS4B $\Delta$ 118-260, or NS4B $\Delta$ 1-117. Changes in IFN- $\beta$  transcriptional responses are reported as fold increase compared to the values for controls lacking RIG-I or TBK1. Asterisks indicate statistical significance ( $P < 0.05$ ) as determined by Student's *t* test.

NS4B proteins from DENV1, DENV2, and DENV4 and analyzed their ability to regulate RIG-I- and TBK1-directed IFN responses. Similar to responses observed for DENV4 proteins, NS2A and NS4B proteins from DENV1 (Fig. 9A and C) and DENV2 (Fig. 9B and D) inhibited RIG-I- and TBK1-directed IFN- $\beta$  induction >80%. While NS4A proteins from DENV2 and DENV4 were unable to regulate IFN- $\beta$  responses, we were surprised to find that DENV1 NS4A potently inhibited RIG-I- and TBK1-directed IFN- $\beta$  induction (>80%) (Fig. 9A and C). A direct comparison of ST1, 2, and 4 NS4A proteins (NS4As) further demonstrated that only the DENV1 NS4A protein regulated TBK1-directed induction of an IFN- $\beta$  luciferase reporter (Fig. 10A). Consistent with this, only the DENV1 NS4A protein reduced total and phospho-IRF3 levels in response to RIG-I activation (Fig. 10B). Our findings indicate that DENV1 uniquely encodes an NS4A protein with the ability to regulate RIG-I/TBK1-directed IFN induction. These unique regulatory responses may be directed by a single domain within the DENV1 NS4A protein that contains at least 6 uniquely charged residues (asterisks) from that of DENV2 and DENV4 NS4As (Fig. 10C). These findings suggest serotype-specific differences in IFN regulation that may contribute to unique DENV virulence and spread.

## DISCUSSION

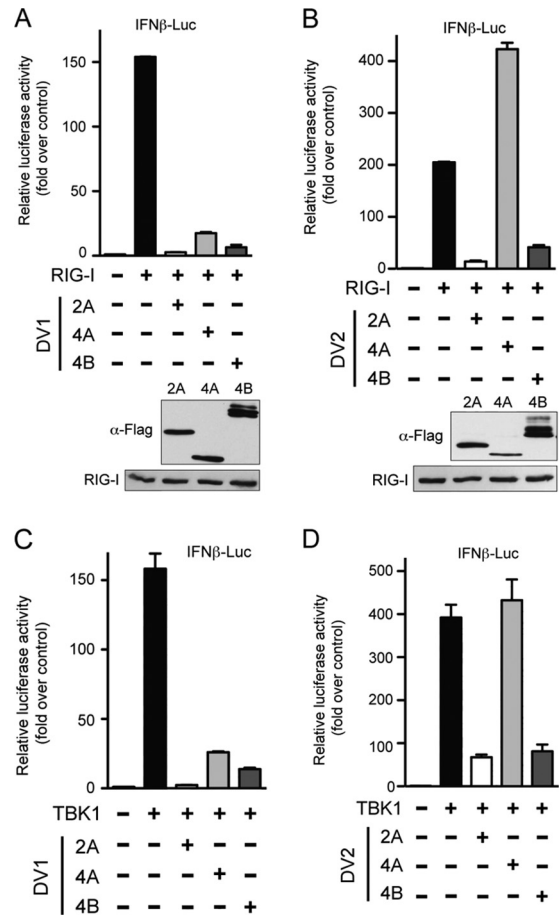
Viral pathogens evade innate host defenses by antagonizing cell signaling pathways that induce interferon or respond to the activation of IFN- $\alpha/\beta$  receptors (IFNARs). IFNAR activation directs the production of antiviral ISGs by activating JAK/STAT signaling pathways. DENV proteins reportedly regulate JAK/STAT signaling responses at various steps downstream of IFNAR activation (48, 49, 57–62). DENV NS4B and NS5 block STAT1/2 phosphorylation and direct STAT degradation, respectively, while NS2A and NS4A reportedly decrease STAT1/2 levels and cellular ISG



**FIG 8** West Nile virus NS4B inhibits RIG-I- and TBK1-directed IFN- $\beta$  induction. (A and B) HEK293T cells were cotransfected with IFN- $\beta$  reporters as described in the legend to Fig. 7A and RIG-I-CARD (A) or TBK1 (B) expression plasmids in the presence of constant or increasing amounts of plasmid expressing WNV (NY99 strain) NS4B (0.5, 1, or 2  $\mu$ g) or empty vector. Luciferase activity was measured and analyzed as described in the legend to Fig. 1 and reported as fold induction over the value for the control lacking RIG-I or TBK1. Asterisks indicate statistical significance ( $P < 0.05$ ) as determined by Student's  $t$  test. (C) HEK293 cells were cotransfected with TBK1 plasmid in the presence or absence of plasmid expressing the NS4B protein from WNV or DENV4 as indicated. Cells were harvested 24 h posttransfection. Phosphorylation of TBK1 was analyzed by Western blot analysis using anti-pTBK1 (Ser172). WNV NS4B, DENV4 NS4B, and total TBK1 levels were detected using anti-FLAG and anti-TBK1 antibodies, respectively, with  $\beta$ -actin expression as a loading control.

responses to secreted IFN (57–60, 63). DENV also reportedly inhibits type I IFN induction directed by NS2B-directed cleavage of STING, a DNA-triggered innate immunity effector (52, 53). RNA viruses generate small amounts of double-stranded RNA (dsRNA) with 5' triphosphate moieties that activate cytoplasmic RIG-I/MDA5 innate signaling pathways (22–25). Activation of RIG-I-mediated signaling was recently shown to inhibit DENV infection via a MAVS/TBK1/IRF3-dependent pathway (54), and MAVS-deficient mice have no initial type I interferon or antiviral immunity during DENV infection (45). These findings suggest that DENVs encode a mechanism for regulating this response in order to replicate successfully.

In the present study, we analyzed RIG-I/MDA5/MAVS/TBK1 responses that are regulated by DENV NS proteins. We determined that NS2A and NS4B proteins from ST 1, 2, and 4 DENVs inhibit the induction of IFN- $\beta$  by blocking RIG-I-, MAVS-, and TBK1 (or IKK $\epsilon$ )-directed signaling responses and preventing TBK1 phosphorylation and activation. Neither NS2A nor NS4B blocked transcription directed by a constitutively active IRF3



**FIG 9** Comparison of DENV1 and DENV2 NS protein inhibition of RIG-I/TBK1 signaling. (A to D) HEK293T cells were cotransfected as described in the legend to Fig. 1 with RIG-I-CARD (A and B) or TBK1 (C and D) plasmids, IFN- $\beta$  luciferase reporter, and *Renilla* luciferase plasmids, and as indicated, plasmids expressing NS2A, NS4A and NS4B from DENV1 (DV1) (A and C) or DENV2 (DV2) (B and D). Luciferase activity was measured 24 h posttransfection, normalized to *Renilla* luciferase activity, and reported as fold increase compared to the control values. Results are reported from duplicate samples and were similar in at least three independent experiments.

(IRF3-5D), indicating that inhibition occurs upstream of IRF3 and at the level of the TBK1 complex. In contrast, we found that neither NS2A nor NS4B blocked STING-directed responses and that NS2B3 failed to inhibit TBK1/IKK $\epsilon$ - or IRF3-5D-mediated IFN- $\beta$  induction. These findings indicate that DENV NS2A and NS4B act downstream of Toll-like receptor (TLR) and RIG-I/MDA5/MAVS signaling responses that commonly direct TBK1/IKK $\epsilon$  activation, IRF3/5/7 phosphorylation, and IFN induction.

Our findings demonstrate that NS2A and NS4B inhibition of IFN- $\beta$  induction is conserved across serotypes 1, 2, and 4, which share ~60 to 80% identical residues, respectively. The ability of the DENV NS2A to inhibit IFN- $\beta$  induction is consistent with a report that WNV NS2A inhibits the induction of type I IFN induction and that modifying this function alone attenuated WNV virulence in mice (35). However, DENV NS4B shares only 40% identity with the WNV NS4B protein and has negligible sequence homology to HCV NS4B (64, 65). In a comparison of DENV and WNV NS4B functions, we found that WNV NS4B inhibits RIG-I/TBK1-directed IFN induction and similarly blocks TBK1 phos-







enhanced DENV1 replication may also skew vaccine seroconversion toward DENV1 responses (2, 8, 14). In fact, DENV1 antagonizes DENV2 seroconversion and is immunologically dominant in monovalent and tetravalent vaccine formulations (8). Our data suggest the potential for increased TBK1 inhibition by DENV1-specific NS4A to temper IFN induction and enhance DENV1 virulence. This could alter the protective efficacy of tetravalent DENV vaccines by confounding immune responses to coadministered DENV2-4 STs (2, 8). Thus, unique IFN regulation and serotype-specific virulence of DENV1 may need to be considered for attenuating DENVs and for inducing equivalent ST responses to tetravalent DENV vaccines (2, 9, 14).

Our results suggest that DENV regulation of innate immune responses results from both conserved and serotype-specific NS protein functions and further suggest the potential for serotype-specific differences in DENV virulence. We have identified new roles for DENV NS2A and NS4B in antagonizing type I IFN induction and defined signaling pathway targets of NS protein regulation. The redundant targeting of innate cellular pathways by flavivirus NS proteins may ensure an early blockade of IFN induction during DENV infections. Since RIG-I/MDA5 signaling pathways and type I IFN addition restrict DENV replication (44–46, 54, 78), the transient regulation of early IRF3-directed ISG responses and IFN induction is likely to be crucial to dengue pathogenesis. In support of this, both IRF3 and IRF7 are reportedly necessary for the control of early stages of DENV infection (45, 54, 79). DENV NS protein regulation of pathways that induce ISGs suggest new potential virulence determinants and potential targets for therapeutically restricting dengue virus infections.

## MATERIALS AND METHODS

**Cells and antibodies.** HEK293T cells were maintained in supplemented Dulbecco's modified Eagle medium (DMEM) at 37°C and 5% CO<sub>2</sub>; the medium was supplemented with 8% fetal bovine serum (FBS), gentamicin (50 µg/ml), and amphotericin B (50 µg/ml). Polyclonal anti-Flag, anti-hemagglutinin (anti-HA), anti-IRF3, anti-pIRF3 (Ser396), anti-TBK1, and anti-pTBK1 (Ser172) were purchased from Cell Signaling. Monoclonal anti-Flag M2 is from Agilent, anti-V5 is from Stratagene, and anti-β-actin is from Roche. Horseradish peroxidase (HRP)-conjugated sheep anti-mouse and goat anti-rabbit immunoglobulin G (H+L) are from GE Healthcare.

**Plasmids.** DENV4 cDNA clone was provided by C.-J. Lai (NIH, NIAID, Laboratory of Infectious Diseases [LID]) (80, 81), and DENV1 and -2 cDNA clones were a gift from Louis Markoff (FDA) (82, 83). DENV1/2/4 NS2A, NS2B3, NS4A, and NS4B were PCR amplified from cDNA clones using BamHI and MluI restriction sites and C-terminal HA or Flag epitope tag-containing primers. DNA was cloned into a lentivirus expression plasmid (pLenti; Addgene) containing a cytomegalovirus (CMV) early promoter and Kozak sequence to drive expression (84). Where appropriate, leader and signaling sequences for specific genes were also PCR amplified. Truncated NS4B protein constructs were generated as described above by amplifying NS4B regions containing residues 1 to 117 or 118 to 260 with C-terminal HA tags and inserting the regions into the pLenti plasmid (*NS4BΔ1-117* and *NS4BΔ118-260*). Constitutively active RIG-I-CARD-Flag (residues 1 to 284) was provided by Michael Gale (85), human IKKε-Flag plasmid was obtained from Chris Basler (86), and human STING-V5 was a gift from Nancy Reich. pCMV-IRF3-T7 and IRF3-5D plasmids were obtained from John Hiscott (87). The following constructs were purchased from Addgene: human TBK1-flag, human MAVS-flag, and human MDA5-flag (88). Plasmid pUC57-WNV4B-Flag, expressing West Nile virus (WNV) NY99 strain (GenBank accession no. AF260967) nonstructural 4B protein (WNV NS4B) with a C-terminal Flag epitope tag, was synthesized and cloned by Genscript and used for

PCR amplification to generate pLenti-WNV4B-Flag by ligation into pLenti-puromycin vector (Addgene). Firefly luciferase ISRE, NF-κB, and IFN-β reporter plasmids were from Clontech; pRL-null *Renilla* reporter was from Promega.

**Transfections and luciferase reporter assays.** Transfections were performed in duplicate using polyethylenimine (PEI) at a 3:1 PEI/DNA ratio and 60% confluent HEK293T cells with a constant amount of total plasmid DNA as previously described (89, 90). HEK293T cells were plated on 12-well plates in supplemented DMEM and incubated overnight at 37°C. IFN-β promoter or ISRE-driven firefly luciferase reporter plasmids (Clontech), *Renilla* luciferase plasmid (pRL-null; Promega), inducer expression plasmid (RIG-I, MAVS, MDA5, TBK1, IRF3-5D), and empty vector or vectors expressing NS proteins were combined in DMEM (un-supplemented) and PEI transfected into cells for 24 h at 37°C (89). Cells were lysed in 250 µl of 1× luciferase lysis buffer (25 mM HEPES [pH 8.0], 15 mM MgSO<sub>4</sub>, 4 mM EGTA, 1% Triton X-100), and 10 µl of each sample was assayed for luciferase activity using a dual-luciferase assay kit according to the manufacturer's instructions (Promega). Assays measured firefly luciferase expression under control of the IFN-β or ISRE-driven promoters. Each assay measurement was controlled for transfection efficiency by standardizing to *Renilla* luciferase expression per the manufacturer's instructions. Fold induction over empty vector uninduced controls was calculated using Excel and graphed using GraphPad Prism. Each assay was performed at least three times. Error bars denote the standard deviations from the negative control values. Asterisks specify statistical significance determined by Student's *t* test (GraphPad Prism software) with the *P* values listed in figure legends.

**TBK1 and IRF3 analysis.** HEK293T cells were plated on 6-well plates, incubated overnight, and subsequently transfected with the desired plasmids expressing DENV and cellular TBK1 or IRF3 proteins by the PEI method described above. In the indicated experiment, cells were supplemented with 20 µM MG132 5 h prior to cell lysis. After 24 h, cells were washed in phosphate-buffered saline (PBS) and lysed in 0.5% SDS lysis buffer (150 mM NaCl, 40 mM Tris, 2 mM EDTA, 5 mM NaF, 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5% SDS, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1× protease inhibitor). After clarification by centrifugation at 14,000 rpm for 30 min, supernatant proteins were separated by 10% SDS-PAGE (90) and analyzed by Western blotting.

**Western blot analysis.** A constant amount of total protein from cell lysates, as determined by a bicinchoninic acid (BCA) assay kit (Pierce), was separated by SDS-PAGE, transferred to nitrocellulose, and Western blotted with antibodies to actin, Flag, pTBK1 (Ser172), HA, IRF3, or pIRF3 (Ser396) at a 1:1,000 to 1:10,000 dilution as specified in the figure legends. Proteins were detected using horseradish peroxidase-conjugated secondary anti-sheep, anti-mouse, or goat anti-rabbit immunoglobulin G and detected by chemiluminescence using the Luminata Forte system (Millipore).

## ACKNOWLEDGMENTS

We thank C.-J. Lai, Lew Markoff, and Nancy Reich for insightful discussions and for providing dengue virus clones, virus, and IFN signaling constructs. We thank Aleksandr Nasonov for technical support.

This work was supported by grants AI097951 and U54AI57158 (NBC-Lipkin) from the National Institutes of Health.

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