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Selective Activation of Type II Interferon Signaling by Zika Virus NS5 Protein

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ABSTRACT Severe complications of Zika virus (ZIKV) infection might be caused by inflammation, but how ZIKV induces proinflammatory cytokines is not understood. In this study, we show opposite regulatory effects of the ZIKV NS5 protein on interferon (IFN) signaling. Whereas ZIKV and its NS5 protein were potent suppressors of type I and type III IFN signaling, they were found to activate type II IFN signaling. Inversely, IFN-γ augmented ZIKV replication. NS5 interacted with STAT2 and targeted it for ubiquitination and degradation, but it had no influence on STAT1 stability or nuclear translocation. The recruitment of STAT1-STAT2-IRF9 to IFN-β-stimulated genes was compromised when NS5 was expressed. Concurrently, the formation of STAT1-STAT1 homodimers and their recruitment to IFN-γ-stimulated genes, such as the gene encoding the proinflammatory cytokine CXCL10, were augmented. Silencing the expression of an IFN-γ receptor subunit or treatment of ZIKV-infected cells with a JAK2 inhibitor suppressed viral replication and viral induction of IFN-γ-stimulated genes. Taken together, our findings provide a new mechanism by which the ZIKV NS5 protein differentially regulates IFN signaling to facilitate viral replication and cause diseases. This activity might be shared by a group of viral IFN modulators.

IMPORTANCE Mammalian cells produce three types of interferons to combat viral infection and to control host immune responses. To replicate and cause diseases, pathogenic viruses have developed different strategies to defeat the action of host interferons. Many viral proteins, including the Zika virus (ZIKV) NS5 protein, are known to be able to suppress the antiviral property of type I and type III interferons. Here we further show that the ZIKV NS5 protein can also boost the activity of type II interferon to induce cellular proteins that promote inflammation. This is mediated by the differential effect of the ZIKV NS5 protein on a pair of cellular transcription factors, STAT1 and STAT2. NS5 induces the degradation of STAT2 but promotes the formation of STAT1-STAT1 protein complexes, which activate genes controlled by type II interferon. A drug that specifically inhibits the IFN-γ receptor or STAT1 shows an anti-ZIKV effect and might also have anti-inflammatory activity.

KEYWORDS Zika virus, NS5 protein, type II interferon, STAT1, STAT2

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Zika virus (ZIKV) is a member of the *Flaviviridae* family with a positive-sense RNA genome of about 10 kb (1). Similar to other flaviviruses, such as yellow fever virus, dengue virus, and Japanese encephalitis virus, ZIKV expresses a single polypeptide which is proteolytically processed into 3 structural (C, prM, and E) and 7 nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. ZIKV is an arbovirus transmitted to humans through the bites of mosquitoes of the species *Aedes aegypti* and, to a lesser extent, *Aedes albopictus* (2). Since its discovery in 1947 (3), ZIKV had been known to cause only mild and self-limiting febrile diseases, with a majority of patients being

unnoticed. This concept changed after a series of explosive outbreaks, since 2007, in Micronesia, the South Pacific, the Americas, and, more recently, Southeast Asia (2, 4). The numbers of people infected in these outbreaks were unprecedentedly high. More severe complications, including birth defects and neurological disorders, were observed (5, 6). New modes of transmission, including blood-borne, transplacental, and sexual transmission, were also found (1, 7). In particular, the causal relationship between ZIKV infection in pregnant women and birth defects, such as microcephaly and other brain anomalies in infants, was recently established (8–10). The association of ZIKV infection with Guillain-Barré syndrome, a neurological complication of autoimmune nature, was also identified (11). In addition, the teratogenic and neuropathogenic effects of ZIKV infection were demonstrated in cellular, organoid, and animal models (12–18).

Although it remains to be determined whether mutations have enabled ZIKV to become more pathogenic and transmissible (19), innate immunity is thought to govern both viral replication and pathogenesis (6, 20). Indeed, innate antiviral effectors, such as type I and type III interferons (IFNs), are capable of suppressing ZIKV replication (21, 22). However, aberrant activation of innate immunity also results in inflammation, apoptosis, and autophagy, which might facilitate ZIKV spread and account for the cytopathic effects in placental cells and neural progenitors (15, 22–24). Inflammation at mosquito bite sites may aid in ZIKV replication and dissemination *in vivo* by recruiting myeloid cells and passing on the virus to them (25). Pyroptosis, as a result of inflammation, and other forms of programmed cell death, including apoptosis and necroptosis, cause tissue damage and the release of a large number of infectious virions, thereby facilitating ZIKV spread (26). A proviral effect of autophagy, plausibly mediated through mobilization of lipid stores, has also been shown for ZIKV and dengue virus (15, 22, 27). During evolution, viruses have developed countermeasures to modulate different branches of innate immune signaling (28). Viral antagonists of type I IFN production and signaling are well described. On the other hand, overproduction of proinflammatory cytokines in infected cells and tissues is a hallmark of severe disease associated with viral infection (29). It is plausible that viruses hijack the host proinflammatory response for their own benefit. During ZIKV infection, both antiviral IFNs and proinflammatory cytokines are robustly induced (17, 22, 24, 30, 31).

One important function of flaviviral nonstructural (NS) proteins is to subvert the host innate immune response (32). Consistent with this notion, the ZIKV NS5 protein was recently shown to suppress type I IFN signaling by targeting STAT2 for degradation (33, 34). STAT2 is known to form a heterotrimeric complex with STAT1 and IRF9 to activate IFN-stimulated response elements (ISRE) in IFN-stimulated genes (ISGs) regulated by type I and type III IFNs (35). On the other hand, IFN-γ-activated sites (GAS) in ISGs are bound by STAT1-STAT1 homodimers (36). As such, a dynamic interaction of STAT1 with STAT1 and STAT2 might dictate the relative activities of ISRE and GAS in different ISGs (37, 38). ZIKV NS5 specifically interacts with STAT2, resulting in its proteasomal degradation (33, 34). This might affect STAT1 activity by tipping the intracellular balance between STAT1-STAT2-IRF9 and STAT1-STAT1 complexes to the latter. Indeed, IFN-γ-stimulated genes, such as the CXCL10 gene, are induced in ZIKV-infected cells (17, 22, 30). It will therefore be of great interest to clarify the activation status of IFN-γ signaling in relation to ZIKV and its NS5 protein. In this study, we compared the impacts of the ZIKV NS5 protein on the activation of STAT1 and STAT2. We found opposite effects of NS5 on type I and type II IFN signaling.

RESULTS

Selective activation of IFN-γ signaling by ZIKV. IFNs are known to have antiviral activity (37), but IFN-γ is more directly and critically involved in immune regulation and proinflammatory responses (39). In addition, the antiviral effect of IFNs might not be seen when ZIKV replication is robust (34). In view of this, we sought to determine how IFN-β and IFN-γ might affect ZIKV replication in JEG3 choriocarcinoma and SF268 glioblastoma cells. Both cell lines are highly susceptible to ZIKV infection (12).

In addition, they might be physiologically more relevant to the placental and neurological effects of ZIKV infection. Both cell lines have also been used extensively in the study of virus-host interactions and, in particular, interferon responses (40, 41). When JEG3 cells were pretreated with IFN-β for 12 h and then infected with ZIKV, viral RNA replication was blocked almost completely (Fig. 1A, bar 2 versus bar 1). In contrast, pretreatment with IFN-γ did not inhibit but instead boosted ZIKV replication (Fig. 1A, bar 3 versus bar 1). Interestingly, the antiviral activity of IFN-β in ZIKV-infected JEG3 cells was not seen when the 12-h treatment with IFN-β started 12 h after infection. Actually, the level of viral RNA was slightly elevated in these cells treated with IFN-β (Fig. 1A, bar 4 versus bar 1). To our surprise, when we treated ZIKV-infected JEG3 and SF268 cells with IFN-γ, viral RNA replication was augmented (Fig. 1A, bar 5 versus bar 1, and B, bar 2 versus bar 1). Thus, IFN-γ might promote ZIKV replication in infected cells.

Although ZIKV and its NS5 protein were shown to suppress the induction of ISGs by IFN-α (32, 33), the levels of some ISGs were still elevated in ZIKV-infected cells (17, 22, 24, 30, 31). The suppression of type I IFN signaling by ZIKV might be incomplete. Alternatively, ZIKV might exert differential effects on different subsets of ISGs and selectively induce some of them. In support of the latter model, some IFN-γ-induced ISGs, such as the CXCL10 gene, are indeed upregulated in ZIKV-infected cells (22). To clarify the impacts of ZIKV on type I and type II IFN signaling, we next asked how ZIKV infection might affect the induction of representative ISGs by IFN-β and IFN-γ in JEG3 and SF268 cells.

The transcription of the MxA, OAS1, and ISG15 genes, which are regulated primarily by type I IFNs and are critically involved in antiviral responses, was slightly induced in ZIKV-infected JEG3 cells (Fig. 1C to E, bars 2 versus bars 1 and 3). Consistent with previous findings obtained with the ZIKV Uganda strain in HEK293T cells (33) or the ZIKV PLCal strain in A549 cells (34), infection of JEG3 cells with a clinical isolate of ZIKV effectively suppressed IFN-β-induced activation of MxA, OAS1, and ISG15 gene transcription (Fig. 1C to E, bars 4 versus bars 3). In stark contrast, ZIKV infection not only induced the expression of IRF1 and CXCL10 mRNAs in JEG3 and SF268 cells (Fig. 1F to H, bars 2 versus bars 1) but also potentiated IFN-γ-induced expression of these transcripts (after deduction of the stimulatory effect of IFN-γ on viral replication) (Fig. 1F to H, bars 4 versus bars 3). The potentiating effect was most prominent in the case of CXCL10 mRNA in SF268 cells (Fig. 1G), implicating a role for this chemokine in neuropathogenesis. For comparison, we used Sendai virus as a control. STAT1 is a primary IFN signaling target of Sendai virus (42). Both IRF1 and CXCL10 were induced by Sendai virus in SF268 cells (Fig. 1F and G, bars 5 versus bars 1). It remained to be seen whether this induction might be mediated by IFN-γ or other pathways, such as NF-κB (43). However, IFN-γ-induced expression of IRF1 and CXCL10 was weakened in Sendai virus-infected SF268 cells (Fig. 1F and G, bars 6 versus bars 2 and 5). Thus, selective augmentation of IFN-γ signaling was unique to ZIKV and was not seen in Sendai virus-infected cells. This augmentation was also observed in JEG3 (Fig. 1H) and HFL (Fig. 1I) cells. Since HFL cells are normal fibroblasts derived from human embryonic lung tissue (12), the stimulatory effect of ZIKV is not due to abnormal activation of IFN-γ signaling in human cancer cell lines. Taken together, these data show that ZIKV exerts opposite effects on IFN-β and IFN-γ signaling.

To verify the impact of IFN-γ on ZIKV replication, we harnessed AG490 (44), a small-molecule inhibitor of JAK2, a protein which specifically mediates IFN-γ signaling but is not required for type I IFN signaling (37, 45). When we treated ZIKV-infected JEG3 cells with AG490, the steady-state level of viral RNA decreased (Fig. 2A, bar 2 versus bar 1). In view of the possibility that JAK2 might be involved in other JAK-STAT signaling pathways (45), we further verified our result by using RNA interference (RNAi). Two independent and prevalidated small interfering RNAs (siRNAs) directed against IFNGR2, a key component of the IFN-γ receptor (37, 46), were employed to knock down IFN-γ signaling more specifically. Effective knockdown of IFNGR2 mRNA by these two siRNAs (Fig. 2B) resulted in attenuation of ZIKV RNA replication (Fig. 2C). These results lend

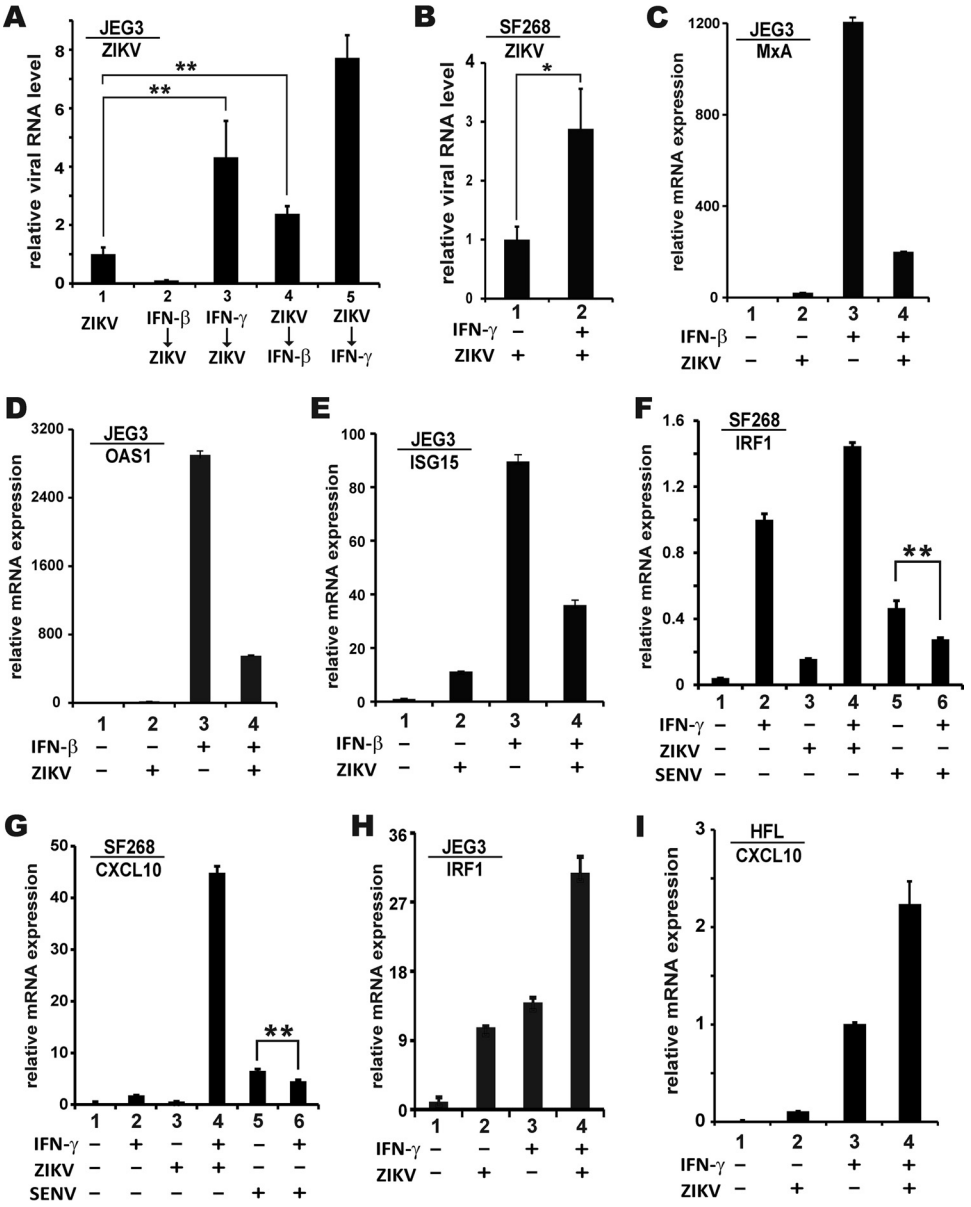

FIG 1 Opposite effects of ZIKV on IFN-β and IFN-γ signaling. (A) Effects of IFN-β and IFN-γ on ZIKV RNA replication in JEG3 cells. Cells were pretreated with 1,000 U/ml of IFN-β (bar 2) or 50 ng/ml of IFN-γ (bar 3) for 12 h and then infected with ZIKV at an MOI of 2 for 36 h. Alternatively, cells were infected with ZIKV for 24 h and then treated with IFN-β (bar 4) or IFN-γ (bar 5) for 12 h. The level of viral RNA was measured by quantitative RT-PCR and normalized to that of GAPDH mRNA. Bars represent the means for three biological replicates, and error bars indicate SD. The differences between bars 2 and 3 and between bars 2 and 4 were statistically significant (**) by Student's *t* test, with *P* values of 0.0029 and 0.0053, respectively. (B) Effect of IFN-γ on ZIKV RNA replication in SF268 cells. Cells were infected for 24 h and then treated with IFN-γ for 12 h. A statistically significant difference (*) between bars 1 and 2 was found by Student's *t* test ($P = 0.016$). (C to E) Suppression of IFN-β signaling by ZIKV. JEG3 cells were infected with ZIKV at an MOI of 2 for 24 h and then treated with 1,000 U/ml of IFN-β for 12 h. ISG transcripts were analyzed by quantitative RT-PCR. The level of ISG mRNA was normalized to that of GAPDH mRNA. Results represent the means $\pm$ SD for three biological replicates. (F to I) Augmentation of IFN-γ signaling by ZIKV. SF268, JEG3, and HFL cells were infected with ZIKV for 24 h at MOIs of 6, 2, and 3, respectively. Infected cells were then treated with 50 ng/ml of IFN-γ for 12 h. The level of RNA expression was normalized to that of GAPDH mRNA. The levels of IRF1 and CXCL10 transcripts were further normalized to that of ZIKV RNA. Infection with Sendai virus (SENV) was performed at an MOI of 10. The differences between bars 5 and 6 in panels F and G were statistically significant (**) as judged by Student's *t* test, with *P* values of 0.0017 and 0.001, respectively.

further support to the notion that IFN-γ signaling exerts a positive effect on ZIKV replication.

Both the IRF1 and CXCL10 genes play an important role in the proinflammatory response (47, 48), and their expression was induced by IFN-γ (Fig. 1F to I). To determine

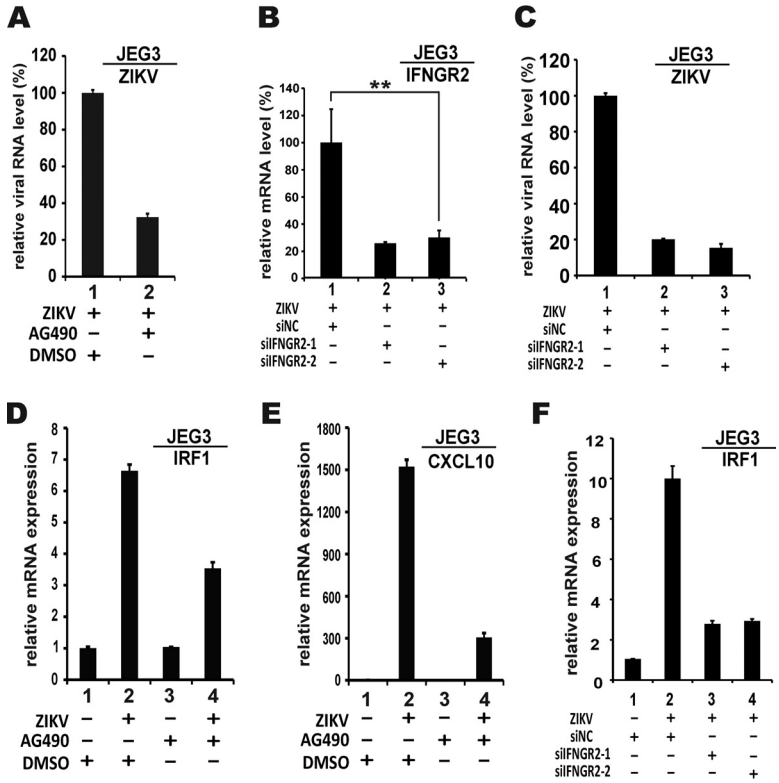

FIG 2 Blocking of IFN-γ signaling inhibits ZIKV RNA replication and ZIKV-induced activation of ISGs. (A) Effect of AG490 on ZIKV replication. JEG3 cells were infected with ZIKV at an MOI of 2 for 24 h and then treated with 50 μM AG490 for 12 h. Viral RNA was measured by quantitative RT-PCR. (B and C) Effect of siIFNGR2 on ZIKV replication. Two independent siRNAs (siIFNGR2-1 and siIFNGR2-2) were transfected into JEG3 cells. After 72 h, cells were infected with ZIKV at an MOI of 2. IFNGR2 mRNA and viral RNA were measured by quantitative RT-PCR. The difference between the values for bars 1 and 3 in panel B was statistically significant (**) by Student's *t* test ($P = 0.0011$). (D to F) Effects of AG490 and siIFNGR2 on ISG induction. The levels of IRF1 and CXCL10 transcripts in infected cells were further normalized to the amount of ZIKV RNA. All data points represent the means for three biological replicates, with error bars denoting SD.

whether ZIKV-induced expression of the IRF1 and CXCL10 genes was mediated by IFN-γ, JEG3 cells were treated with AG490, and the levels of IRF1 and CXCL10 transcripts relative to viral RNA levels were determined. Treatment with AG490 dampened ZIKV-induced activation of IRF1 and CXCL10 gene transcription (Fig. 2D and E, bars 4 versus bars 2), indicating that ZIKV activated these genes through IFN-γ. Similar results were also obtained with the two IFNGR2-silencing siRNAs (Fig. 2F, bars 3 and 4 versus bar 2). Thus, IFN-γ signaling was activated in ZIKV-infected cells.

Opposite effects of ZIKV NS5 protein on type I and type II IFN signaling. The differential impacts of ZIKV infection on type I and type II IFN signaling prompted us to identify the viral proteins that mediate these impacts. Because one primary function of flaviviral NS proteins is to subvert the host defense (32), we started with the seven NS proteins of ZIKV. Generally consistent with a recent report (34), NS5 was the most potent inhibitor of IFN-β-induced activation of ISRE-dependent luciferase (ISRE-Luc) activity among all NS proteins (Fig. 3A, bar 16). In addition, NS1 and NS2B might also have weak to moderate suppressive effects on ISRE-Luc (Fig. 3A, bars 4 and 8). Interestingly, NS5 was also the most prominent activator of IFN-γ-induced activation of GAS-Luc activity (Fig. 3B, bars 15 and 16), although NS2A and NS4B might also have weak stimulatory effects. Hence, expression of NS5 alone was sufficient for inhibition of IFN-β signaling and concurrent activation of IFN-γ signaling.

The suppressive effect of ZIKV NS5 on IFN-β-induced activation of ISRE activity was dose dependent (Fig. 4A) and was also comparable to that of severe fever with

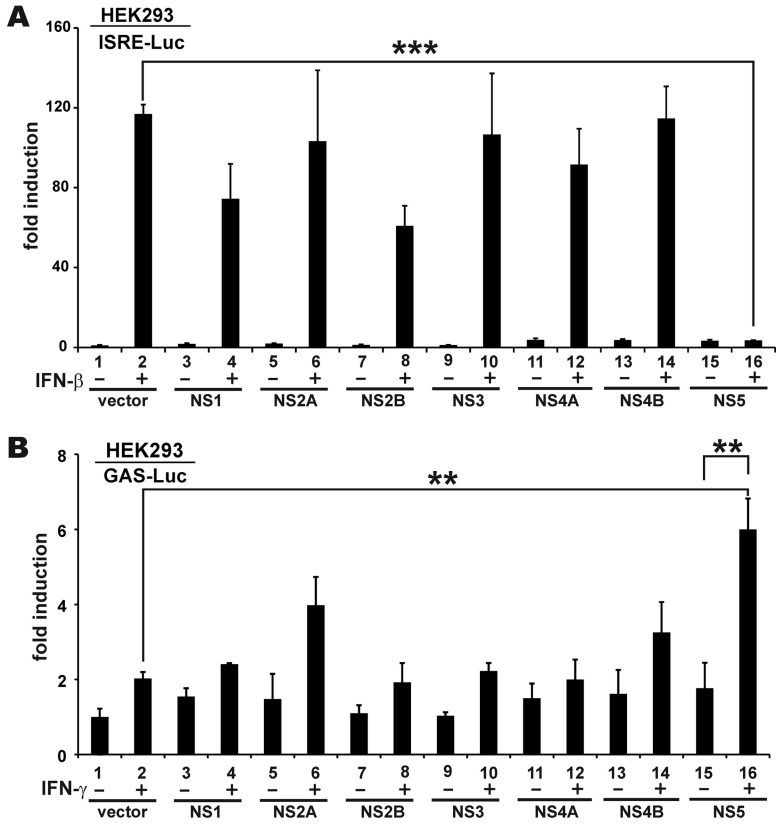

FIG 3 Influence of ZIKV NS proteins on IFN-β and IFN-γ signaling. (A) Influence of ZIKV NS proteins on IFN-β signaling. HEK293 cells were transfected with an ISRE-Luc reporter plasmid and expression vectors for ZIKV NS proteins. Cells were treated with 1,000 U/ml of IFN-β 24 h after transfection, for an additional 24 h, and then a dual-luciferase reporter assay was performed. Bars represent the means for three biological replicates, and error bars indicate SD. The difference between bars 2 and 16 was statistically significant (***) by Student's *t* test ($P = 0.000002$). (B) Influence of ZIKV NS proteins on IFN-γ signaling. HEK293 cells were transfected with a GAS-Luc reporter plasmid and treated with 50 ng/ml of IFN-γ. The differences between bars 2 and 16 and between bars 15 and 16 were statistically significant (**) by Student's *t* test ($P = 0.0012$ and $P = 0.0017$, respectively).

thrombocytopenia syndrome virus (SFTSV) NSs (Fig. 4B, bar 4 versus bar 5), a known inhibitor of type I and type III IFN signaling (49). Type III IFNs, including IFN-λ1, IFN-λ2, and IFN-λ3, are known to play an important role in antiviral responses, particularly in ZIKV infection (21). They also share with type I IFNs the same signaling pathway to activate target genes (50). We therefore investigated whether ZIKV NS5 might also suppress IFN-λ1 signaling. Indeed, ZIKV NS5 effectively blunted IFN-λ1-induced activation of ISRE (Fig. 4C, bar 4 versus bar 2). This suppressive activity was also comparable to that of the SFTSV NSs protein (Fig. 4C, bars 4 and 5). Interestingly, ZIKV NS5 and SFTSV NSs also shared the ability to potentiate IFN-γ-induced activation of GAS-Luc activity (Fig. 4D, bars 3 and 4). In light of the importance of NF-κB activation in innate immunity and viral infection, we extended our analysis to address whether ZIKV NS5 might also modulate NF-κB signaling. We found that NS5 had no influence on tumor necrosis factor alpha (TNF-α)-induced activation of NF-κB activity in HEK293 cells (Fig. 4E). That is, the differential effect of ZIKV NS5 on IFN signaling is specific, and the induction of ISGs, such as the IRF1 and CXCL10 genes, is unlikely to be mediated through NF-κB. Collectively, our results indicate that ZIKV NS5 is capable of suppressing type I and type III IFN signaling but activating type II IFN signaling.

ZIKV NS5 induces STAT2 ubiquitination and destabilization. STAT1 and STAT2 are the master transcriptional activators that mediate type I and type II IFN signaling (37, 45). To investigate the mechanism by which ZIKV NS5 differentially modulates type I and type II IFN signaling, we examined the steady-state expression of STAT1 and

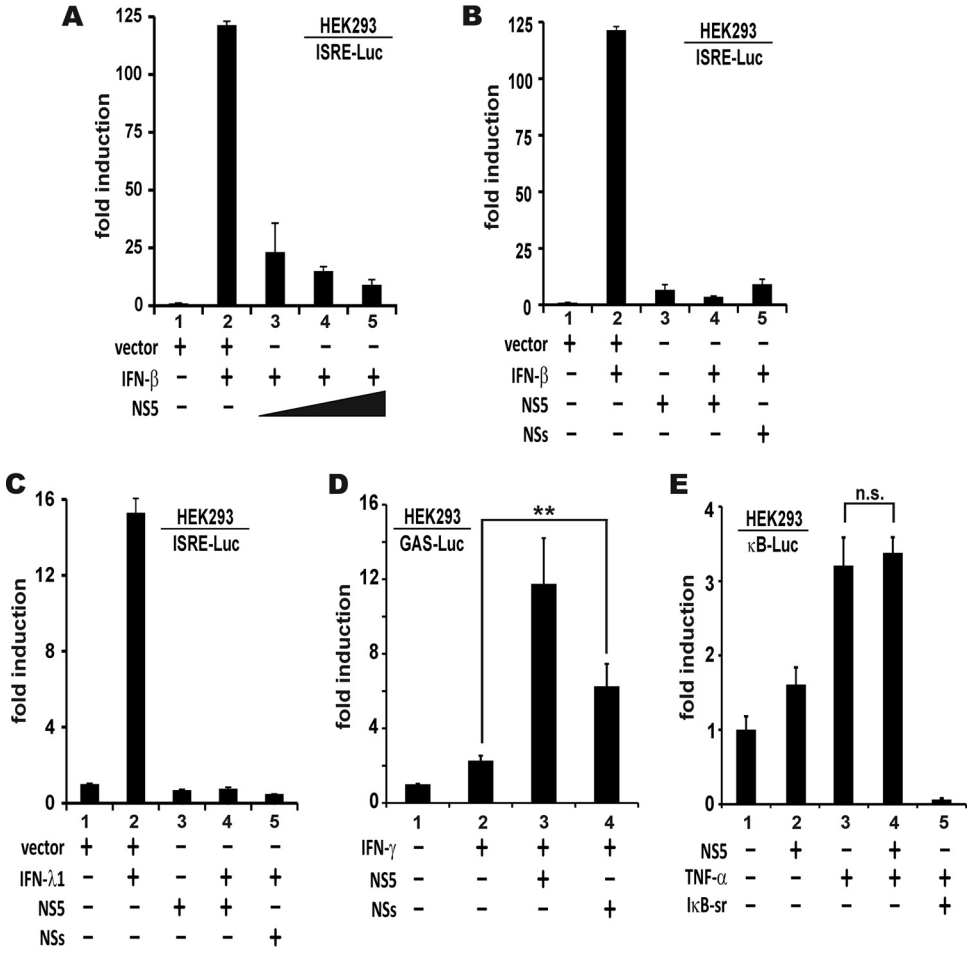

FIG 4 Differential modulation of IFN-β and IFN-γ signaling by ZIKV NS5 protein. (A to C) Suppression of IFN-β and IFN-λ1 signaling by NS5. (A) Progressively increasing doses of NS5 plasmid were used. (B) SFTSV NSs served as a positive control. (C) Cells were treated with 100 ng/ml IFN-λ1 for 24 h. (D) Augmentation of IFN-γ signaling by NS5. Cells were treated with 50 ng/ml IFN-γ for 24 h. A statistically significant difference (**) was found between bars 2 and 4 ($P = 0.0023$). (E) Influence of NS5 on NF-κB activation. Cells were transfected with pκB-Luc. Cells were cotransfected with the NS5 plasmid, treated with 10 ng/ml of TNF-α, or cotransfected with the IκB-sr plasmid for 24 h. IκB-sr served as a positive control in this assay. The difference between bars 3 and 4 was statistically not significant (n.s.) as judged by Student's *t* test ($P = 0.57$). All results are the means $\pm$ SD for three biological replicates.

STAT2 in NS5-expressing HEK293 cells. Generally consistent with previous findings (33, 34), NS5 selectively destabilized STAT2 but had no influence on STAT1 protein stability (Fig. 5A, lane 2 versus lane 1). The destabilizing effect on STAT2 occurred at the protein level, since NS5 did not affect the steady-state amount of STAT2 mRNA (Fig. 5B, bar 2 versus bar 1) and the treatment of cells with the proteasome inhibitor MG132 reversed the phenotype (Fig. 5C, lane 4 versus lane 2). Immunoprecipitation assay confirmed the association of NS5 with STAT2 but not STAT1 (Fig. 5D, lane 2 versus lane 1).

The existing results supported a model in which NS5 might affect ubiquitination of STAT2. To provide direct evidence, an *in vivo* polyubiquitination assay was performed. NS5 was immunoprecipitated using anti-V5. Both NS5 and STAT2 were present in the precipitate (Fig. 5E). To assess whether NS5-associated STAT2 was polyubiquitinated, myc-tagged ubiquitin was probed with anti-myc. While basal polyubiquitination of STAT2 was undetectable, a polyubiquitination smear was evident when NS5 was expressed (Fig. 5E, lane 3 versus lane 1). In addition, the K0 and K48R mutants of ubiquitin could not support NS5-induced polyubiquitination of STAT2, whereas the polyubiquitination ladder was still visible when the K63R mutant of ubiquitin was expressed (Fig. 5E, lane 6 versus lanes 4 and 5). These results are consistent with the notion that NS5 induces K48- but not K63-linked polyubiquitination of STAT2.

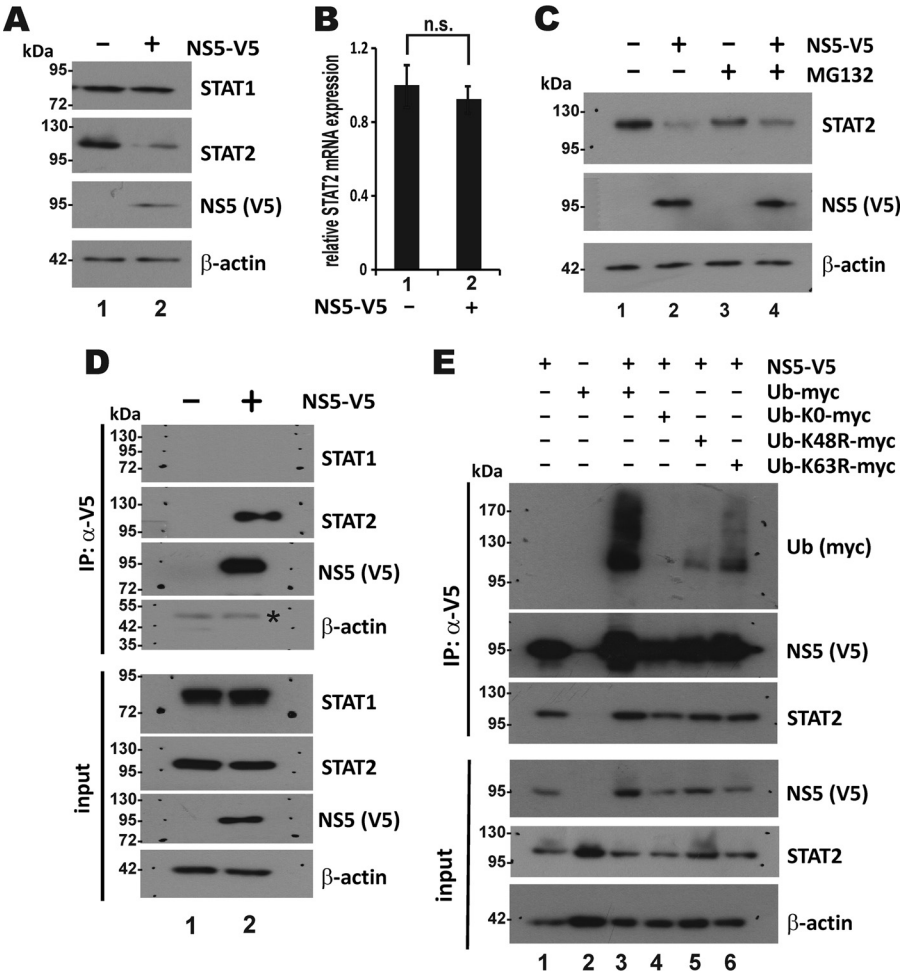

FIG 5 ZIKV NS5 protein selectively induces STAT2 ubiquitination and degradation. (A to C) STAT2 destabilization by NS5. ZIKV NS5 was overexpressed in HEK293 cells for 48 h. Cell lysates were then collected for STAT1 and STAT2 protein analysis by Western blotting (A) and for STAT2 mRNA measurement by quantitative RT-PCR (B). (C) Cells were treated with 10 μM MG132 for 6 h before harvest. The difference between bars 1 and 2 in panel B was statistically not significant (n.s.) as judged by Student's *t* test ($P = 0.067$). (D) Interaction of NS5 with STAT2. Cell lysates were collected for immunoprecipitation (IP) with an anti-V5 antibody. Input lysates and precipitates were analyzed by Western blotting. The band for the immunoglobulin heavy chain is highlighted with an asterisk. (E) NS5 induces K48-linked polyubiquitination of STAT2. The indicated proteins were expressed in HEK293 cells for 48 h. Immunoprecipitation (IP) of NS5-containing protein complexes was performed with an anti-V5 antibody. Input lysates and precipitates were analyzed by Western blotting. Results are representative of four biological replicates.

We next compared the activation levels of STAT1 and STAT2 in JEG3 and HeLa cells expressing ZIKV NS5. In these cells treated with IFN-β, STAT1 and STAT2 were activated and translocated into the nucleus (Fig. 6A and B). Whereas the intensity of nuclear STAT1 staining was unaffected when NS5 was also expressed (Fig. 6A and B, panels 1 and 4, transfected versus nontransfected cells), STAT2 disappeared from NS5-expressing cells (Fig. 6A and B, panels 5 and 8, transfected versus nontransfected cells). Likewise, for cells treated with IFN-γ, nuclear staining of STAT1 was even more prominent in NS5-expressing cells (Fig. 6C, panels 5 and 8 versus panels 1 and 4, transfected versus nontransfected cells in the same panel), whereas no staining of STAT2 was seen (data not shown). Thus, NS5 promoted degradation of STAT2 but had no influence on STAT1 stability or nuclear translocation.

ZIKV NS5 promotes STAT1 homodimerization and recruitment to ISG promoters. The STAT1 protein can form both heterotrimeric and homodimeric complexes to activate ISRE and GAS, respectively (37, 45). An ambient balance between these two complexes might be reached inside the cell but broken in response to type I and type

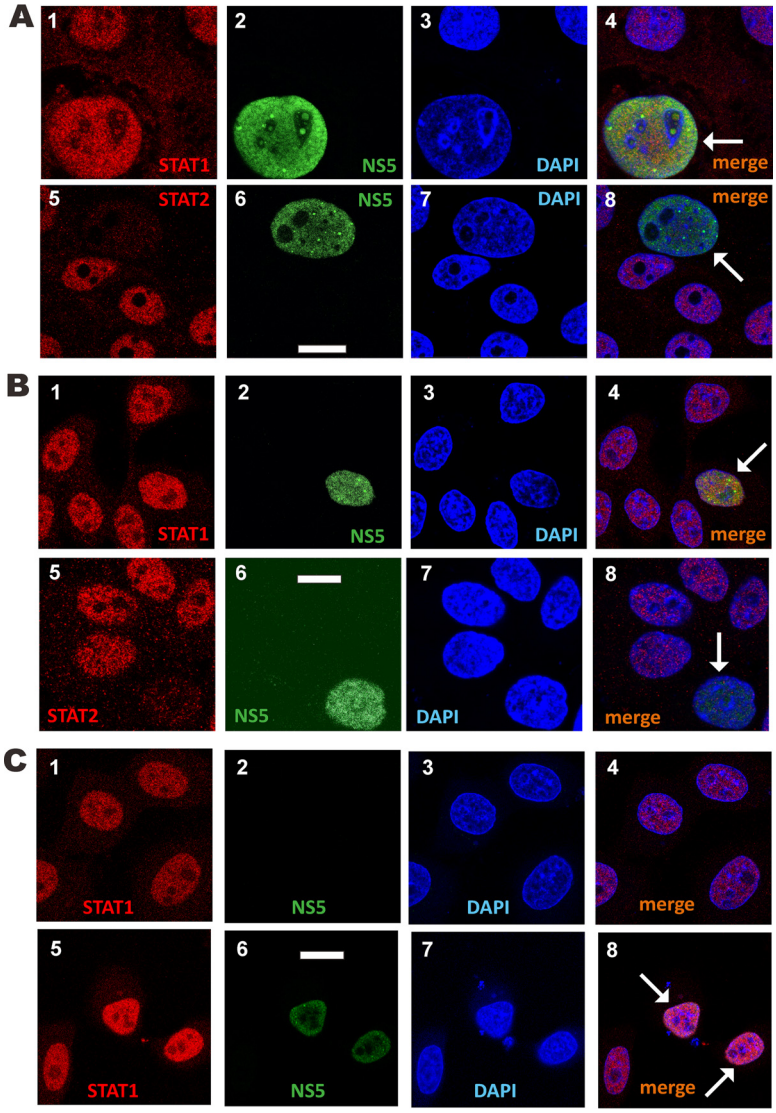

FIG 6 Influence of ZIKV NS5 protein on STAT1 and STAT2 nuclear translocation. (A and B) NS5 was expressed in JEG3 (A) and HeLa (B) cells for 24 h and then treated with 1,000 U/ml of IFN-β for 30 min. (C) HeLa cells were mock transfected or transfected with NS5 plasmid for 24 h and then treated with 50 ng/ml of IFN-γ for 30 min. Cells were fixed and stained with anti-V5 for NS5, anti-STAT1, and anti-STAT2. Fluorescence signals of different colors were merged with DAPI staining for detection of nuclear morphology. Arrows highlight transfected cells. Bars, 20 μm. Results are representative of three independent experiments.

II IFNs (38). Whereas a decrease in STAT2 protein level would certainly affect the formation and activity of the STAT1-STAT2-IRF9 complex that activates ISRE, it might also tip the balance between STAT1-STAT2-IRF9 and STAT1-STAT1 complexes to the latter, leading to activation of IFN-γ signaling. To determine whether this might explain the activation of IFN-γ signaling by ZIKV NS5, we expressed differentially tagged STAT1 and V5-tagged NS5 proteins in HEK293T cells and performed a coimmunoprecipitation assay to check for STAT1-STAT1 complex formation. Expression of NS5 correlated with destabilization of endogenous STAT2 but did not affect the steady-state levels of exogenously expressed STAT1-myc or STAT1-Flag (Fig. 7A, lanes 3 to 5). When we pulled down STAT1-Flag with anti-Flag, STAT1-myc was detected in the precipitate only when cells were treated with IFN-γ (Fig. 7A, lane 2 versus lane 1). In addition, when we increased the expression of NS5, increasing amounts of STAT1-myc were found in the precipitate with a fixed amount of STAT1-Flag (Fig. 7A, lanes 3 to 5). Thus, NS5

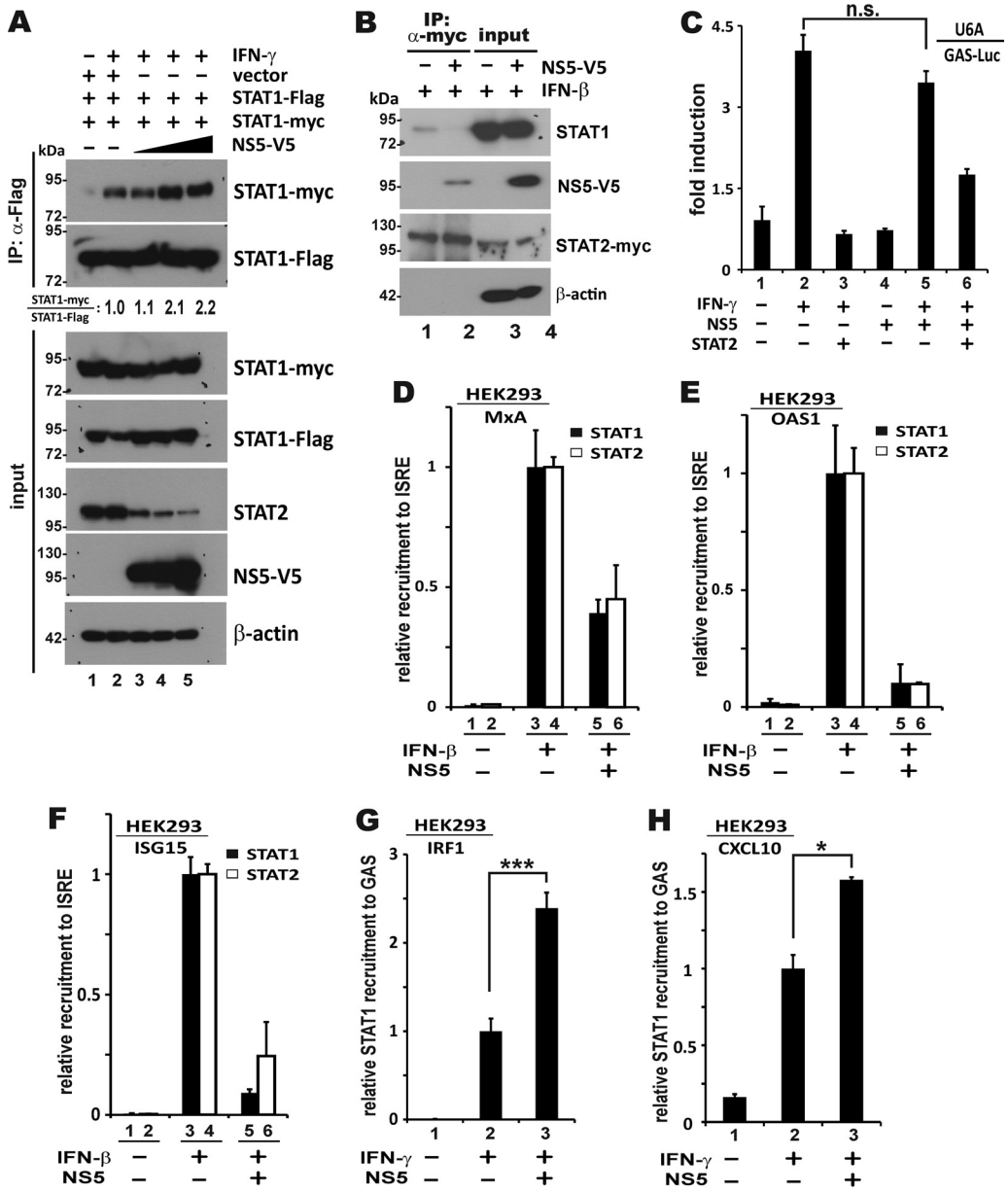

FIG 7 Differential effect of ZIKV NS5 on STAT1 and STAT2 recruitment and activity. (A) NS5 promotes STAT1-STAT1 homodimerization. NS5 and differentially tagged STAT1 proteins were expressed in HEK293T cells for 24 h. Cells were treated with 50 ng/ml IFN-γ for another 24 h. Cell lysates were collected, and immunoprecipitation (IP) was performed with anti-Flag. Input lysates and precipitates were analyzed by Western blotting. Relative ratios of STAT1-myc to STAT1-Flag were determined by densitometry and are indicated below the top two blots. Results are representative of four independent experiments. (B) NS5 affects STAT1-STAT2 complex formation. NS5 and STAT2-myc were expressed in HEK293T cells for 24 h. Cells were treated with 1,000 U/ml of IFN-β for another 24 h. IP was carried out with anti-myc, and precipitates were probed for STAT1 and NS5. (C) Effect of NS5 on GAS activity in STAT2-deficient U6A cells. Cells were transfected with the indicated reporter and expression plasmids for 24 h. Cells were then treated with 50 ng/ml of IFN-γ for 24 h, and a dual-luciferase reporter assay was performed. The difference between bars 2 and 5 was statistically not significant (n.s.) by Student's *t* test ($P = 0.081$). (D to H) ZIKV NS5 differentially affects STAT1 and STAT2 recruitment to ISGs. (D to F) The recruitment of STAT1 and STAT2 to ISRE in the MxA, OAS1, and ISG15 gene promoters in IFN-β-treated HEK293 cells was analyzed by ChIP with anti-STAT1 and anti-STAT2. (G and H) STAT1 recruitment to GAS in the IRF1 and CXCL10 gene promoters in IFN-γ-treated HEK293 cells was also assessed. Anti-GFP was used for normalization. The differences between bars 2 and 3 in panels G and H were statistically significant by Student's *t* test, with *P* values of 0.00045 (***) and 0.012 (*), respectively. All data points are the means ± SD for three biological replicates.

promoted the interaction of STAT1-Flag with STAT1-myc. In other words, STAT1-STAT1 homodimerization was potentiated.

In contrast, when we treated HEK293T cells with IFN-β and pulled down the STAT2-containing complex, STAT1 and NS5 appeared to be mutually exclusive in this

complex. As such, a weak STAT1 band was seen in the absence of NS5, whereas STAT1 was not detected when NS5 was found (Fig. 7B, lane 2 versus lane 1). That is, NS5 blocked IFN-β-induced formation of the STAT1-STAT2 complex but facilitated IFN-γ-induced assembly of the STAT1-STAT1 complex.

To address how STAT1 activity was affected in the absence of STAT2, we harnessed STAT2-deficient U6A cells (51). The activation of GAS-Luc activity by IFN-γ in U6A cells was robust and was ablated by reexpression of STAT2 (Fig. 7C, bar 3 versus bar 2). The inhibitory effect of STAT2 on IFN-γ signaling observed here is consistent with recent findings reported in the literature (52). Interestingly, the expression of NS5 in U6A cells did not enhance IFN-γ-induced activation of GAS-Luc (Fig. 7C, bar 5 versus bar 2). These results are compatible with a model in which NS5 boosts IFN-γ signaling by destabilizing STAT2. Consistent with this, the potentiating effect of NS5 on IFN-γ activation of GAS was seen when STAT2 was overexpressed (Fig. 7C, bar 6 versus bar 3). Collectively, our results suggest that NS5 destabilizes STAT2 to potentiate STAT1-mediated activation of IFN-γ signaling.

The above model predicts that IFN-β-induced recruitment of STAT1-STAT2-IRF9 to ISRE is compromised, whereas IFN-γ-induced recruitment of STAT1-STAT1 to GAS is augmented, in NS5-expressing cells. To verify this, chromatin immunoprecipitation (ChIP) was performed with anti-STAT1 and anti-STAT2 antibodies. Indeed, the recruitment of STAT1 and STAT2 to ISRE in the MxA, OAS1, and ISG15 gene promoters in IFN-β-treated HEK293 cells was dampened when NS5 was expressed (Fig. 7D to F, bars 5 and 6 versus bars 3 and 4). In sharp contrast, the recruitment of STAT1 to the GAS in the IRF1 and CXCL10 gene promoters in IFN-γ-treated HEK293 cells expressing NS5 was boosted (Fig. 7G and H, bars 3 versus bars 2). Thus, our results consistently support the hypothesis that NS5 exerts opposite effects on type I and type II IFN signaling.

DISCUSSION

In this study, we demonstrated selective activation of IFN-γ signaling by ZIKV and its NS5 protein. ZIKV infection had opposite effects on IFN-β- and IFN-γ-induced activation of ISGs (Fig. 1), and this phenomenon was ascribed to the NS5 protein (Fig. 2 and 3). NS5 interacted with and destabilized STAT2 but not STAT1 (Fig. 4 and 5). Compromising STAT2 had a significant impact on the formation of not only STAT1-STAT2-IRF9 but also STAT1-STAT1 complexes, leading to differential modulation of type I and type II IFN signaling (Fig. 6). These findings, together with other recent findings reported in a related paper (52), are depicted in Fig. 8. In this model, STAT2 binds to unphosphorylated and phosphorylated STAT1 to prevent its nuclear translocation, leading to inhibition of IFN-γ signaling.

Although the suppression of STAT1 and STAT2 signaling by flaviviral NS5 proteins and other viral IFN antagonists has been well described (32–34, 53, 54), ZIKV NS5 is the first example of a viral protein that concurrently suppresses type I and type III IFN signaling but activates type II IFN signaling. This reveals another level of complexity in the interaction between viruses and host immunity. As a result, the expression of different subsets of ISGs will be affected differentially in ZIKV-infected cells, leading to preferential induction of some immunomodulatory and proinflammatory ISGs. Our ongoing mRNA profiling and transcriptomic studies in infected cells and animals should provide further evidence to clarify this issue. Our results also indicated the same ability of another viral IFN antagonist, i.e., SFTSV NSs, to differentially modulate type I to type III IFN signaling. In view of the similarity between ZIKV NS5 and its counterparts in dengue viruses (33, 55), it is not too surprising that they might also share this ability to exert opposite regulatory effects on type I and type II IFN signaling. Further investigations will elucidate whether this is a common feature of additional RNA and DNA viruses. The balance between type I and type II IFN signaling in virus-infected cells was previously recognized and was shown to be tightly regulated by cellular protein kinases, such as IKKε (56). The interplay between ZIKV NS5 and IKKε merits further analysis.

In our study, we did not find any major difference between the Puerto Rican and

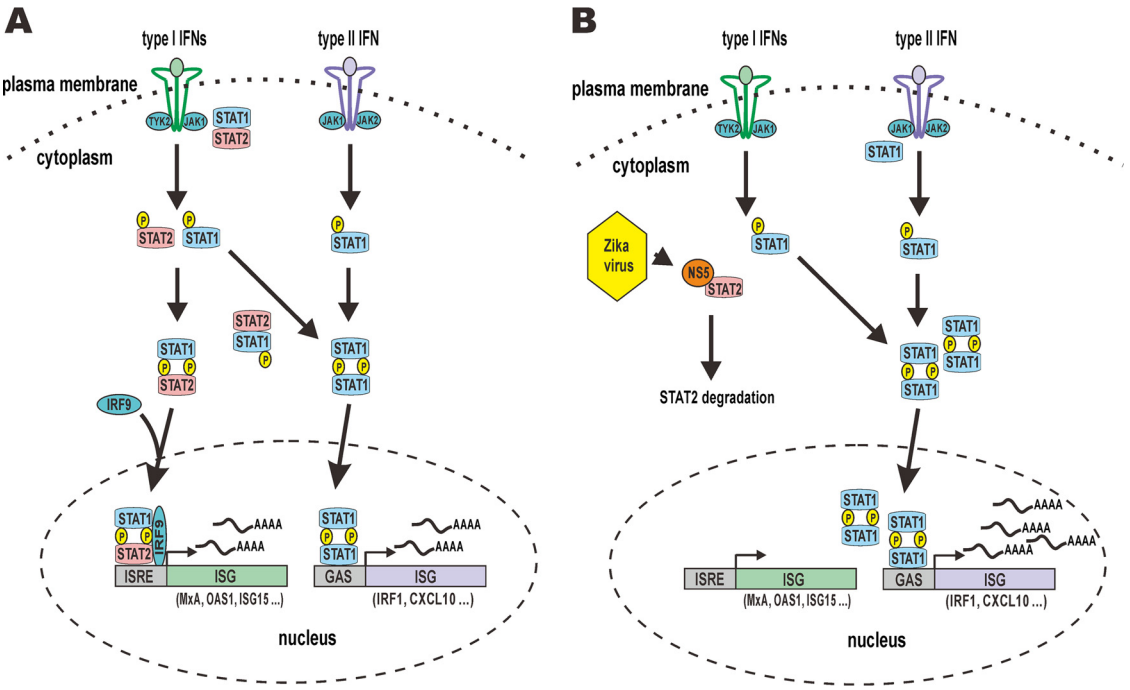

FIG 8 Working model for differential regulation of type I and II IFN signaling by ZIKV NS5 protein. (A) Uninfected cell. STAT1 and STAT2 phosphorylation is induced by type I and type II IFNs, leading to nuclear translocation and transcriptional activation of ISGs under the control of ISRE and GAS. Unphosphorylated STAT2 can also bind to unphosphorylated and phosphorylated STAT1 to prevent its nuclear translocation (52). (B) ZIKV-infected cell. Degradation of STAT2 by ZIKV NS5 relieves the inhibition of STAT1, leading to augmentation of STAT1 homodimerization, nuclear translocation, and selective transcriptional activation of ISGs under the control of GAS.

Ugandan strains of ZIKV (data not shown), but emerging findings in the literature have begun to reveal strain differences in replication kinetics, proinflammatory cytokine induction, and pathogenicity (57, 58). On the other hand, tissue and cell type differences of ZIKV infection have been highlighted by the differential activation patterns of IFN signaling, JAK-STAT signaling, and proinflammatory responses in human maternal decidual tissues and dendritic cells (57, 59). Full documentation of these differences and their biological significance in human infection awaits further study.

IFN-γ is an important mediator of immune and proinflammatory responses (39). IFN-γ is secreted by activated immune cells but acts on various tissues and cells, including those targeted by ZIKV, particularly trophoblastic epithelium (60). IFN-γ has also been shown to be overproduced in ZIKV-infected animals (61, 62). ZIKV replicates robustly in AG129 mice deficient for both IFN-α and IFN-γ (63). We found, surprisingly, that treatment of JEG3 and SF268 cells with IFN-γ before and after ZIKV infection augmented viral RNA replication. In addition, treatment of JEG3 cells with AG490 or siIFNGR2 showed the opposite effect. Thus, AG490 and other specific inhibitors of JAK2 and IFN-γ signaling are anti-ZIKV agents that might prove useful in the treatment of ZIKV infection. This should be evaluated further, as in our recent work on bromocriptine (64). In a recent study, pretreatment of A549 cells with IFN-γ for 6 h before ZIKV infection was found to inhibit viral replication by 50%, but treatment with IFN-γ for 12 h starting from 6 h after infection had no inhibitory effect on viral replication (34). Whether a lower multiplicity of infection (MOI) and the different cell lines used in our study might allow observation of the moderate stimulatory effect of IFN-γ on ZIKV replication remains to be determined. IFN-γ is known to induce the expression of some cellular factors that facilitate ZIKV replication. In particular, the ZIKV entry factors AXL, Tyro3, and DC-SIGN are encoded by IFN-γ-stimulated genes (22, 65). In addition, inflammation and cell death induced by IFN-γ may also facilitate the dissemination of ZIKV *in vivo* (25, 26). Elucidation of the mechanism by which IFN-γ augments ZIKV infection will pave the way for further research and drug discovery.

Suppression of the antiviral response and aberrant induction of proinflammatory cytokines are characteristic of different viruses that cause severe diseases (29, 53). To hit two birds with one stone, ZIKV might employ NS5 both to evade the type I IFN-dependent antiviral response and to exacerbate IFN-γ-mediated inflammation. In addition, selective activation of IFN-γ signaling by NS5 might also have an impact on other IFN-γ-regulated immune functions, such as macrophage activation and Th1 responses. A better understanding of the implications of NS5-induced activation of IFN-γ signaling in ZIKV biology and pathogenesis might provide new strategies for therapeutic intervention. In particular, it will be of interest to determine whether inhibition of IFN-γ signaling by use of JAK2 inhibitors, such as AG490, or other agents, including siRNAs, might have antiviral, anti-inflammatory, and other beneficial effects in severe cases of ZIKV infection.

Our study not only unravels a previously unrecognized role of ZIKV NS5 in selective activation of IFN-γ signaling but also provides a new mechanism by which compromising STAT2 results in the promotion of STAT1 homodimerization. STAT2 is known to play a unique role in the antiviral response and to be targeted by viruses (66, 67). We showed that a reduction or complete loss of STAT2 in NS5-expressing or STAT2-deficient cells had a potentiating effect on STAT1 homodimerization and recruitment to GAS. Although this phenotype was not recognized or reported in the original study on Stat2$^{-/-}$ mice (68), our reanalysis of the published data revealed that IFN-γ signaling might indeed be enhanced in the absence of STAT2. For example, the IGTP and IRF1 bands in the Northern blots in Fig. 3B of that report were more pronounced in Stat2$^{+/-}$ and Stat2$^{-/-}$ cells than in Stat2$^{+/+}$ cells. In Fig. 4B of that report, IRF1, CXCL10, and MIG transcripts were also more abundant in Stat2$^{-/-}$ cells. Thus, STAT2 deficiency should be a general mechanism by which IFN-γ signaling is augmented. As such, STAT1 homodimerization and IFN-γ signaling would also be activated in cells infected with other viruses capable of downregulating STAT2. On the contrary, our finding that overexpression of STAT2 sufficiently inhibits IFN-γ signaling is generally in keeping with the new model in which unphosphorylated STAT2 binds to unphosphorylated and phosphorylated STAT1 to prevent its nuclear translocation and activation (52).

ZIKV was recently found to replicate well in Stat2$^{-/-}$ mice and hamsters (58, 69). Viral infection of different organs and some of the neurological symptoms seen in human infection can be recapitulated in these models. Since IFN-γ signaling will be selectively activated by ZIKV in these animals, they might provide a unique opportunity to assess how this activation contributes to viral pathogenesis.

Another study showing the inhibition of type I IFN production and signaling by ZIKV and its NS5 protein was published recently (34). Pretreatment of cells with IFN-α or IFN-β was found to prevent ZIKV replication in both studies. IFN-α had no inhibitory effect on ZIKV replication when A549 cells were treated 6 h after ZIKV infection in that study. In our work, a slight increase in ZIKV RNA level was observed when JEG3 cells were treated with IFN-β 12 h after infection. One plausible interpretation of these results is that the antiviral effects of IFN-α and IFN-β could not be seen when viral replication was robust. Since ZIKV replicates better in JEG3 cells than in A549 cells (12), viral RNA continued to increase slightly in the presence of IFN-β in our experiment. Although it was not explicitly stated or discussed in the previous study, infection of A549 cells with ZIKV at an MOI of 3 was found to result in a >5-fold activation of the induction of IFIT1 transcripts by IFN-γ, as shown in Fig. 2B of that study. In that setting, cells were treated with 10 U/ml of IFN-γ for 12 h. IFN-γ induction of IFIT1 is known to be mediated primarily through GAS (70). Hence, this new piece of data lent further support to our notion that ZIKV further activates IFN-γ signaling. However, when the authors checked for the impact of ZIKV on IFN-γ-induced activation of GAS-Luc activity, as shown in Fig. 2C of their study, A549 cells were treated with 10 U/ml of IFN-γ for only 2 h. In that scenario, infection with ZIKV at an MOI of 5 for 6 h neither activated nor suppressed the activity of IFN-γ on GAS-Luc. Unfortunately, there was no control to show the activation of GAS by IFN-γ in that experiment. In addition, it remained to be seen whether the time for treatment with IFN-γ was too short to have any effect on

luciferase expression. Indeed, when we repeated the same experiment in our A549 cells, in which ZIKV replication was less robust than in JEG3 and SF268 cells, as previously reported (12), treatment with IFN-γ for 12 h potently activated GAS-Luc, and GAS-Luc was further activated by ZIKV when cells were infected for 24 h at an MOI of 2 before IFN-γ treatment (data not shown). Thus, ZIKV further augmented the activation of GAS-Luc by IFN-γ in A549 cells in our setting. Further investigations are warranted to clarify the discrepancies in the two studies.

MATERIALS AND METHODS

Cell culture and transfection. HEK293, HEK293T, HeLa, and SF268 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. U6A cells were obtained from Public Health England and grown in DMEM with 5% L-glutamine and 10% FBS. JEG3, HFL, and Vero cells were propagated in minimum essential medium (MEM) with 10% FBS. Cells were transfected using GeneJuice (Novagen).

Virus. ZIKV Puerto Rico strain PRVABC59, originally isolated from a patient in the South American epidemic (71), was kindly provided by Brandy Russell and Barbara Johnson from the Centers for Disease Control and Prevention. For virus propagation, Vero cells grown for 24 h were infected with ZIKV for 1 h and maintained in MEM with 1% FBS. When cytopathic effects were evident on day 3 after infection, virus was harvested and spun at 2,500 $\times$ *g* for 15 min to remove cell debris. The clarified viral supernatant was aliquoted and frozen at −80°C. JEG, SF268, and HFL cells were infected with ZIKV for 1 h at MOIs of 2, 6, and 3, respectively. Infected cells were then maintained in culture medium with 1% FBS for 24 to 36 h. SF268 cells were infected with Sendai virus at an MOI of 10.

Plasmids, antibodies, IFNs, and chemicals. All seven NS genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) of ZIKV were PCR amplified from viral cDNA and cloned into the pCAGEN vector. STAT1 and STAT2 constructs (35) were kindly provided by James Darnell from Rockefeller University. pISRE-Luc, pGAS-Luc, and pκB-Luc were obtained from Clontech. An expression plasmid for an IκBα superrepressor (IκB-sr) with the serines at positions 32 and 36 replaced by alanines was obtained from EMD Millipore. Mouse anti-β-actin, rabbit and mouse anti-myc, and mouse anti-Flag antibodies were purchased from Sigma-Aldrich. Mouse anti-V5 was purchased from Invitrogen. Rabbit anti-STAT1 and anti-STAT2 antibodies were purchased from Santa Cruz Biotechnology. IFN-β was obtained from PBL Assay Science, IFN-λ1 and IFN-γ were from PeproTech, MG132 was from Sigma-Aldrich, and AG490 was from InvivoGen.

Luciferase reporter assay. Cells were harvested 48 h after transfection. A dual-luciferase reporter assay was performed as described previously (49, 72), using reagents supplied by Promega. The pSV-RLuc reporter was used as an internal control to normalize for transfection efficiency. Three independent experiments were carried out, and standard deviations (SDs) were calculated.

RNAi. siRNAs against IFNGR2 were purchased from Ambion (siRNAs s7197 and s7198). They were transfected into JEG3 cells 72 h before ZIKV infection by use of Lipofectamine 2000. The sequences of the siRNAs were 5′-CAACAUAUCUUGCUACGAAtt-3′ (sense) and 5′-UUCGUAGCAAGAUAUGUUGct-3′ (antisense) for IFNGR2-1 and 5′-CAUUAUCUCGUUUCCGGAAtt-3′ (sense) and 5′-UUCCGGAAACGAGAUAA UGga-3′ (antisense) for IFNGR2-2.

Western blotting and immunoprecipitation. Cells were harvested in immunoprecipitation buffer (50 mM Tris-Cl, pH 7.4, 250 mM NaCl, 1 mM EDTA, 1% NP-40, and 0.2% Triton X-100). Cell lysates were centrifuged and incubated with mouse anti-Flag and anti-V5 bound to protein G agarose (Invitrogen) overnight at 4°C. Antigen-antibody complexes were collected and washed three times with immunoprecipitation buffer. Proteins were resuspended in sample buffer (50 mM Tris-Cl, 2% SDS, 5% glycerol, 1% β-mercaptoethanol, and 0.002% bromophenol blue) and analyzed by Western blotting as described previously (49, 73).

ChIP. ChIP was performed as described previously (74, 75) 48 h after transfection. In brief, HEK293 cells were cross-linked with 1% formaldehyde for 10 min at room temperature. The cross-linking was stopped by the addition of 1 M glycine for 5 min. Cells were washed and then lysed by sonication in the presence of a protease inhibitor cocktail. Cell debris was removed by centrifugation. The DNA-protein complex was immunoprecipitated, and the DNA was extracted by use of phenol-chloroform. The ISRE sequences in the promoters of the MxA, OAS1, and ISG15 genes were analyzed by quantitative PCR with the primers 5′-GCAGAAATGAAACCGAAACTG-3′ and 5′-AAACACGGGCCTCAGGAT-3′ for MxA, 5′-TGCAA AAGGAAAGTGCAAAG-3′ and 5′-CAACAGAACTGCCTCCCAGA-3′ for OAS1, and 5′-TCCCTGTCTTTCGGTC ATT-3′ and 5′-CTTCAGTTTCGGTTTCCCTTT-3′ for ISG15. The GAS sequences in the promoters of the IRF1 and CXCL10 genes were analyzed with the primers 5′-GCTCTACAACAGCCTGATTTCC-3′ and 5′-CCAAAC ACTTAGCGGGATTC-3′ for IRF1 and 5′-AGCCAGCAGGTTTTGCTAAG-3′ and 5′-GGTGCTGAGACTGGAGG TTC-3′ for CXCL10. Relative recruitment levels were expressed as fold enrichment. The input of each sample was normalized to the signal obtained with anti-green fluorescent protein (anti-GFP). Fold enrichment was derived using the normalized input.

Quantitative reverse transcription-PCR (RT-PCR). Total RNA was extracted using RNAiso Plus reagents (TaKaRa). cDNA was synthesized with a Transcriptor first strand cDNA synthesis kit (Roche), using random hexamer primers. Real-time PCR was performed with SYBR premix *Ex Taq* reagents (TaKaRa) and the StepOne real-time PCR system (Applied Biosystems). The normalized value for each sample was derived from the relative quantity of target mRNA divided by the relative quantity of glyceraldehyde-3-phosphoate dehydrogenase (GAPDH) mRNA. The relative mRNA expression level was derived from the threshold cycle. The primers for OAS1, ISG15, CXCL10, and GAPDH transcripts have

been described previously (49, 76, 77). Other primers were 5′-GGTGGTCCCCAGTAATGTGG-3′ and 5′-CGTCAAGATTCCGATGGTCCT-3′ for MxA, 5′-CTGTGCGAGTGTACCGGATG-3′ and 5′-ATCCCCACATGACTTCCTCTT-3′ for IRF1, 5′-AAAAGACAGCTTAGGAGAACAAGA-3′, 5′-CCGCTGCCCAACACAAG-3′, and 5′-CCACTAACGTTCTTTTGCAGACAT-3′ for ZIKV, and 5′-GAAGGAGCTGAAGGGACTGA-3′ and 5′-GACGCTGTAGCAACTCTGTGA-3′ for STAT2.

Confocal immunofluorescence microscopy. JEG3 cells and HeLa cells were fixed with 4% paraformaldehyde 48 h after transfection. Cells were permeabilized with methanol-acetone (1:1) and blocked with 3% bovine serum albumin. Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI). Confocal microscopy was performed with a Carl Zeiss LSM710 microscope as described previously (78).

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V.C., K.-S.Y., J.F.-W.C., K.-Y.Y., and D.-Y.J. conceptualized and designed the study. V.C. and K.-S.Y. performed all experiments, with help from Ching-Ping Chan, P.-H.W., and J.-P.C. All authors contributed to data analysis. D.-Y.J. wrote the manuscript with input from all authors.

We declare that we have no competing financial interests.

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