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Results

NS4B suppressed RIG-I, Cardif, and STING-mediated activation of IFN-β expression signaling

First, we performed a reporter assay using a luciferase reporter plasmid regulated by native IFN-β promoter. Consistent with our previous study (19), overexpression of NS4B, as well as NS3/4A, inhibited the IFN-β promoter activation that was induced by ΔRIG-I and Cardif, respectively (***Figure 1A***). We next studied whether NS4B targets STING and inhibits RIG-I pathway-mediated activation of IFN-β production. Expression of NS4B protein significantly suppressed STING-mediated activation of the IFN-β promoter reporter, whereas expression of NS3/4A showed no effect on STING-induced IFN-β promoter activity (***Figure 1A***). To study whether NS4B blocks STING- mediated DNA-sensing pathway, we performed a reporter assay using a luciferase reporter plasmid cotransfection with poly (dA:dT), which is a synthetic analog of B-DNA and has been reported to induce STING-mediated IFN-β production, and NS4B. NS4B significantly blocked poly (dA:dT)-induced IFN-β promoter activation, suggesting that NS4B may block STING signaling in the DNA-sensing pathway (***Figure 1A***).

Activation of RIG-I signaling induces phosphorylation of IRF-3, which is a hallmark of IRF3 activation (32). Thus, we examined the effects of NS3/4A and NS4B expression on phosphorylation of IRF3 by immunoblotting analysis. As shown in ***Figure 1B***, overexpression of ΔRIG-I, Cardif, or STING in HEK293T cells increased phopho-IRF3 (pIRF3) levels. Expression of NS4B impaired the IRF3 phosphorylation that was induced by ΔRIG-I, Cardif, or STING. NS3/4A also blocked production of pIRF3 induced by ΔRIG-I or Cardif. Intriguingly, NS3/4A did not block STING-induced pIRF3 production. These results demonstrate that both NS3/4A and NS4B suppress RIG-I-mediated IFN-β production but they do so by targeting different molecules in the signaling pathway.

Subcellular localization of NS4B, Cardif, and STING

We next studied the subcellular localization of NS4B following its overexpression and measured the colocalization of NS4B with Cardif and STING in both HEK293T cells and Huh7 cells by indirect immunofluorescence microscopy. NS4B was localized predominantly in the ER, which is consistent with previous reports (33) (***Figure 2A***). Cardif was localized in mitochondria but did not colocalize with the ER-resident host protein PDI. Interestingly, Cardif and NS4B colocalized partly at the boundary of the 2 proteins, although their original localization was different (***Figure 2A and 2C***). STING was localized predominantly in the ER (20, 21) (***Figure 2B and 2D***). STING colocalized partly with Cardif, which is consistent with a previous report by Ishikawa et al.(20) (***Figure 2B and 2D***). In cells cotransfected with NS4B and STING expression plasmids, NS4B colocalized precisely with STING (***Figure 2B and 2D***). To examine the region of NS4B-STING interaction, we next observed the two proteins by performing staining for them along with mitochondria-associated ER membrane (MAM), which is a physical association with mitochondria (34) and has been reported the site of Cardif-STING association (24). Both NS4B and STING were adjacent to and partially colocalized with fatty acid-CoA ligase long chain 4 (FACL4), which is a MAM marker protein (35) (36) ***(Figure2E).*** These findings suggest that NS4B might interact with STING on MAM more strongly than with Cardif.

Protein-protein interaction between NS4B, Cardif and STING

Knowing that NS4B was colocalized strongly with STING and only partly with Cardif, we next analyzed direct protein-protein interactions between NS4B, Cardif, and STING. To detect those interactions in living cells, we performed BiFC assays (37, 38). We constructed NS4B, Cardif, and STING expression plasmids that were N- or C-terminally fused with

truncated mKG proteins, respectively. First, we cotransfected several different pairs of NS4B- and STING-expression plasmids that were fused with complementary pairs of N- or C-terminally truncated mKG. Strong fluorescence by mKG complexes (BiFC signal) was detected in all pairs of cotransfections, suggesting significant molecular interaction (***Figure 3A***). In flow cytometry, all pairs of NS4B- and STING-mKG fusion proteins were positive for strong BiFC signal ***(Figure 3B)***. The percentages of cells positive for BiFC signal were significantly higher in STING- and NS4B-mKG fusion complexes than in corresponding controls ***(Figure 3C)***. These results demonstrate that HCV-NS4B and STING proteins interact with each other strongly and specifically in cells. Fluorescence microscopy indicated that N- and C-terminal fusion of mKG onto NS4B and STING did not affect subcellular localization ***(Figure3D)***.

We next studied the molecular interaction between NS4B and Cardif by BiFC assay using NS4B and Cardif fusion plasmids that were tagged with complementary pairs of truncated mKG. Weak fluorescence was detected in cells transfected with the pairs N-Cardif and NS4B-C, N-Cardif and C-NS4B, C-Cardif and NS4B-N, and C-Cardif and N-NS4B ***(Figures 4A and B)***. The percentage of cells positive for BiFC signal increased with the combination of N-Cardif and NS4B-C, and C-Cardif and NS4B-N ***(Figure 4C)***. Fluorescence microscopy indicated that mKG-Cardif, but not Cardif-mKG, was partially colocalized with mitochondria, possibly due to disruption of mitochondria anchor domain by C-terminal fusion with mKG ***(Figure4D)***. These results indicate the lack of significant molecular interactions between NS4B and Cardif.

Binding of NS4B to STING blocks molecular interaction between Cardif and STING

It has been reported that STING binds Cardif directly (20, 22). Thus, we hypothesized that NS4B, through a competitive interaction with STING, may hinder the direct molecular

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interaction between Cardif and STING. To verify this hypothesis, we performed immunoprecipitation assays. First, we transfected plasmids that expressed NS4B and Cardif, or NS4B and STING, in HEK293T cells or Huh7 cells, and performed immunoprecipitation. NS4B strongly bound to STING in both HEK293T cells and Huh7 cells, suggesting specific molecular interactions, while NS4B and Cardif did not show any obvious interaction (***Figure 5A and 5C***). Consistent with previous reports, STING and Cardif showed significant interaction (***Figure 5B and 5D***). Interestingly, those interactions were decreased by co-expression of NS4B, depending on its input amount, and finally blocked completely in both HEK293T and Huh7 cells (***Figure 5B and 5D***). Collectively, the results above demonstrate that NS4B disrupts the interaction between Cardif and STING possibly through competitive binding to STING.

Effects on HCV infection and replication levels by STING knock down and NS4B overexpression

We next studied the impact of STING-mediated IFN production and its regulation by NS4B on HCV infection and cellular replication. First, we transfected three STING-targeted siRNAs into Huh7/Feo cells (***Figure 6A***). As shown in ***Figure 6B***, STING knock down cells conferred significantly higher permissibility to HCV replication. We next transfected HCV-JFH1 RNA into Huh7 cells that were transiently transfected with NS4B. As shown in ***Figure 6C***, HCV core protein expression was significantly higher in NS4B-overexpressed cells. Furthermore, HCV replication was increased significantly in Huh7/Feo cells overexpressing NS4B ***(Figure 6D)***. Taken together, the results above demonstrate that STING and NS4B may negatively or positively regulate cellular permissiveness to HCV-replication.

The N-terminal domain of NS4B is essential for suppressing IFN-β promoter activity

mediated by RIG-I, Cardif, and STING

It has been reported that the N-terminal domain of several forms of flaviviral NS4B shows structural homology with STING (24). We therefore investigated whether the STING-homology domain in NS4B is responsible for suppression of IFN-β production. We constructed 2 truncated NS4B expression plasmids, which covered the N-terminus (NS4Bt1-84, amino acids 1 through 84) containing the STING-homology domain and the C-terminus (NS4Bt85-261, amino acids 85 through 261), respectively ***(Figure 7A)***. Immunoblotting showed that NS4Bt1-84 and NS4Bt85-261 yielded protein bands of ~9 kDa and ~20 kDa, respectively. Aberrant bands in the truncated NS4B may be due to alternative posttranslational processing. HEK293T cells were transfected with ΔRIG-I, Cardif, or STING, and NS3/4A or the truncated NS4B, along with IFN-β-Fluc plasmid, and a reporter assay was performed. NS4Bt1-84 significantly suppressed RIG-I, Cardif, and STING-induced IFN-β promoter activity, whereas NS4Bt85-261 did not ***(Figure 7B)***. These results suggest that the N-terminal domain of NS4B is responsible for association with STING. Fluorescent microscopy indicated that both NS4Bt1-84 and NS4Bt85-26 colocalized with ER and STING ***(Figure 7C)***.

NS4B suppresses IFN production signaling cooperatively with NS3/4A

It has been reported that HCV NS3/4A serine protease cleaves Cardif between Cys-508 and His-509, releases Cardif from the mitochondrial membrane, and blocks RIG-I-induced IFN-β production. We next assessed whether NS4B suppresses IFN-β production in the presence of Cardif cleaved by NS3/4A protease (Cardif1-508, ***Figure 8A***). The truncation of Cardif-C-terminal residue abolished mitochondrial localization but still colocalized with STING ***(Figure 8B)***. The reporter assay showed that Cardif1-508 induced weak IFN-β activation. Interestingly, NS4B completely blocked the residual function of the Cardif1-508

protein to activate IFN-β expression, suggesting an additive effect of NS3/4A and NS4B on the RIG-I activating pathway ***(Figure 8C)***.

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Discussion

It has been reported that viruses, including HCV, target IFN signaling to establish persistent replication in host cells (39). We have reported that NS4B blocks the transcriptional activation of ISRE induced by overexpression of RIG-I and Cardif, but not by TBK1 or IKKε (19). In the present study, we have shown that NS4B directly and specifically binds STING, an ER-residing scaffolding protein of Cardif and TBK1 and an inducer of IFN-β production ***(Figures 3 and 5),*** and blocked the interaction between STING and Cardif (***Figure 5B and 5D***) resulting in strong suppression of RIG-I-mediated phosphorylation of IRF3 and expressional induction of IFN-β ***(Figure 1)***. Furthermore, HCV replication was increased by knock down of STING or overexpression of NS4B ***(Figure 6).*** Taken together, our results demonstrate that HCV-NS4B strongly blocks virus-induced, RIG-I-mediated activation of IFN-β production signaling through targeting STING, which constitutes a novel mechanism of viral evasion from innate immune responses and establishment of persistent viral replication.

Our results also showed that the effects of NS4B on the RIG-I signaling were independent of NS3/4A-mediated cleavage of Cardif. Reporter assays showed that a cleaved form of Cardif (Cardif1-508) partially retained activity for the induction of IFN-β promoter activation. The residual IFN-β promoter activation was suppressed almost completely by NS4B but not by NS3/4A ***(Figure 8C)***. These findings show that there are at least 2 mechanisms by which HCV can abrogate RIG-I-mediated IFN production signaling to accomplish abrogation of cellular antiviral responses.

NS4B and STING are ER proteins (20, 21, 40), while Cardif is localized on the outer mitochondrial membrane (9). Consistent with those reports, our immunostaining experiments demonstrated that most NS4B protein colocalized with STING ***(Figure 2),*** and their association was localized on MAM ***(Figure2E).*** In addition to the significant colocalization

of STING and NS4B, STING partially colocalized with Cardif at the boundary region of the 2 proteins ***(Figure 2B)***. Furthermore, immunoprecipitation experiments showed that overexpression of NS4B completely blocked the interaction of STING with Cardif ***(Figure 5B).*** Ishikawa et al. reported that STING could associate with Cardif by MAM interaction (24). Castanier et al. reported that Cardif-STING interaction was enhanced in cells with elongated mitochondria (41). In addition, Horner et al. observed NS3/4A targeting of MAM-anchored synapse and cleavage of Cardif at MAM but not in mitochondria (42, 43). These results led us to speculate that interaction between STING and Cardif was enhanced by altering their subcellular localization during viral infection and that NS4B inhibits Cardif activation by interfering with the association between STING and Cardif on MAM like NS3/4A behavior against host innate immunity.

HCV-NS4B is an ER-localized 27-kDa protein with several functions in the HCV lifecycle. Cellular expression of NS4B induces convolution of the ER membrane and formation of a membranous web that harbors HCV replicase complex (44, 45). NS4B also has RNA-binding capacity (46). In addition, several point mutations of NS4B were found to alter viral replication activity (33, 46, 47). The studies above indicate that NS4B provides an important protein-protein or protein-RNA interaction platform within the HCV replication complex and is essential for viral RNA replication. However, there are few reports on the involvement of NS4B with antiviral immune responses. Consistent with our previous study, Moriyama et al. reported that NS4B partially inhibited dsRNA-induced but not TRIF-induced activation of IFN-β (48). In NS4B-expressing cells, IFN-α induced activation of STAT1 was suppressed (49). The present study has demonstrated that NS4B functions against the host IFN response, such that NS4B directly interacts with STING and suppresses downstream signaling, resulting in the induction of IFN production.

STING contains a domain homologous to the N-terminus of NS4B derived from several

flaviviruses including HCV. In our previous NS4B truncation assay, the NS4B N-terminal domain (amino acids 1 through 110) was important for suppression of RIG-I induced IFN-β expression (19). Consistently with these results, N-terminally truncated NS4B (NS4Bt1-84) significantly suppressed STING and Cardif-induced IFN-β promoter activation, while the C-terminus of NS4B (NS4Bt85-261) did not ***(Figure** 7)*. These results reinforce our hypothesis that NS4B binds STING at its homology domain and blocks the ability of STING to induce IFN-β production.

A small molecule inhibitor of NS4B has been developed and is under preliminary clinical trials (50). Einav et al. identified clemizole hydrochloride, an H1 histamine receptor antagonist, as an inhibitor of the RNA-binding function of NS4B and HCV RNA replication (51). A phase 1B clinical trial of clemizole in hepatitis C patients has been completed (52). Other two NS4B inhibitors which are a compound of amiloride analog and anguizole are under preclinical development (53, 54). The possibility remains that such NS4B inhibitors may suppress HCV replication partly through inhibiting the ability of NS4B to suppress IFN-β production and restore cellular antiviral responses.

In conclusion, IFN production signaling induced by HCV infection and mediated by RIG-I is suppressed by NS4B through a direct interaction with STING. These virus-host interactions help to elucidate the mechanisms of persistent HCV infection and constitute a potential target to block HCV infection.

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Figure Legends

Figure 1. NS4B suppressed IFN-β signaling mediated by RIG-I, Cardif, or STING.

A. Plasmids expressing ΔRIG-I, Cardif, or STING or poly (dA:dT) as well as NS3/4A or NS4B were cotransfected with pIFN-β-Fluc and pRL-CMV into HEK293T cells. After 24 hours, dual luciferase assays were performed. Plasmids expressing RIG-IKA, ΔCARD, or an empty plasmid (pcDNA) were used as a corresponding negative control. The experiments were performed more than three times and yielded consistent results. The Y-axis indicates relative IFN-β-Fluc activity. Assays were done in triplicate and error bars indicate mean ± SD. Asterisks indicate P-values less than 0.05.

B. HEK293T cells were cotransfected with indicated plasmids. On the day after transfection, the cells were lysed and immunoblot analyses were performed. No Tf denotes transfection-negative controls. pIRF3 and IRF3 denote phosphorylated and total IRF3, respectively.

Figure 2. Subcellular localization of NS4B, Cardif, and STING

Subcellular localization of NS4B, Cardif, and STING in 293T (A and C) and Huh7 (B and D) **cells**; **A and C**. NS4B-myc (first, second and fifth panels of A and third panel of C) was transfected and after 24 hours later the cells were fixed and immunostained with anti-myc. In third, fourth and fifth panels of A, and first and second panels of C, endogenous Cardif was detected with anti-Cardif antibody. ER was immunostained with anti PDI antibody (first and third panels of A and first panel of C). Mitochondria were stained using Mitotracker (second and fourth panels of A and second panel of C). Nuclei were stained with DAPI. **B and D.** STING-HA (all panels) and NS4B-myc (third panels) were transfected and after 24 hours the cells were fixed and immunostained with anti-HA or anti-myc, respectively. In the fourth panels, endogenous Cardif was detected with anti-Cardif antibody. ER was immunostained

with anti-PDI antibody (first panels). Mitochondria were stained using Mitotracker (second panels). Nuclei were stained with DAPI. **E.** NS4B-myc and STING-HA were transfected into Huh7 cells and after 24 hours the cells were fixed and immunostained with anti-HA, anti-myc, and anti-FACL4 (MAM) antibody. Cells were visualized by confocal microscopy. Scale bars indicate 5 μm. In each microscopic image, the grade of protein colocalization in a single cell was quantified and is shown in the graphs at the bottom of each panel. Values are shown as the average colocalization ratio in 8 cells. Error bars indicate mean+S.D.

Figure 3. BiFC assays of STING and NS4B.

The complementary pairs of N- or C- terminally mKG-fused NS4B and STING expression plasmids were cotransfected in HEK293T cells. After 24 hours, the cells were fixed and observed by confocal microscopy (A) or subjected to flow cytometry to measure mKG-emitted fluorescence (BiFC signal) and to count BiFC signal-positive cells (B, C). Plasmids expressing p65-mKGN and p50-mKGC individually were used as a BiFC-positive control and plasmids expressing N- or C terminally mKG fused Rluc were used as a negative control. Letters 'N' and 'C' denote complimentary N- and C-terminal fragments of mKG, respectively. Assays were done in triplicate and error bars indicate mean ± SD. Scale bars indicate 10 μm (A). Asterisks indicate P-values less than 0.05 compared with corresponding negative controls.

C. Plasmids expressing mKG fragment-fused STING or NS4B were transfected in HEK293T cells. After 24 hours, the cells were fixed and immunostained with anti-mKG and anti-PDI (ER) antibody. Nuclei were stained with DAPI. Cells were observed by confocal microscopy. Scale bars indicate 5 μm.

Figure 4. BiFC assays of Cardif and NS4B.

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The complementary pairs of N- or C- terminally mKG-fused NS4B and Cardif expression plasmids were cotransfected in HEK293T cells. After 24 hours, the cells were fixed and observed by confocal microscopy (A) or subjected to flow cytometry to measure mKG-emitted fluorescence (BiFC signal) and to count BiFC signal-positive cells (B, C). Plasmids expressing p65-mKGN and p50-mKGC individually were used as a BiFC positive control and plasmids expressing N- or C terminally mKG-fused Rluc were used as a negative control. Letters 'N' and 'C' denote complimentary N- and C-terminal fragments of mKG, respectively. Assays were done in triplicate and error bars indicate mean ± SD. Scale bars indicate 10 μm (A). Asterisks indicate P-values less than 0.05 compared with corresponding negative controls.

C. Plasmids expressing mKG fragment-fused STING or NS4B were transfected in HEK293T cells. After 24 hours, the cells were fixed and immunostained with anti-mKG antibody. Mitochondria were stained using Mitotracker and nuclei were stained with DAPI. Cells were observed by confocal microscopy. Scale bars indicate 5 μm.

Figure 5. Binding of NS4B to STING blocks molecular the interaction between Cardif and STING.

A, C. NS4B expression plasmid was cotransfected with STING or Cardif expression plasmid into HEK293T cells (A) or Huh7 cells (C). After 24 hours, cell lysates were subjected to immunoprecipitation using anti-HA or anti-Flag and were immunoblotted with anti-myc.

B, D. Cardif and STING expression plasmids were cotransfected with various amounts of NS4B plasmid in HEK293T cells (B) or Huh7 cells (D). After 24 hours, cells lysates were subjected to immunoprecipitation using anti-Flag and were immunoblotted with anti-HA

Figure 6. Effects on HCV replication levels by STING knock down and NS4B overexpression

A. Effects of siRNA knock down of STING by siRNA. Huh7 cells were transfected with STING- targeted siRNAs (siRNA STING-1, -2 and -3, respectively) or negative control siRNA (siRNA NTC). Seventy-two hours after transfection, cells were harvested and expression levels of STING protein were detected by immunoblotting. The tf(-) denotes transfection-negative control.

B. Huh7 cells expressing HCV-Feo subgenomic replicon (Huh7/Feo) (27, 28) were transfected with STING-targeted siRNAs or negative control siRNA. Seventy-two hours after transfection, cells were harvested and internal luciferase activities were measured. The Y-axis indicates luciferase activity shown as a ratio of transfection-negative control. Assays were carried out in triplicate and error bars indicate mean+SD. Asterisks indicate P-values less than 0.05 compared with corresponding negative controls.

C. Empty plasmid or plasmid expressing NS4B was transfected into Huh7 cells. After 24 hours, HCV-JFH1 RNA was transfected into these cells. Seventy-two hours after virus transfection, HCV core antigen levels in culture medium were measured. Assays were carried out in triplicate and error bars indicate mean+SD. Asterisks indicate P-values less than 0.05 compared with corresponding negative controls. The tf(-) denotes transfection-negative control.

D. Huh7 cells expressing HCV-Feo replicon (Huh7/Feo) (27, 28) were transfected with NS4B expressing plasmid or empty plasmid (pcDNA). Forty-eight hours after transfection, internal luciferase activities were measured. The Y-axis indicates luciferase activity shown as a ratio of the transfection-negative control. Assays were carried out in triplicate and error bars indicate mean+SD. Asterisks indicate P-values less than 0.05 compared with corresponding negative controls.

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Figure 7. The N-terminal domain of NS4B is essential for suppressing IFN-β promoter activity induced by RIG-I, Cardif, or STING.

A. Immunoblotting of NS4B and truncated NS4B; NS4B t1-84, NS4Bt85-216. HEK293T cells were transfected with NS4B or truncated NS4B. After 24 hours, the cells were lysed and immunoblot assays were performed. The band indicated by '#' is a truncated NS4B, probably generated via alternative posttranslational processing.

B. Plasmids expressing ΔRIG-I, Cardif, or STING as well as NS3/4A or the indicated truncated form of NS4B were cotransfected with pIFN-β-Fluc and pRL-CMV in HEK293T cells. Dual luciferase assays were performed 24 hours after transfection. Plasmids expressing RIG-IKA, ΔCARD, or pcDNA were used as negative controls. The Y-axis indicates IFN-β-Fluc activity shown as relative values. Assays were carried out in triplicate and error bars indicate mean ± SD. Asterisks indicate P-values less than 0.05 compared with corresponding negative controls.

C. Plasmids expressing NS4Bt1-84-myc of NS4Bt85-261-myc were transfected with or without plasmids expressing HA-STING in HEK293T cells. After 24 hours, the cells were fixed and immunostained. Nuclei were stained with DAPI. Cells were observed by confocal microscopy. Scale bars indicate 5 μm.

Figure 8. NS4B suppressed IFN-β production pathway independently of and cooperatively with NS3/4A.

A. Immunoblotting of Cardif and truncated Cardif (Cardif1-508). HEK293T cells were transfected with Cardif or truncated Cardif (Cardif1-508). After 24 hours, the cells were lysed and immunoblot assays were performed.

B. Subcellular localization of Cardif and truncated Cardif (Cardif1-508). HEK293T cells

were immunostaind with anti-Cardif antibody or HEK293T cells were transfected with myc-tagged truncated Cardif (Cardif1-508-myc) and after 24 hours the cells were immunostained with anti-myc. Mitochondria were stained with Mitotracker (red) and nuclei were stained with DAPI (blue). Plasmid expressing myc-tagged truncated Cardif (Cardif1-508) and plasmid expressing HA-tagged STING were transfected into HEK293T cells. The cells were immunostained with anti-myc and anti-HA antibodies and analyzed by confocal laser microscopy. Scale bars indicate 10 μm.

C. Plasmids expressing Cardif or truncated Cardif (Cardif1-508) and pIFN-β-Fluc and pRL-CMV were transfected with or without plasmid expressing NS3/4A or NS4B into HEK293T cells as indicated. Dual luciferase assays were performed 24 hours after transfection. Plasmid expressing ΔCARD or pcDNA was used as a negative control. The Y-axis indicates IFN-β-Fluc activity shown as relative values. Assays were carried out in triplicate and error bars indicate mean ± SD. Asterisks indicate P-values less than 0.05.

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