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Zika Virus Proteins NS2A and NS4A Are Major Antagonists that Reduce IFN-β Promoter Activity Induced by the MDA5/RIG-I Signaling Pathway

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Zika virus (ZIKV) is a mosquito-transmitted, emerging Flavivirus that causes Guillain-Barré syndrome and microcephaly in adults and fetuses, respectively. Since ZIKV was first isolated in 1947, severe outbreaks have occurred at various places worldwide, including Yap Island in 2007, French Polynesia in 2013, and Brazil in 2015. Although incidences of ZIKV infection and dissemination have drastically increased, the mechanisms underlying the pathogenesis of ZIKV have not been sufficiently studied. In addition, despite extensive research, the exact roles of individual ZIKV genes in the viral evasion of the host innate immune responses remain elusive. Besides, it is still possible that more than one ZIKV-encoded protein may negatively affect type I interferon (IFN) induction. Hence, in this study, we aimed to determine the modulations of the IFN promoter activity, induced by the MDA5/RIG-I signaling pathway, by over-expressing individual ZIKV genes. Our results show that two nonstructural proteins, NS2A and NS4A, significantly down-regulated the promoter activity of IFN-β by inhibiting multiple signaling molecules involved in the activation of IFN-β. Interestingly, while NS2A suppressed both full-length and constitutively active RIG-I, NS4A had inhibitory activity only on full-length RIG-I. In addition, while NS2A inhibited all forms of IRF3 (full-length, regulatory domain-deficient, and constitutively active), NS4A could not inhibit constitutively active IRF3-5D. Taken together, our results showed that NS2A and NS4A play major roles as antagonists of MDA5/RIG-I-mediated IFN-β induction and more importantly, these two viral proteins seem to inhibit induction of the type I IFN responses in differential mechanisms. We believe this study expands our understanding regarding the mechanisms via which ZIKV controls the innate immune responses in cells and may pave the way to development of ZIKV-specific therapeutics.

Keywords: Zika virus, interferon, evasion

Introduction

Zika virus (ZIKV) was first isolated from a febrile sentinel rhesus monkey (Rhesus 766) of the Zika Forest in Uganda in 1947 [1]. The first serious outbreak in Yap Island in 2007 was followed by the 2013 outbreak in French Polynesia and the outbreaks throughout Central and South America and the Caribbean regions [2–4]. ZIKV commonly causes mild symptoms such as rash, fever, arthralgia and conjunctivitis; however, the outbreak in Brazil warned that ZIKV infection can be associated with microcephaly in fetuses and the Guillain-Barre syndrome in adults [5–8]. ZIKV is mainly transmitted to humans by two *Aedes* mosquitoes, *Aedes aegypti* and *Aedes albopictus* [9]. New transmission routes of ZIKV have recently been determined in several cases, for example, from mother to fetus during pregnancy and

breastfeeding after birth, and from one human to another via sexual intercourse or blood transfusion [10].

ZIKV is a single-stranded, positive sense RNA virus belonging to the genus *Flavivirus* of the family Flaviviridae [9]. The 11-kb genome of ZIKV encodes three structural proteins: the capsid (C), precursor membrane protein (prM), and envelope (E), and seven nonstructural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.

Interferon (IFN) production is an important element of the host immune response against viral infection and replication [11, 12]. The viral genome, especially of RNA viruses, is recognized by two pattern recognition receptors (PRRs) named 'melanoma differentiation-associated gene 5' (MDA5) and 'retinoic acid-inducible gene 1' (RIG-I). Recognition of cognate ligands by and activation of PRRs lead to expression of type I IFNs [12, 13] and subsequently induce the production of pro-inflammatory cytokines by stimulating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [14]. The activation of the downstream signaling molecules of the MDA5/RIG-I pathway, including the mitochondrial antiviral adaptor protein (MAVS), inhibitor of kappa-B kinase epsilon (IKKε), TANK-binding kinase 1 (TBK1), and interferon regulatory factor 3 (IRF3), plays a crucial role in modulating the expression of type I IFN genes. Type 1 IFNs in turn activate the IFN-stimulated response element (ISRE) in an autocrine and paracrine manner and initiate a cascade of signaling events leading to IFN-stimulated genes (ISGs), which function to hinder virus replication.

In this study, we demonstrate that the ZIKV NS2A and NS4A proteins play major roles in antagonizing IFN-β production. NS2A and NS4A suppressed IFN-β promoter activities through not only down-regulation of RIG-I-like receptors (RLRs) but also the downstream components of the MDA5/RIG-I signaling pathway. Furthermore, ZIKV NS1 also contributed to moderate reduction in IFN-β levels by down-regulation of MDA5, constitutively active RIG-I (RIG-I-1-228), MAVS, TBK1, and IKKε although the effect was not as strong as those of NS2A or NS4A. Better understanding of the strategies used by ZIKV to overcome the host innate immunity will pave the way to development of antivirals and preventive vaccines.

Materials and Methods

Cells

Human embryonic kidney 293T (HEK293T) cells and Vero-E6 cells were obtained from the American Type Culture Collection (ATCC) and maintained in high-glucose (4,500 mg/ml) Dulbecco's modified Eagle's medium (DMEM) (Welgene, Korea) supplemented with 10% fetal bovine serum (FBS) (Welgene) and 1% penicillin/streptomycin (Gibco, USA) [15–17]. The cells were incubated at 37°C in a 5% CO_2 humidifying incubator. Trypsin-EDTA (0.05%) and Dulbecco's phosphate-buffered saline (DPBS) were also purchased from Welgene.

Antibodies [18-22].

A mouse monoclonal anti-FLAG antibody (M2) (1:5000) was purchased from Millipore Sigma, USA. HA-tagged (6E2) mouse monoclonal antibody (1:1000), horseradish peroxidase (HRP)-conjugated rabbit monoclonal anti-GAPDH (14C10) (1:2000), HRP-conjugated anti-rabbit IgG antibody (1:2000) and HRP-conjugated anti-mouse IgG antibody (1:2000) were purchased from Cell Signaling, USA.

Reagents

Opti-MEM Minimal Essential Medium (MEM) (Gibco), polyethylenimine (PEI; Millipore Sigma) and cOmplete Mini Protease Inhibitor Cocktail (Millipore Sigma) were purchased and used for plasmid transfection. The luciferase assay system and Beta-Glo Assay System were obtained from Promega, USA. 4× Laemmli sample buffer and 2-mercatoethanol were purchased from Bio-Rad, USA. Amersham ECL Prime western blotting detection reagent, Amersham ECL western blotting detection reagent, and Amersham Protran 0.45 nitrocellulose (NC) blotting membrane were purchased from GE Healthcare Life Sciences, USA. EcoRI-HF, BamHI-HF, NotI-HF, NheI-HF, PmeI, PmlI, XhoI, XbaI, and T4 DNA ligase were purchased from New England Biolabs (NEB), USA. MAX Efficiency DH5a competent cells were purchased from Thermo Fisher Scientific, USA. Pfu Plus DNA polymerase was purchased from Elpisbio, Republic of Korea.

Plasmids

Generation of the pcDNA3.1-Neo-JY3 vector has been described previously [23]. The pcDNA3.1-Neo-JY3-3× FLAG-N vector was modified from pcDNA3.1-Neo-JY3. Briefly, the pcDNA3.1-Neo-JY3 vector was digested using NheI-HF and PmeI. The linearized vector was ligated with a linker containing a 3× FLAG N-terminus tag (5'-GCTAGCGCCACCATGGACTACAAGGACCACGACGGT GACTACAAGGACCACGACATCGACTACAAGGACGACGAC GACAAGCTTATCGATATCACCGGTACCTCAGGATCCTGCA GGCCGGCCCGTACGCGGCCGCTCGAGGTTAACTCTAGAGG GCCCGTTTAAAC-3'). ZIKV RNA was extracted from the supernatant of ZIKV-infected Vero-E6 cells using TRiZol LS (Thermo Fisher Scientific) following the manufacturer's instructions. Reverse transcription (RT) was performed using a primer targeting the 3'-end of the ZIKV genome. Each ZIKV gene was amplified using Pfu Plus DNA polymerase (Elpisbio) under the following conditions: 95°C, 20 s; 58°C, 20 s; 72°C, 10 s/kb for 30 cycles. The amplicons were purified using the FavorPrep GEL/PCR Purification Kit (Favorgen, Taiwan) according to the manufacturer's instructions. All the primers used are listed in Table 1. Linearized vectors were

Table 1. Primers for cloning of ZIKV genes.

Name	Sequence (5′-3′)
ZIKV-C-F	CCGGAATTCAAAAACCCAAAAAAGAAATCCGGAGG
ZIKV-C-R	CCGCTCGAGTTATCGTCTCTTCTTCTCCTTCCTAGC
ZIKV-prM-F	CCGGAATTCGCGGAGGTCACTAGACGTGGG
ZIKV-prM-R	CCGCTCGAGTTAGCTGTATGCCGGGGCAATCAG
ZIKV-E-F	CCGGAATTCATCAGGTGCATAGGAGTCAGCAATAG
ZIKV-E-R	CCGCTCGAGTTAAGCAGAGACGGCTGTGGATAAG
ZIKV-NS1-F	CCGGAATTCATCAGGTGCATAGGAGTCAGCAATAG
ZIKV-NS1-R	CGCTCGAGTTATGCAGTCACCATTGACCTTACTAAGTTGC
ZIKV-NS2A-F	CCGGAATTCGGATCAACTGATCACATGGACCACTTC
ZIKV-NS2A-R	CCGCTCGAGTTACCGCTTCCCACTCCTTGTGAGC
ZIKV-NS2B-F	CCGGAATTCAGCTGGCCCCCTAGCGAAGTAC
ZIKV-NS2B-R	CCGCTCGAGTTACCTTTTTCCAGTCTTCACGTATACGTACC
ZIKV-NS3-F	CCGGAATTCAGTGGTGCTCTATGGGATGTGCC
ZIKV-NS3-R	CCGCTCGAGTTATCTTTTCCCAGCGGCAAACTCCTTG
ZIKV-NS4A-F	CCGGAATTCGGAGCGGCTTTTGGAGTGATGG
ZIKV-NS4A-R	CCGCTCGAGTTATCTTTGCTTTTCTGGCTCAGGTATGAGC
ZIKV-NS4B-F	CCGGAATTCAATGAACTCGGATGGTTGGAGAGAAC
ZIKV-NS4B-R	CCGCTCGAGTTAACGTCTCTTGACCAAGCCAGC
ZIKV-NS5-F	CCGGAATTCGGGGGTGGAACAGGAGAGACC
ZIKV-NS5-R	CCGCTCGAGTTACAGCACTCCAGGTGTAGACCCTTC

dephosphorylated using calf intestinal alkaline phosphatase (CIP; NEB) at 37°C for 60 min. The ligated products were transformed into DH5α cells. The constructions of the pcDNA3.1-Hygro-JY4-HAN-GS3 vector and plasmids harboring genes involved in the type I interferon pathway, such as *MDA5, RIG-I, MAVS, IKKε, TBK1*, and *IRF3* have been described previously [24, 25]. RIG-I-1-228 and IRF3-1-390 were cloned into pcDNA3.1-Hygro-JY4-HAN-GS3 using conventional cloning methods. In addition, a part of *IRF3* (Table 2) was synthesized at Bionics (Republic of Korea) to generate a constitutive active form named 'IRF3-5D' (S396D, S398D, S402D, T404D, and S405D). The synthesized *IRF3*-5D fragment was replaced with the corresponding region of the pcDNA3.1-Hygro-JY4-HAN-GS3-IRF3 construct using PmlI and XhoI (NEB).

Transfection and Luciferase Assay

HEK293T cells (8×10^5) per well were seeded on a 6-well plate 24 h prior to transfection [26]. A mixture containing 500 ng interferon (IFN)-β-luc plasmid, 100 ng β-gal-expression plasmid, 500 ng each immune gene expression plasmid and 1,000 ng individual ZIKV gene-encoding plasmid were transfected using the PEI reagent in the ratio of 1:2 (DNA:PEI). At 24 h post-transfection, transfected cells were lysed with lysis buffer supplemented with the protease inhibitor cocktail. After incubation on ice for 10 min, the lysed cells were cleared at 15,000 ×*g*, 4°C, for 15 min. Twenty-five microliters of the sample supernatants were mixed with 25 μl assay substrate and either the luciferase assay or Beta-Glo assay was performed according to the manufacturer's instructions. Firefly luciferase activities of the samples were first normalized to the internal control (β-gal), and then the fold changes over the empty vector control were calculated. Paired two-tailed Student's t-test was performed. p-value < 0.05 was considered statistically significant.

Table 2. Synthesized fragment for IRF3-5D substitution (Solid underline: mutations; amino acid substitutions are indicated below).

Name	Sequence
IRF3-5D	GCCTCCTCCCTGGAGAATACTGTGGACCTGCACATT**GAT**AAC**GAC**CA S→D S→D CCCACTC**GAT**CTC**GACGAT**GACCAGTACAAGGCCTACCTGCAGGAC S→D TS→DD TTGGTGGAGGGCATGGATTTCCAGGGCCCTGGGGAGAGCTGA

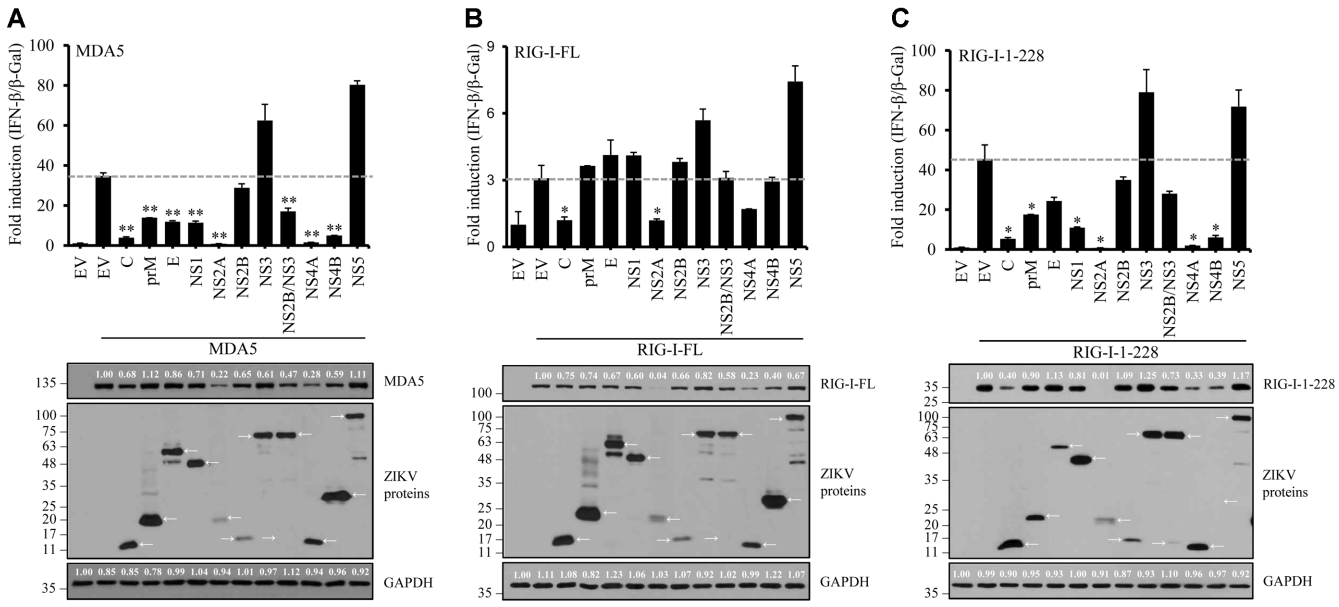

Fig. 1. ZIKV-encoded proteins suppressed the cytoplasmic RNA sensors, MDA5 and RIG-I.
HEK293T cells were co-transfected with plasmids encoding individual ZIKV protein, IFN-β-luc, β-Gal, and MDA5 (**A**), RIG-I-FL (**B**), and RIG-I-1-228 (**C**). At 24 h post-transfection (hpt), the cells were lysed and used for luciferase assay (top panel). Protein expression was analyzed by western blotting (bottom panel) with anti-HA antibody (for MDA5, RIG-I, and RIG-I-1-228), anti-FLAG antibody (for ZIKV proteins), and anti-GAPDH antibody (for human GAPDH). A representative data of two independent experiments is shown. Statistical significance was determined by Student's two-tailed *t*-test $^{**}p < 0.01$, $^{*}p < 0.05$. The sizes of the proteins are indicated on the left (kDa). Arrows indicate the expected protein bands.

Results

ZIKV-Encoded C, NS2A and NS4A Strongly Inhibit Both MDA5- and RIG-I-Induced Activation of the IFN-β Promoter Activities

To screen out the potential targets of individual ZIKV genes that affect IFN-β production, we first tested MDA5 and RIG-I as they are cytoplasmic dsRNA sensors (Fig. 1). MDA5, full-length RIG-I (RIG-I-FL), or constitutively active RIG-I-1-228 encoding plasmids was co-transfected into HEK293T cells together with expression plasmids of each individual ZIKV gene and IFN-β-luc. Transfected cells were assayed for luciferase activity at 24 h post-transfection (hpt). Results showed that ZIKV C and NS2A significantly inhibited the IFN promoter activities induced by all forms of RLRs used (MDA5, RIG-I-FL, and the RIG-I-1-228 (Fig. 1)). ZIKV prM, NS1, NS4A, and NS4B decreased IFN-β promoter activation by targeting MDA5 and RIG-I-1-228, but not RIG-I-FL (Figs. 1A and 1C vs 1B). Of note, C, NS2A, and NS4A dramatically decreased both luciferase expression driven by the IFN-β promoter and the protein levels of RLRs. These results suggest that these ZIKV-encoded proteins are capable of suppressing induction of the IFN-β promoter activitiy by down-regulating RNA sensors of the IFN-β pathway.

Many ZIKV-Encoded Proteins Significantly Inhibit MAVS-, TBK1-, and IKKε-Induced Activation of the IFN-β Promoter

Next, we attempted to identify ZIKV proteins that can inhibit IFN-β promoter activity via the downstream players of MDA5 and RIG-I, including MAVS, TBK1, and IKKε. The MAVS-, TBK1-, or IKKε-expressing plasmids were co-transfected together with each individual ZIKV gene, and activation of the IFN-β promoter activities was assessed by luciferase assay (Fig. 2). Interestingly, NS2A and NS4A demonstrated as strong inhibitory effects on MAVS-, TBK1-, and IKKε-induced activation of the IFN-β promoter as they did on that of RLRs. Of note, NS2A-mediated inhibition seemed to be associated with its down-regulation of protein levels of MAVS, TBK1, and IKKε (Figs. 2A–2C, respectively). NS4A also moderately reduced the protein levels of TBK1 as well (Fig. 1B). Targeting of MAVS by ZIKV C, prM, NS1, NS2B, NS2B-NS3 complex, and NS4B also led to inhibition of IFN-β promoter activity without affecting protein expression levels of MAVS (Fig. 2A). All ZIKV proteins significantly suppressed TBK1-mediated

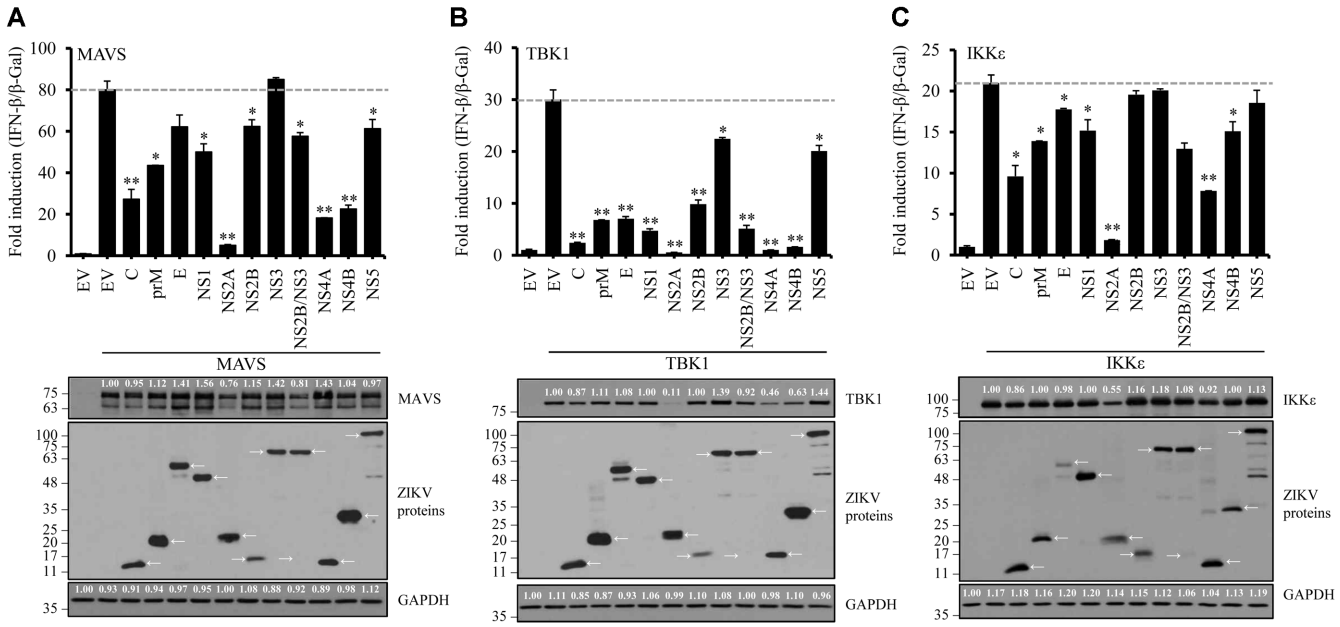

Fig. 2. ZIKV-encoded proteins antagonized activation of the IFN-β promoter induced by MAVS, TBK1 and IKKε.
HEK293T cells were co-transfected with plasmids encoding ZIKV proteins, IFN-β-luc, β-Gal, and MAVS (**A**), TBK1 (**B**), or IKKε (**C**). At 24 hpt, the cells were lysed and used for the luciferase assay (top panel). Protein expression was analyzed by western blotting (bottom panel) with anti-MAVS antibody or anti-HA antibody (for TBK1 and IKKε), anti-FLAG antibody (for ZIKV proteins), or anti-GAPDH antibody (for human GAPDH). The results presented are a representative of two independent experiments. Statistical significance was determined using Student's two-tailed *t*-test $^{**}p < 0.01$, $^{*}p < 0.05$. The size of the proteins is indicated on the left (kDa). Arrows indicate the expected protein bands.

activation of the IFN-β promoter; however, only NS2A substantially abated protein expression of TBK1 (roughly 89% reduction) while NS4A and NS4B decreased TBK1 protein levels by 54% and 37%, respectively (Fig. 2B). Although IKKε-mediated IFN-β promoter activity was down-regulated by three structural (C, prM, and E) and four nonstructural (NSA1, NS2A, NS4A, and NS4B) proteins, the protein levels of IKKε remained unperturbed except for NS2A, expression of which reduced IKKε protein levels by 45% (Fig. 2C).

ZIKV NS2A Is Competent to Significantly Inhibit Induction of the IFN-β Promoter When Activated by All Forms of IRF3 (IRF3-FL, IRF3-1-290, and IRF3-5D)

IRF3 plays an important role in the expression of IFN-β upon virus infection [27]. Phosphorylation of IRF3 by TBK1 or IKKε results in phosphorylation and homo-dimerization of IRF3, which enables its translocation into the nucleus to activate IFN-β transcription and thus initiation of innate immune responses [28]. In this study, three IRF3-expressing constructs, including regulatory domain-deleted (IRF3-1-390) and a constitutive active (IRF3-5D) and full-length IRF3 (IRF3-FL) were employed to investigate whether ZIKV-encoded proteins are competent to inhibit activation of the IFN-β promoter induced by any of these IRF3's (Fig. 3). Surprisingly, NS2A demonstrated inhibitory activities on IFN-β promoter induction by all forms of IRF3's. NS4A decreased IFN-β induction by the expression of IRF3-FL and IRF3-1-390, but not IRF3-5D (Figs. 3A and 3B). Interestingly, ZIKV E, NS1, NS2B, and NS4A significantly down-regulated IFN-β expression only when induced by IRF3-1-390 (Fig. 3B) but not by the other two IRF3 forms (IRF3-5D and IRF3-FL).

ZIKV NS2A and NS4A Inhibit MDA5-Mediated Induction of the IFN-β Pathway in a Dose-Responsive Manner

Varying amounts (0, 0.1, 0.3, or 1 μg) of plasmid expressing NS2A (Fig. 4A) or NS4A (Fig. 4B) were transfected into HEK293T cells, and luciferase assay and western blotting were performed at 24 hpt. As shown in the bottom panels of Figs. 4A and 4B, NS2A or NS4A protein levels increased depending on the amounts of expression plasmids transfected. As the protein levels increased, the levels of MDA5 were decreased in a NS2A or NS4A dose-dependent manner. Accordingly, luciferase activities were decreased with inverse relationship with

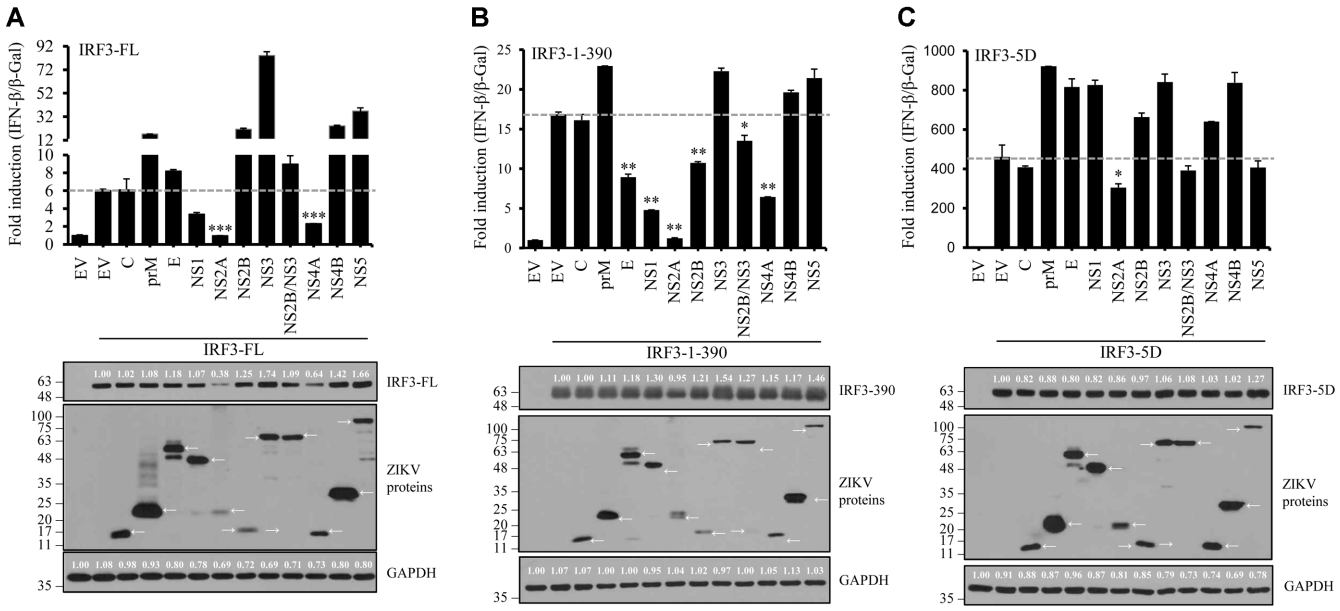

Fig. 3. ZIKV NS2A inhibits activation of the IFN-β promoter induced by all forms of IRF3's (IRF-FL, IRF3-1-390, and IRF3-5D). (**A**) HEK293T cells were co-transfected with plasmids encoding ZIKV proteins, IFN-β-luc, β-Gal, and IRF3-FL (**A**), IRF3-390 (**B**), IRF3-5D (**C**). At 24 hpt, the cells were lysed and used for luciferase assay (top panel). Protein expression was analyzed using western blotting (bottom panel) with anti-HA antibody (for IRF3-FL, IRF3-1-390, and IRF3-5D), anti-FLAG antibody (for ZIKV proteins), and anti-GAPDH antibody (for human GAPDH). Results are representative of two independent experiments. Statistical significance was determined by the Student's two-tailed *t*-test ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. The size of the proteins is indicated on the left (kDa). Arrows indicate the expected protein bands.

NS2A or NS4A, clearly suggesting that ZIKV NS2A and NS4A are bona fide antagonists of MDA5-mediated activation of the IFN-β promoter. In addition, it is likely that NS2A and NS4A activities may be responsible for the inhibition of IFN-β promoter activity and reduction in the protein levels of MDA5.

Discussion

The aim of our study is to investigate which ZIKV-encoded proteins inhibit activation of the IFN-β promoter via the MDA5/RIG-I signaling pathway. The data presented here demonstrated that ZIKV NS2A and NS4A are the main antagonists of induction of IFN-β by suppressing almost all the components of the MDA5/RIG-I signaling pathway with or without their effects on the protein levels of the signaling molecules involved.

The ZIKV genome encodes a single polyprotein which is proteolytically cleaved to three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The polyprotein is cleaved by either host or viral proteases [29]. The C protein binds to the viral RNA genome to build a nucleocapsid, while prM is required for virus maturation and egression. The E protein is involved in virus-host attachment, membrane fusion, and virus entry [30]. Nonstructural proteins are mainly involved in polyprotein processing and viral genome replication and modulation of host immune responses as well. ZIKV NS1 glycoprotein, which functions as a homodimer in vivo, is an essential cofactor required for the replication of the RNA genome and viral morphogenesis. In addition, NS1 has also been shown to antagonize the innate immune response [31–33]. The alignment of the NS2A amino acid sequences among members of the Flaviviridae family revealed that the NS2A proteins are highly conserved across multiple species (> 80%) [34]. Flavivirus NS2A proteins, an endoplasmic reticulum (ER) membrane protein, have multiple functions; viral replication, virion assembly and secretion, and inhibition of innate immunity [33, 35-40]. NS3 acts as a serine protease along with NS2B to process the polyprotein into an RNA helicase and triphosphatase [41]. NS4A is involved in the arrangement of the host membrane and the viral replication complex [42, 43]. NS4A is known to interrupt the interaction between RLRs and MAVS [44]. NS4B is involved in the formation of the viral replication

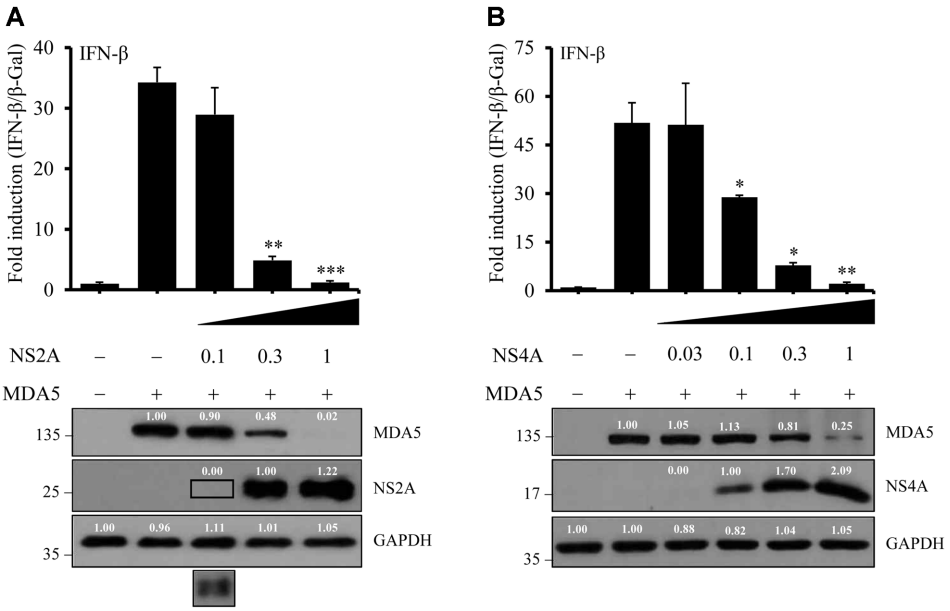

Fig. 4. ZIKV NS2A and NS4A impairs MDA5-induced IFN-β signaling pathway in a dose-dependent manner.
Increasing amounts (0, 0.1, 0.3, or 1 μg) of DNA encoding ZIKV NS2A (**A**) ZIKV NS4A (**B**) were co-transfected with MDA5 (0.5 μg), IFN-β-luc and β-Gal. At 24 hpt, cell lysates were prepared for luciferase assay (top panel). Protein expression was determined by western blotting (bottom panel) with anti-HA antibody (for MDA5), anti-FLAG antibody (for ZIKV proteins), and anti-GAPDH antibody (for human GAPDH). Results are representative of two independent experiments. Statistical significance was determined by the Student's two-tailed *t*-test ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. The size of the proteins is indicated on the left (kDa).

complex [45]. NS4A and NS4B inhibit the Akt-mTOR pathway to induce cellular dysregulation [46]. NS5 plays an important role in viral RNA replication as an RNA-dependent RNA polymerase, and also functions as an N-terminal methyltransferase targeting N-7 and 2′-O methylation of the viral RNA [47–49].

Type I IFNs are one of well-known host defense mechanisms against virus infection [50–53]. IFN secretion is initiated by the recognition of the infecting pathogen via pathogen recognition receptors (PRRs). Viral RNAs are detected by distinct RLRs, including MDA5 and RIG-I [54]. These pattern recognition receptors are key sensors for the genomes of the invading pathogens, leading to induction of the type I IFNs such as IFN-α and IFN-β, and pro-inflammatory cytokines at the early stage of virus infection. Activated RLRs cause conformational changes and then activate MAVS on the mitochondrial membrane. MAVS sequentially trigger activation of TBK1 and IKKε, resulting in the phosphorylation of IRF3. Phosphorylated IRF3 (pIRF3) dimerizes and translocates to the nucleus to induce transcription of type I IFN genes. Therefore, invading viruses should have strategies for successful infections that hinder the IFN-mediated innate immunity.

ZIKV NS2A specifically suppressed RIG-I-, MAVS-, IKKε-, and TBK1-induced IFN-β activity [33]. However, our results showed that all players of the MDA5/RIG-I signaling pathway were down-regulated by ZIKV NS2A (Figs. 1–3). ZIKV NS4A antagonizes IFN response by perturbing RIG-I and MAVS interaction [44, 55]. With the exception of IRF3-5D, ZIKV NS4A was able to down-regulate all the signaling molecules involved in the MDA5/RIG-I signaling pathway (Figs. 1–3). The ZIKV NS3 and NS2B-NS3 complex are known to inhibit MAVS and mediator of IFN regulatory factor 3 activation (MITA) [56]. In contrast, our results showed no significant reduction in MAVS level by the NS3 and the NS2B-NS3 complex (Fig. 2A). Moreover, NS3 and NS2B-NS3 complex did not perturb IRF3-mediated activation of IFN responses (Fig. 3). Interestingly, NS4A demonstrated itself to be another general inhibitor of MDA5-mediated induction of IFN signaling as NS2A. That is, NS4A strongly inhibited MDA5 (Fig. 1), MAVS, TBK1, IKKε (Fig. 2) and IRF3-FL (Fig. 3). Of note, NS4A suppressed IRF3-FL (Fig. 3A) but not IRF3-5D (Fig. 3C), which is a mimic of a phosphorylated, thus active, form of IRF3. These data suggest that NS4A-mediated inhibition of IRF3 may be indirect and occur

through the inhibition of upstream IRF3 kinases (Figs. 2B and 2C): TBK1 and IKKε. Employment of TBK1- and/or IKKε-deficient cell lines would help clarify these possibilities, which is currently underway. NS4B also displays inhibitory effects on upstream signaling molecules (MDA5 (Fig. 1A), MAVS (Fig. 2A), TBK1 (Fig. 2B), and IKKε (Fig. 2C)), but not IRF3-FL (Fig. 3A). Biochemical analysis of NS4B-mediated inhibition is now in progress to determine the exact suppressive mechanisms of NS4B. On the other hand, ZIKV NS1 was found to be a 'minor' inhibitor of the IFN-β pathway. It hampered to a lesser degree a selective set of signaling molecules involved in the signaling event: MDA5 (Fig. 1), MAVS, TBK1, and IKKε (Fig. 2), which is largely consistent with previous studies [32, 33]. In our studies, ZIKV NS5 did not seem to inhibit any signaling molecules of IFN-β pathway, which is in sharp contrast with a previous report [57]. Kumar *et al*. showed that ZIKV NS5 inhibited both IRF3 and IFN-β promoter activities, suggesting that it may function as a ZIKV-encoded inhibitor of IRF3. However, none of three forms of IRF3's was affected by overexpression of NS5 (Fig. 3). It is interesting to note that expression of ZIKV C, NS2A, NS4A and NS4B resulted in low levels of RIG-I protein (Fig. 1B), which suggests that those ZIKV proteins may influence synthesis or direct/indirect degradation of RIG-I protein. Underlying mechanisms are now being sought. On the other hand, ZIKV prM and E protein inhibited IFN-β production without affecting RIG-I protein levels, hinting at differential inhibitory mechanisms from those of ZIKV C, NS2A, and NS4A/4B.

Overall, our data are largely consistent with the current view of the functions of the ZIKV-encoded proteins regarding antagonism of the host innate immunity. However, our results reveal some discrepancies with some of the results of the previous studies. Those discrepancies may have stemmed from multiple reasons: 1) different human or non-human cell lines were employed in many of the studies (cell line-dependent), 2) varying sequences of ZIKV proteins were utilized (ZIKV strain-specific), 3) the size and position of tags fused to the ZIKV proteins were different (tag-dependent). Detailed and careful analysis of these issues will help resolve partial study-to-study discrepancies.

Taken together, the data presented here show that many ZIKV-encoded proteins are involved in the down-regulation of the type I IFN signaling as previously shown with minor discrepancies. More importantly, we demonstrated that ZIKV NS2A and NS4A play major roles in the inhibition of IFN-β promoter activity by inhibiting multiple signaling mediators of the MDA5/RIG-I signaling pathway. Scrutiny of potential mechanisms of NS2A- and NS4A-mediated blockade of the type I IFN responses is currently underway. We believe that our study will add to the current understanding regarding the ZIKV-host interactions and provide clues for developing anti-ZIKV therapeutics.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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