

資料3 ウイルス学的反応の定義

ウイルス学的反応	定義
Rapid virological response (RVR) extended RVR	治療開始後 4 週で血中 HCV RNA 感度以下 治療開始後 4 週・12 週のいずれにおいても血 中 HCV RNA 感度以下
Early virological response (EVR) Complete EVR(cEVR) Partial EVR(pEVR)	cEVR あるいは pEVR 治療開始後 12 週で血中 HCV RNA 感度以下 治療開始後 12 週で血中 HCV RNA が陽性だ が 2 log 以上低下
End-of-treatment response (ETR)	治療終了時血中 HCV RNA 感度以下
Sustained virological response (SVR)	治療終了後 24 週で血中 HCV RNA 感度以下
Breakthrough	治療中にいったん感度以下となった血中 HCV RNA が治療中に再出現
Relapse	治療中にいったん感度以下となった血中 HCV RNA が治療終了後に再出現
Non-responder	治療中に HCV-RNA が感度以下にならず
Null responder	治療開始後 12 週で血中 HCV RNA の減少が 2 log 未満
Partial responder	治療開始後 12 週で血中 HCV RNA が 2 log 以上減少、しかし治療開始後 24 週で血中 HCV RNA が感度以下にならない

注：AASLD から 2009 年に発表された「C型肝炎ガイドライン」¹¹⁶⁾では、「治療開始後 24 週で血中 HCV RNA が感度以下にならない」「治療開始後 24 週で血中 HCV RNA の減少が 2 log 未満」「治療開始後 24 週で血中 HCV RNA が 2 log 以上減少、しかし感度以下にならない」を、それぞれ nonresponder、null responder、partial responder と定義していた。しかし、テラプレビルとボセプレビルの登場を期してアップデートされた 2011 版¹⁰⁾では、nonresponder というカテゴリーは採用されず、null responder、partial responder が「治療開始後 12 週で血中 HCV RNA の減少が 2 log 未満」「治療開始後 12 週で血中 HCV RNA が 2 log 以上減少、しかし治療開始後 24 週で血中 HCV RNA が感度以下にならない」と再定義されている。

本ガイドラインでは 2011 年版の AASLD に準じて null/partial responder を定義し、さらに null/partial responder を包括した“無効”として“Non-responder”を定義する。

資料4 HCV についての外注検査

IL28B SNP、HCV コア領域・NS5A 領域のアミノ酸変異は保険適用外であるものの、外注検査で測定可能である。各施設の検査会社担当者に直接照会されたい。

(1) IL28B SNP 測定

ある検査会社では、専用容器(EDTA-2Na 加)、検体量 5.0 ml、報告日数 12~16 日としている。なお、価格については各施設の検査会社担当者に直接照会されたい。

なお、IL28B 測定はヒトゲノムを検体としており、医療領域では「医療・介護関係事業者における個人情報の適切な取り扱いのためのガイドライン(厚生労働省)」、および「遺伝学的検査に関するガイドライン」(遺伝医学関連 10 学会)、「ファーマコゲノミクス検査の運用指針」(日本臨床検査医学会など)を、また研究領域では「ヒトゲノム・遺伝子解析研究に関する倫理指針」(文部科学省・厚生労働省・経済産業省)を遵守する必要がある。したがって、個人の遺伝情報の保護に十分留意しつつ、IL28B SNP 検査について患者に対して文書による説明を十分に行い、同意を得なければならない。検査会社によっては説明文書・同意書を用意しているところもあるので、参考にされたい。また、施設内に倫理委員会が設置されていれば、IL28B SNP 測定についてあらかじめ倫理委員会に申請し、承認を得るべきである。

(2) HCV コア領域・NS5A 領域のアミノ酸変異測定

ある検査会社によればそれぞれ以下のとおりである。

HCV コア領域 70 番・91 番アミノ酸変異:専用容器、検体量 5.0 ml、報告日数 10~14 日。

HCV NS5A 領域アミノ酸変異(ISDR):専用容器、検体量 5.0 ml、報告日数 10~14 日。

なお、価格については各施設の検査会社担当者に直接照会されたい。

参考資料 平成 23 年度厚生労働省科学研究費肝炎等克服緊急対策研究事業(肝炎分野) ウイルス肝炎における最新の治療法の標準化を目指す研究班による平成 24 年 B 型 C 型慢性肝炎・肝硬変治療ガイドライン

(http://www.jsh.or.jp/medical_date/H24_guideline.pdf)

Treatment Guidelines of Hepatitis C

The Committee for Hepatitis Clinical Guidelines, Japan Society of Hepatology

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The Committee for Hepatitis Clinical Guidelines, JSH

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Hepatitis C Virus NS4B Protein Targets STING and Abrogates RIG-I–Mediated Type I Interferon-Dependent Innate Immunity

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Hepatitis C virus (HCV) infection blocks cellular interferon (IFN)-mediated antiviral signaling through cleavage of Cardif by HCV-NS3/4A serine protease. Like NS3/4A, NS4B protein strongly blocks IFN- β production signaling mediated by retinoic acid-inducible gene I (RIG-I); however, the underlying molecular mechanisms are not well understood. Recently, the stimulator of interferon genes (STING) was identified as an activator of RIG-I signaling. STING possesses a structural homology domain with flaviviral NS4B, which suggests a direct protein-protein interaction. In the present study, we investigated the molecular mechanisms by which NS4B targets RIG-I-induced and STING-mediated IFN- β production signaling. IFN- β promoter reporter assay showed that IFN- β promoter activation induced by RIG-I or Cardif was significantly suppressed by both NS4B and NS3/4A, whereas STING-induced IFN- β activation was suppressed by NS4B but not by NS3/4A, suggesting that NS4B had a distinct point of interaction. Immunostaining showed that STING colocalized with NS4B in the endoplasmic reticulum. Immunoprecipitation and bimolecular fluorescence complementation (BiFC) assays demonstrated that NS4B specifically bound STING. Intriguingly, NS4B expression blocked the protein interaction between STING and Cardif, which is required for robust IFN- β activation. NS4B truncation assays showed that its N terminus, containing the STING homology domain, was necessary for the suppression of IFN- β promoter activation. NS4B suppressed residual IFN- β activation by an NS3/4A-cleaved Cardif (Cardif1-508), suggesting that NS3/4A and NS4B may cooperate in the blockade of IFN- β production. **Conclusion:** NS4B suppresses RIG-I-mediated IFN- β production signaling through a direct protein interaction with STING. Disruption of that interaction may restore cellular antiviral responses and may constitute a novel therapeutic strategy for the eradication of HCV. (HEPATOLOGY 2013;57:46-58)

Type I interferon (IFN) plays a central role in eliminating hepatitis C virus (HCV) both under physiological conditions and when used as a therapeutic intervention.¹⁻³ In experimental acute-resolving HCV infection in chimpanzees, numerous IFN-related genes are expressed during clinical course of infection.⁴ Viruses are recognized by cellular innate immune receptors, such as toll-like receptors, and a family of RIG-I-like receptors, such as retinoic acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA-5); host antiviral responses are then activated, resulting in the

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BiFC, bimolecular fluorescence complementation; CARD, caspase recruitment domain; DAPI, 4',6-diamidino-2-phenylindole; dsRNA, double-stranded RNA; ER, endoplasmic reticulum; FAFL4, fatty acid-CoA ligase, long chain 4; HCV, hepatitis C virus; IFN, interferon; IKK ϵ , I κ B kinase ϵ ; IRF-3, interferon-regulatory factor 3; ISRE, interferon-stimulated response element; MAM, mitochondria-associated ER membrane; mKG, monomeric Kusabira-Green; PDI, protein disulphide-isomerase; pIRF-3, phosphorylated IRF3; poly(dA:dT), poly(deoxyadenylic-deoxythymidylic) acid; RIG-I, retinoic acid-inducible gene I; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling; STAT1, signal transducer and activator of transcription protein-1; STING, stimulator of interferon genes; TBK1, TANK binding kinase 1.

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production of cytokines such as type I and type III IFNs.⁵ RIG-I is activated through recognition of short double-strand RNA (dsRNA) or triphosphate at the 5' end of dsRNA as pathogen-associated molecular patterns,^{6,7} forming a homo-oligomer that binds with the caspase recruitment domain (CARD) of Cardif (also known as MAVS, VISA, or IPS-1).⁸⁻¹¹ Cardif subsequently recruits TANK binding kinase 1 (TBK1) and I κ B kinase ϵ (IKK ϵ) kinases, which catalyze phosphorylation and activation of IFN regulatory factor-3 (IRF-3).¹² Activation of TBK1 and IKK ϵ results in the phosphorylation of IRF-3 or IRF-7, translocation to the nucleus, and induction of IFN- β mRNA transcription.

Several HCV proteins can block host cellular antiviral responses. HCV core protein blocks IFN signaling by interacting with signal transducer and activator of transcription protein-1 (STAT1).¹³ The core protein also induces expression of suppressor of cytokine signaling-1 (SOCS1) and SOCS3, and blocks Janus kinase-STAT signaling.^{14,15} A well-elucidated immune evasion strategy of HCV involves NS3/4A serine protease and its ability to inhibit host IFN signal pathways. Gale and colleagues^{11,16,17} revealed that NS3/4A protease cleaves Cardif at Cys-508 resulting in dislocation of Cardif from mitochondria, and blocks downstream signaling of IFN- β production. On the other hand, Baril et al.¹⁸ reported that Cardif was still able to form a homo-oligomer and to activate downstream IFN production signaling despite delocalization from the mitochondria. These reports suggest that homo-oligomerization of Cardif, and not mitochondrial anchorage, is essential for the activation of downstream IFN signaling and that other virus-derived molecules may cooperate with NS3/4A to abrogate the signaling of IFN production.

We reported previously that HCV-NS4B, as well as NS3/4A, inhibited RIG-I and Cardif-mediated interferon-stimulated response element (ISRE) activation, while TBK1- and IKK ϵ -mediated ISRE activation were not suppressed.¹⁹ These results indicate that NS4B suppresses IFN production signaling by targeting Cardif or other unknown signaling molecules between the level of Cardif and TBK1/IKK ϵ .

Recently, a stimulator of interferon genes (STING, also known as MITA/ERIS/MPYS/TMEM173) was

identified as a positive regulator of RIG-I-mediated IFN- β signaling.²⁰⁻²³ STING is a 42-kDa protein localized predominantly in the endoplasmic reticulum (ER) that binds RIG-I, Cardif, TBK1, and IKK ϵ . STING is thought to act as a scaffold for Cardif/TBK1/IRF-3 complex upon viral infection.²² It has been reported that NS4B of yellow fever virus, which is a member of the flaviviridae family of viruses, inhibits STING activation probably through a direct molecular interaction.²⁴ These reports have led us postulate that HCV-NS4B may also inhibit RIG-I dependent IFN signaling through association with STING.

In the present study, we further investigated the molecular mechanisms by which HCV-NS4B protein inhibits RIG-I-mediated IFN expression signaling. We demonstrated that HCV-NS4B specifically binds STING, blocks the molecular interaction between STING and Cardif, and suppresses the RIG-I-like receptor-induced activation of IFN- β production signaling.

Materials and Methods

Plasmids. The Δ RIG-I and RIG-IKA plasmids express constitutively active and inactive RIG-I, respectively.⁵ Full-length Cardif (Cardif) and CARD-truncated Cardif (Δ CARD) plasmids were provided by J. Tschopp.¹¹ Plasmids expressing STING were provided by G. N. Barber.²⁰ Plasmids expressing HCV NS3/4A, NS4B, and truncated NS4B have been described.²⁵ Plasmid pIFN- β -Fluc was provided by R. Lin.²⁶

Cell Culture. HEK293T and Huh7 cells were maintained in Dulbecco's modified minimal essential medium (Sigma) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37°C with 5% CO₂.

HCV Replicon Constructs and HCV-JFH1 Cell Culture. An HCV subgenomic replicon plasmid, pRep-Feo, expressed fusion protein of firefly luciferase and neomycin phosphotransferase.^{27,28} Huh7 cells were transfected by Rep-Feo RNA, cultured in the presence of 500 μ g/mL of G418, and a cell line that stably expressed Feo replicon was established. For HCV cell culture, the HCV-JFH1 strain was used.^{29,30}

Antibodies. Antibodies used were anti-IRF-3 (FL-425, Santa Cruz Biotechnology), anti-HA (Invitrogen), anti-myc (Invitrogen), mouse anti-PDI (Abcam),

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Additional Supporting Information may be found in the online version of this article.

rabbit anti-PDI (Enzo Life Science), anti-Flag (Sigma Aldrich), anti-Cardif (Enzo Life Science), anti-phospho-IRF-3 (Ser396, Millipore), anti-monomeric Kusabira-Green C- or N-terminal fragment (MBL), and anti-FACL4 (Abgent).

Luciferase Reporter Assay. IFN- β reporter assays were performed as described.^{19,31} The plasmids pIFN- β -Fluc and pRL-CMV were cotransfected with NS3/4A or NS4B, and Δ RIG-I, Cardif, STING or poly(deoxyadenylic-deoxythymidylic) acid [poly(dA:dT)] (Invivo-gen). RIG-IKA, Δ CARD, and pcDNA3.1, respectively, were used as controls. Luciferase assays were performed 24 hours after transfection by using a 1420 Multilabel Counter (ARVO MX PerkinElmer) and Dual Luciferase Assay System (Promega). Assays were performed in triplicate, and the results are expressed as the mean \pm SD.

Immunoblotting. Preparation of total cell lysates was performed as described.^{19,28} Protein was separated using NuPAGE 4%-12% Bis/Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane. The membrane was immunoblotted with primary followed by secondary antibody, and protein was detected by chemiluminescence.

Immunoprecipitation Assay. HEK-293T or Huh7 cells were transfected with plasmids as indicated. Twenty-four hours after transfection, cellular proteins were harvested and immunoprecipitation assays were performed using an Immunoprecipitation Kit according to the manufacturer's protocol (Roche Applied Science). The immunoprecipitated proteins were analyzed by immunoblotting.

Indirect Immunofluorescence Assay. Cells seeded onto tissue culture chamber slides were transfected with plasmids as indicated. Twenty-four hours after transfection, the cells were fixed with cold acetone and incubated with primary antibody and subsequently with Alexa488- or Alexa568-labeled secondary antibodies. Mitochondria were stained by MitoTracker (Invitrogen). Cells were visualized using a confocal laser microscope (Fluoview FV10, Olympus).

BiFC Assay. Expression plasmids of NS4B, Cardif, or STING that was fused with N- or C-terminally truncated monomeric Kusabira-Green (mKG) were constructed by inserting polymerase chain reaction-amplified fragments encoding NS4B, Cardif, or STING, respectively, inserted into fragmented mKG vector (Coral Hue Fluo-Chase Kit; MBL). HEK293T cells were transfected with a complementary pair of mKG fusion plasmids. Twenty-four hours after transfection, fluorescence-positive cells were detected and counted by flow cytometry, or observed by confocal laser microscopy.

Small Interfering RNA Assay. Nucleotide sequences of STING-targeted small interfering RNAs (siRNAs) were as follows: (1) 5'-gcaacagcatctatgagcttctggagaac-3', (2) 5'-gtgcagtgagccagcggctgtatattctc-3', (3) 5'-gctggcatggcatattacatcgatc-3'.²² Stealth RNAi Negative Control Duplex (Medium GC Duplex, Invitrogen) was used. Forty-eight hours after siRNA transfection, expression levels of STING were detected by immunoblotting.

Statistical Analyses. Statistical analyses were performed using unpaired, two-tailed Student *t* test. *P* < 0.05 were considered to be statistically significant.

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 Δ RIG-I and Cardif, respectively (Fig. 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 (Fig. 1A). To study whether NS4B blocks the 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 (Fig. 1A).

Activation of RIG-I signaling induces phosphorylation of IRF-3, which is a hallmark of IRF-3 activation.³² Thus, we examined the effects of NS3/4A and NS4B expression on phosphorylation of IRF-3 by immunoblotting analysis. As shown in Fig. 1B, overexpression of Δ RIG-I, Cardif, or STING in HEK293T cells increased levels of phosphorylated IRF-3 (pIRF-3). Expression of NS4B impaired the IRF-3 phosphorylation that was induced by Δ RIG-I, Cardif, or STING. NS3/4A also blocked production of pIRF-3 induced by Δ RIG-I or Cardif. Intriguingly, NS3/4A did not block STING-induced pIRF-3 production. These results demonstrate that both NS3/4A and

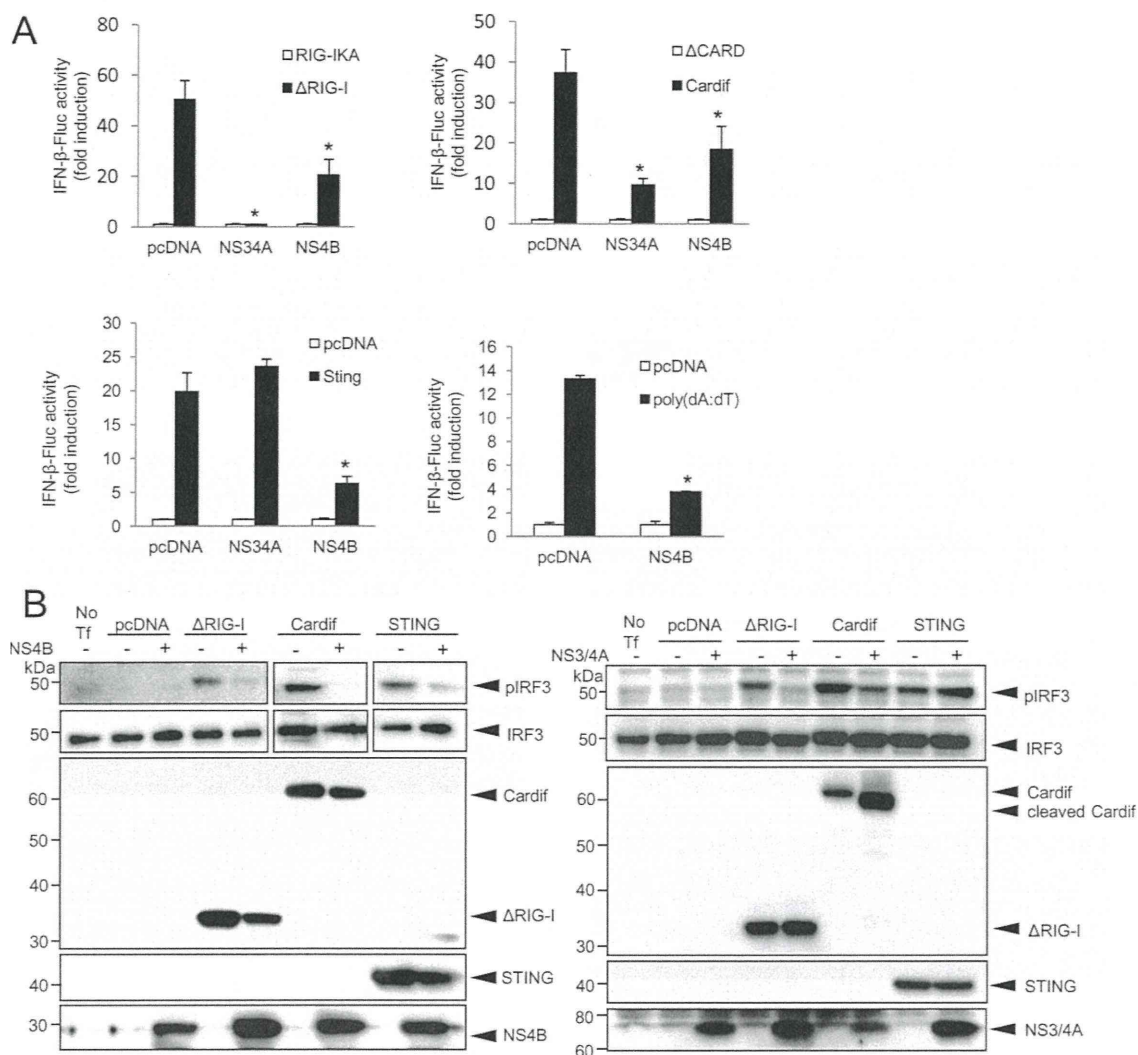


Fig. 1. NS4B suppressed IFN- β signaling mediated by RIG-I, Cardif, or STING. (A) Plasmids expressing Δ RIG-I, Cardif, or STING or poly(dA:dT) as well as NS3/4A or NS4B were cotransfected with pIFN- β -Fluc and pRL-CMV into HEK293T cells. After 24 hours, dual luciferase assays were performed. Plasmids expressing RIG-IKA, Δ CARD, 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 performed in triplicate and error bars indicate mean \pm SD. * $P < 0.05$. (B) HEK293T cells were cotransfected with indicated plasmids. On the day after transfection, the cells were lysed and immunoblot analyses were performed. No Tf, transfection-negative controls. pIRF-3 and IRF-3, phosphorylated and total IRF-3, respectively.

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 reports³³ (Fig. 2A). Cardif was localized in mitochondria but did not colocalize with the ER-resident host protein disulphide-isomerase (PDI). Interestingly, Cardif and NS4B colocalized partly at the boundary of

the two proteins, although their original localization was different (Fig. 2A,C). STING was localized predominantly in the ER^{20,21} (Fig. 2B,D). STING colocalized partly with Cardif, which is consistent with a previous report by Ishikawa and Barber²⁰ (Fig. 2B,D). In cells cotransfected with NS4B and STING expression plasmids, NS4B colocalized precisely with STING (Fig. 2B,D). 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 mitochondria³⁴ and has been reported the site of Cardif-STING association.²⁴ Both NS4B and STING were adjacent to and partially colocalized

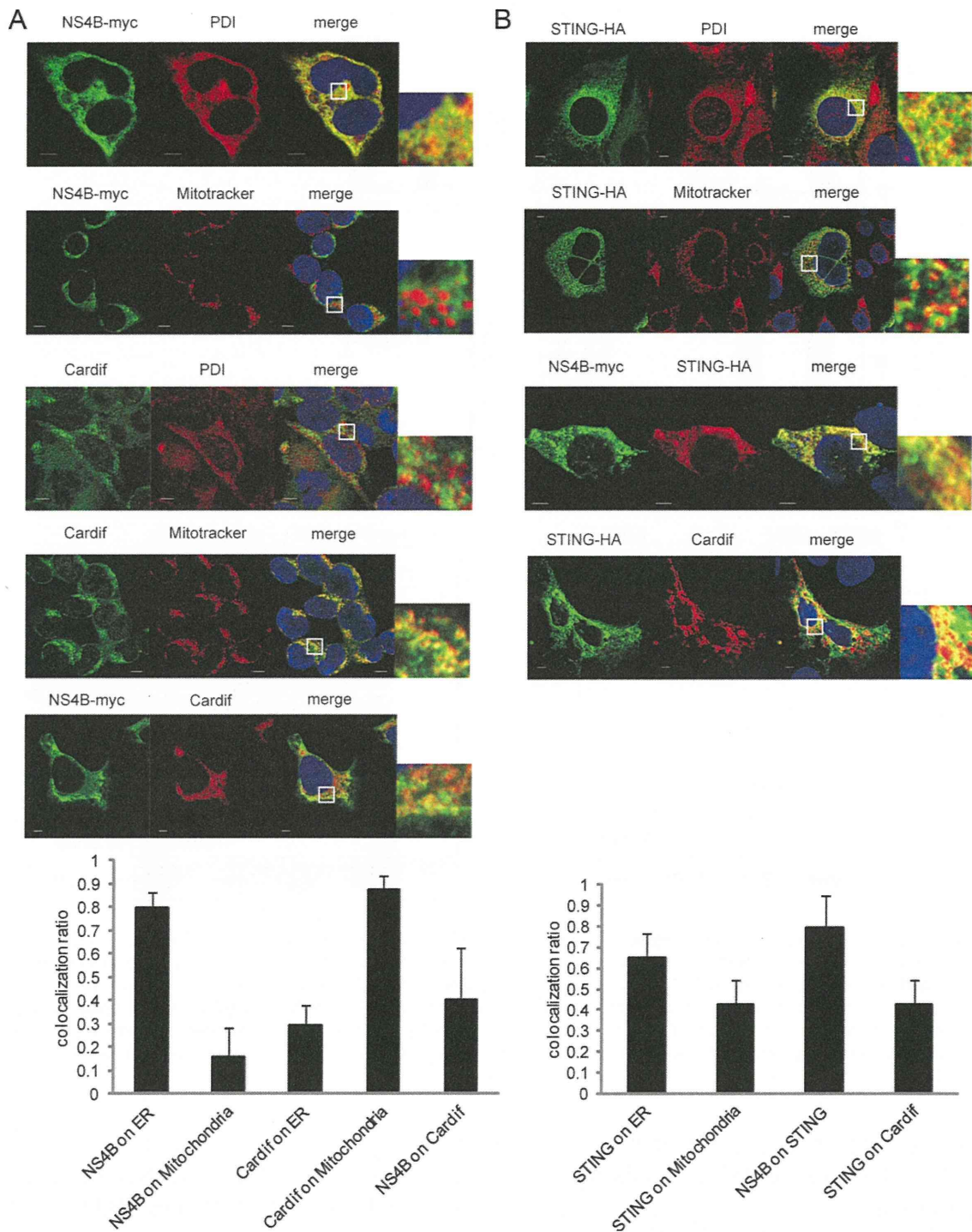


Fig. 2. Subcellular localization of NS4B, Cardif, and STING. (A-D) Subcellular localization of NS4B, Cardif, and STING in 293T (A,C) and Huh7 (B,D) cells. (A,C) NS4B-myc (first, second, and fifth panels of A and third panel of C) was transfected, and 24 hours later the cells were fixed and immunostained with anti-myc. In the third, fourth, and fifth panels of A, and the 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 4',6-diamidino-2-phenylindole (DAPI). (B,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 μ m. 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 the mean + SD.

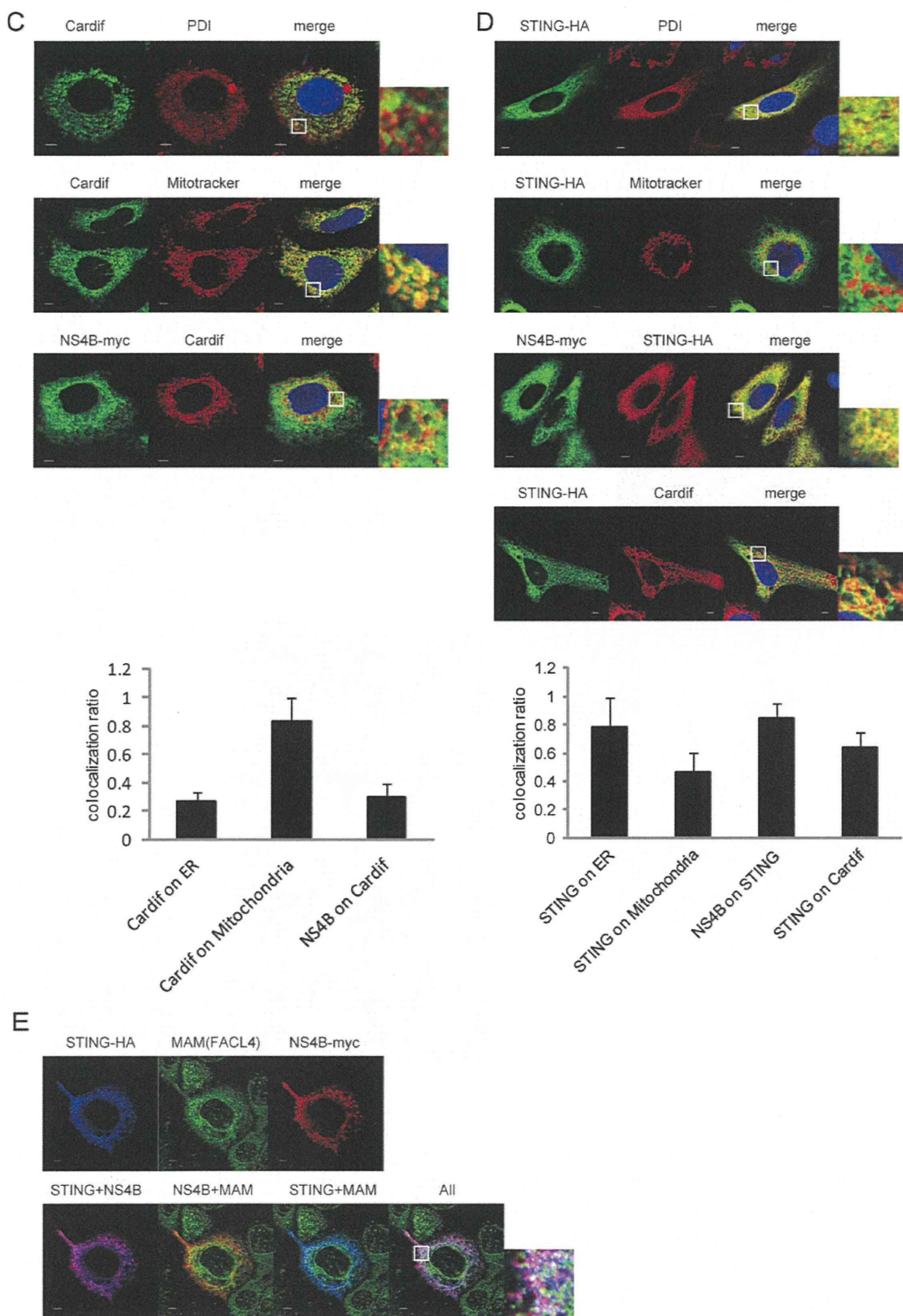


Fig. 2. Continued

with fatty acid-CoA ligase long chain 4 (FACL4), which is a MAM marker protein^{35,36} (Fig. 2E). 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

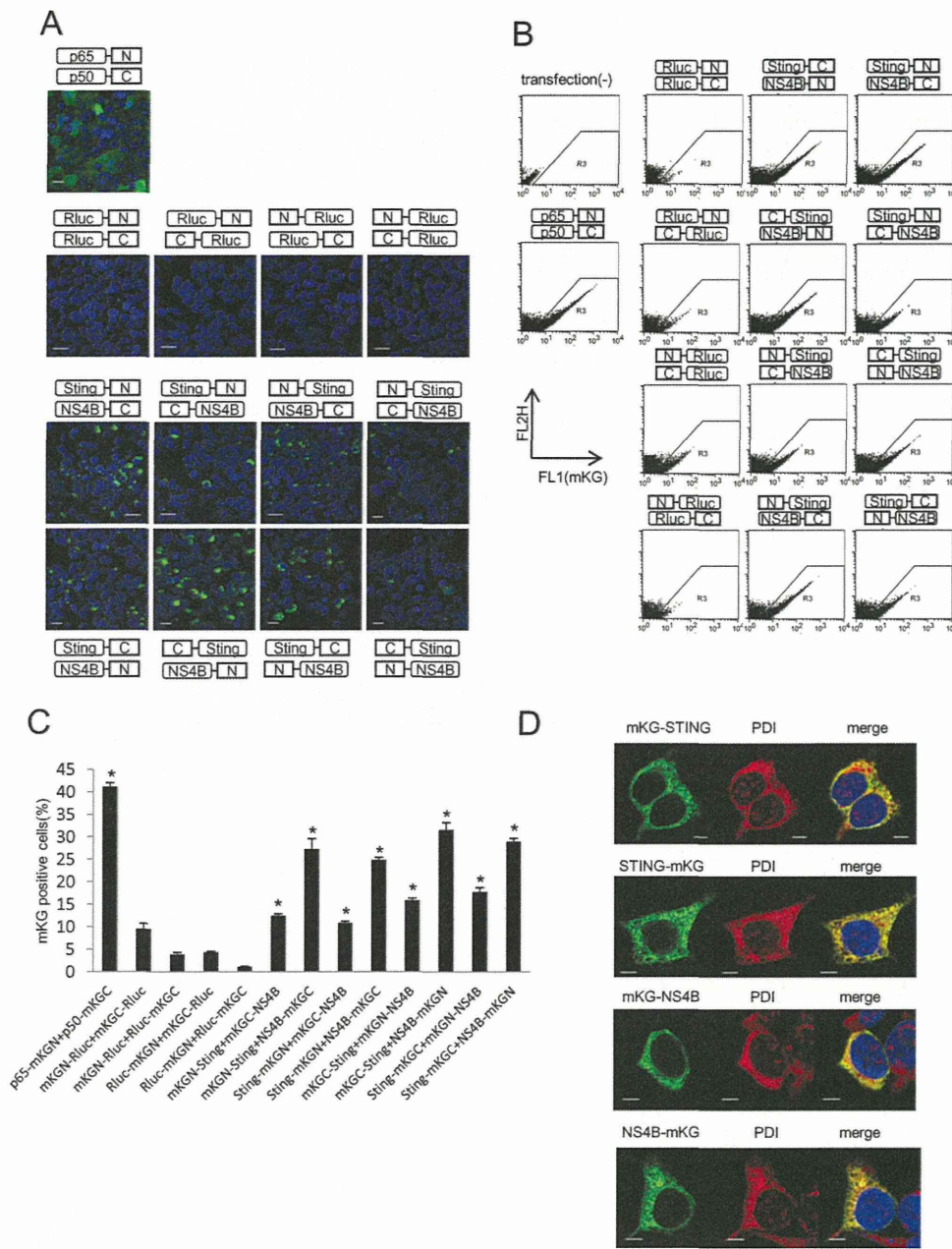


Fig. 3. BiFC assays of STING and NS4B. The complementary pairs of N- or C-terminally mKG-fused NS4B and STING expression plasmids were cotransfected in HEK293T cells. After 24 hours, the cells were fixed and observed by confocal microscopy (A) or subjected to flow cytometry to measure mKG-emitted fluorescence (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. The letters N and C denote complimentary N- and C-terminal fragments of mKG, respectively. Assays were performed in triplicate and error bars indicate the mean \pm SD. Scale bars indicate 10 μ m (A). * $P < 0.05$ compared with corresponding negative controls. (D) 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 = 5 μ m.

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 (Fig. 3A). In flow cytometry, all pairs of NS4B- and STING-mKG fusion proteins were positive for strong BiFC signal (Fig. 3B). The percentages of cells positive for BiFC

signal were significantly higher in STING-mKG and NS4B-mKG fusion complexes than in corresponding controls (Fig. 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 (Fig. 3D).

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

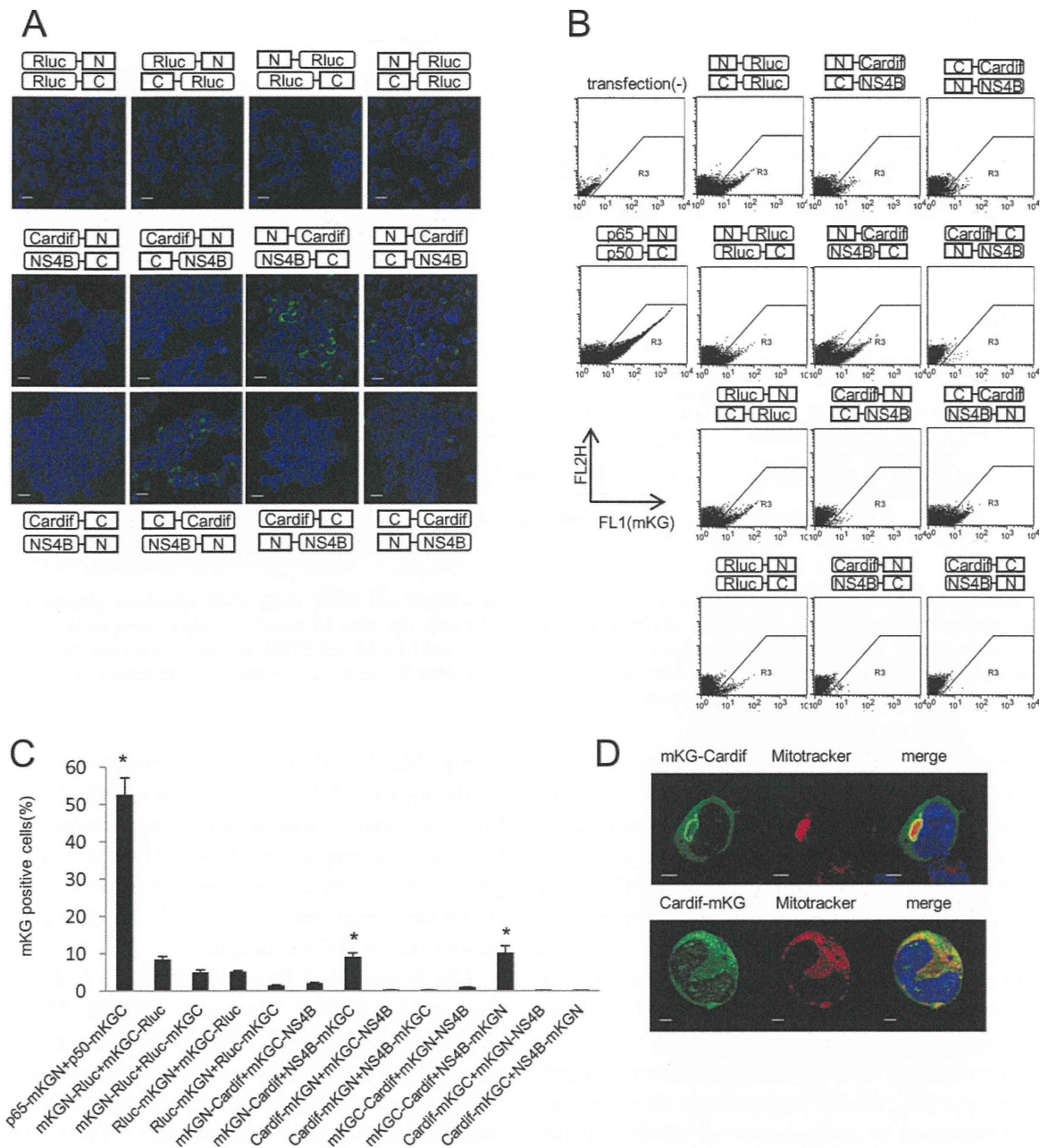


Fig. 4. BiFC assays of Cardif and NS4B. The complementary pairs of N- or C-terminally mKG-fused NS4B and Cardif expression plasmids were cotransfected in HEK293T cells. After 24 hours, the cells were fixed and observed by confocal microscopy (A) or subjected to flow cytometry to measure mKG-emitted fluorescence (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. The letters N and C denote complimentary N- and C-terminal fragments of mKG, respectively. Assays were performed in triplicate, and error bars indicate the mean \pm SD. Scale bars indicate 10 μ m (A). * $P < 0.05$ compared with corresponding negative controls. (D) 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 = 5 μ m.

NS4B-N, and C-Cardif and N-NS4B (Fig. 4A,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 (Fig. 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 (Fig. 4D). 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

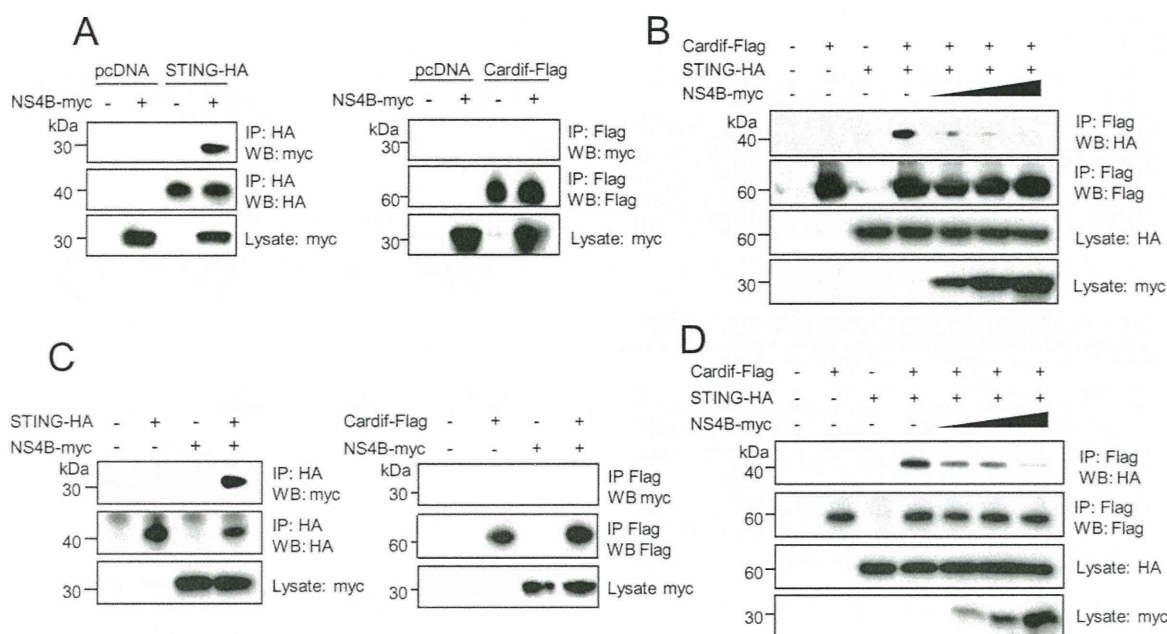


Fig. 5. Binding of NS4B to STING blocks molecular the interaction between Cardif and STING. (A,C) NS4B expression plasmid was cotransfected with STING or Cardif expression plasmid into HEK293T cells (A) or Huh7 cells (C). After 24 hours, cell lysates were subjected to immunoprecipitation using anti-HA 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.

interaction with STING, may hinder the direct molecular 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, whereas NS4B and Cardif did not show any obvious interaction (Fig. 5A,C). Consistent with previous reports, STING and Cardif showed significant interaction (Fig. 5B,D). Interestingly, those interactions were decreased by coexpression of NS4B, depending on its input amount, and finally blocked completely in both HEK293T and Huh7 cells (Fig. 5B,D). 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 Knockdown 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 (Fig. 6A). As shown in Fig. 6B, STING knockdown 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 Fig. 6C, HCV core protein expression was significantly higher in NS4B-overexpressed cells. Furthermore, HCV replication was increased significantly in Huh7/Feo cells overexpressing NS4B (Fig. 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 two 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 (Fig. 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 post-translational processing. HEK293T cells were transfected with Δ RIG-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-

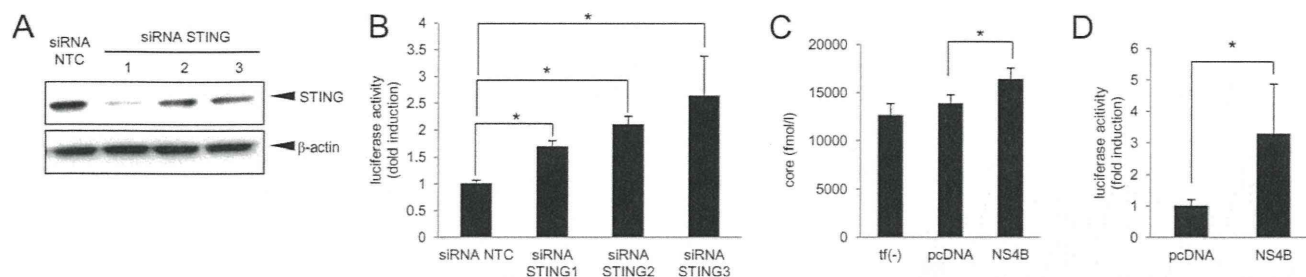


Fig. 6. Effects on HCV replication levels by STING knockdown and NS4B overexpression. (A) Effects of siRNA knockdown of STING by siRNA. Huh7 cells were transfected with STING-targeted siRNAs (siRNA STING-1, -2, and -3, respectively) or negative control siRNA (siRNA NTC). Seventy-two hours after transfection, cells were harvested and expression levels of STING protein were detected by immunoblotting. (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 performed in triplicate, and error bars indicate the mean + SD. **P* < 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 performed in triplicate, and error bars indicate the mean + SD. **P* < 0.05 compared with corresponding negative controls. tf(-), 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 performed in triplicate, and error bars indicate the mean + SD. **P* < 0.05 compared with corresponding negative controls.

induced IFN- β promoter activity, whereas NS4Bt85-261 did not (Fig. 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 NS4Bt85-261 colocalized with ER and STING (Fig. 7C).

NS4B Suppresses IFN Production Signaling Cooperatively with NS3/4A. It has been reported that

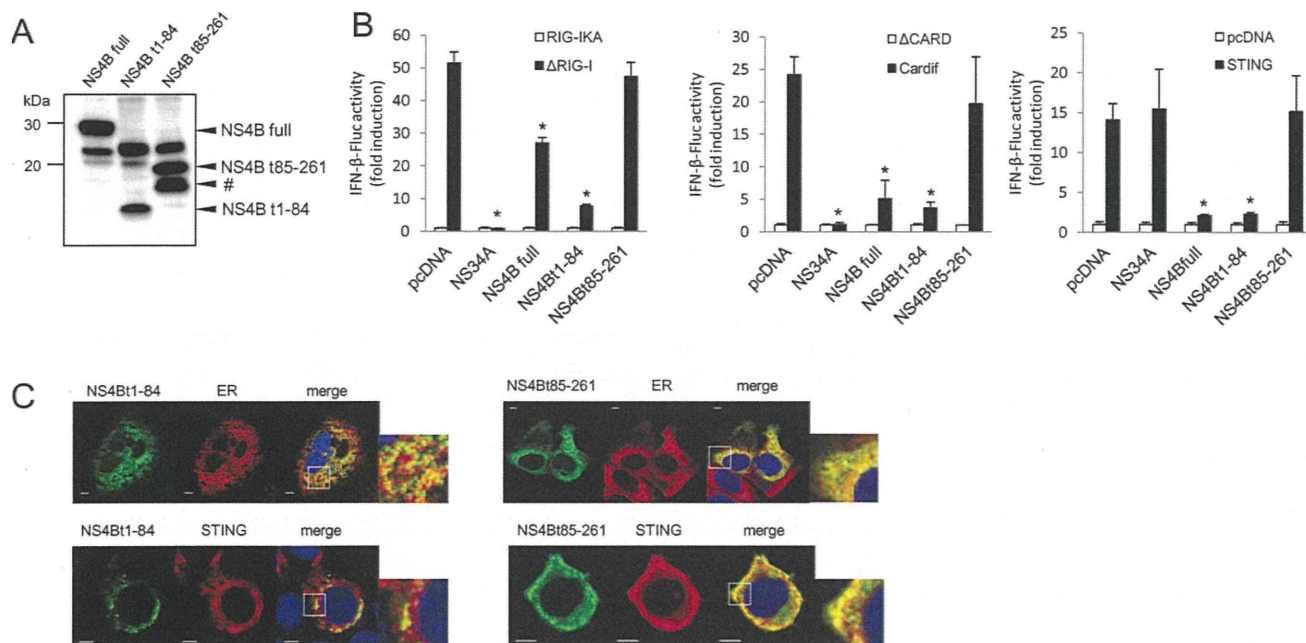


Fig. 7. The N-terminal domain of NS4B is essential for suppressing IFN- β promoter activity induced by RIG-I, Cardif, or STING. (A) Immunoblotting of NS4B and truncated NS4B, NS4B t1-84, and NS4Bt85-261. HEK293T cells were transfected with NS4B or truncated NS4B. After 24 hours, the cells were lysed and immunoblot assays were performed. The band indicated by the pound sign (#) is a truncated NS4B, probably generated via alternative posttranslational processing. (B) Plasmids expressing Δ RIG-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, Δ CARD, or pcDNA were used as negative controls. The y axis indicates IFN- β -Fluc activity shown as relative values. Assays were performed in triplicate, and error bars indicate the mean \pm SD. **P* < 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 μ m.

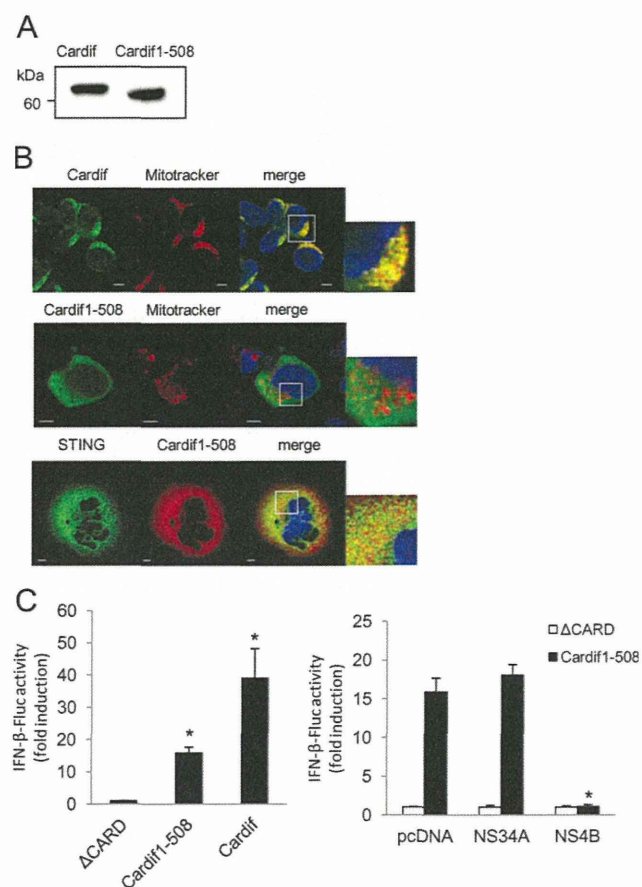


Fig. 8. NS4B suppressed IFN- β production pathway independently of and cooperatively with NS3/4A. (A) Immunoblotting of Cardif and truncated Cardif (Cardif1-508). HEK293T cells were transfected with Cardif or truncated Cardif (Cardif1-508). After 24 hours, the cells were lysed and immunoblot assays were performed. (B) Subcellular localization of Cardif and truncated Cardif (Cardif1-508). HEK293T cells were immunostained 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 = 10 μ m. (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 Δ CARD or pcDNA was used as a negative control. The y axis indicates IFN- β -Fluc activity shown as relative values. Assays were performed in triplicate, and error bars indicate the mean \pm SD. * P < 0.05.

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, Fig. 8A). The truncation of Cardif-C-terminal residue abolished mitochondrial localization but still colocalized with

STING (Fig. 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 (Fig. 8C).

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 IKK ϵ .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 (Figs. 3 and 5), and blocked the interaction between STING and Cardif (Fig. 5B,D) resulting in strong suppression of RIG-I-mediated phosphorylation of IRF-3 and expressional induction of IFN- β (Fig. 1). Furthermore, HCV replication was increased by knock-down of STING or overexpression of NS4B (Fig. 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 (Fig. 8C). These findings show that there are at least two 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} whereas Cardif is localized on the outer mitochondrial membrane.⁹ Consistent with those reports, our immunostaining experiments demonstrated that most NS4B protein colocalized with STING (Fig. 2), and their association was localized on MAM (Fig. 2E). In addition to the significant colocalization of STING and NS4B, STING partially colocalized with Cardif at the boundary region of the two proteins (Fig. 2B). Furthermore, immunoprecipitation experiments showed that overexpression of NS4B completely blocked the interaction of STING with Cardif (Fig. 5B). Ishikawa et al.²⁴ reported

that STING could associate with Cardif by MAM interaction. Castanier et al.⁴¹ reported that Cardif-STING interaction was enhanced in cells with elongated mitochondria. In addition, Horner et al.^{42,43} observed NS3/4A targeting of MAM-anchored synapse and cleavage of Cardif at MAM but not in mitochondria. 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 life cycle. 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.⁴⁸ reported that NS4B partially inhibited dsRNA-induced but not TRIF-induced activation of IFN- β . 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 1-110) was important for suppression of RIG-I-induced IFN- β expression.¹⁹ Consistent with these results, N-terminally truncated NS4B (NS4Bt1-84) significantly suppressed STING and Cardif-induced IFN- β promoter activation, whereas the C terminus of NS4B (NS4Bt85-261) did not (Fig. 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.⁵¹ identified clemizole hydrochloride, an H1 histamine receptor antagonist, as an inhibitor of the RNA-binding function of NS4B and HCV RNA replication. A phase 1B clinical trial of clemizole in hepatis

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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Special Report

Guidelines for the Management of Hepatitis C Virus Infection

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Editors of the Drafting Committee for Hepatitis Management Guidelines: The Japan Society of Hepatology*[†]****1. INTRODUCTION**

THE JAPAN SOCIETY of Hepatology (JSH) has, until now, produced “A Management Guide for Chronic Hepatitis and Liver Cirrhosis”, “A Management Guide for NASH and NAFLD”, and “A Treatment Manual for Hepatocellular Carcinoma”. The only official guidelines produced by the Society have been the “Clinical Practice Guidelines for Hepatocellular Carcinoma Based on Scientific Evidence”, however, and we had not yet developed guidelines for hepatitis.

As a scientific body that promotes hepatology research, we considered it necessary to publish our official position on the diagnosis and treatment of hepatitis. The regular JSH board meeting on 19 October 2011

approved the establishment of the Drafting Committee for Hepatitis Management Guidelines.

The Committee decided that our first priority was the production of guidelines for the management of hepatitis C, most urgently needed by Society members, so we began with the production of these “Guidelines for the Management of Hepatitis C Virus Infection (First Edition)”. We hope and anticipate that these guidelines will be used throughout Japan in the management of hepatitis C.

This is a field that changes rapidly with the accumulation of new evidence, accompanied by changes in the level of evidence, so we have elected not to show evidence levels. We plan to revise these guidelines at appropriate intervals, as new evidence comes to hand.

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2. GENERAL STRATEGY AGAINST HEPATITIS C VIRUS INFECTION

FOLLOWING THE IDENTIFICATION of the hepatitis C virus (HCV) by Choo *et al.* in the USA in 1989,¹ it became clear that over 90% of patients previously diagnosed with non-A non-B hepatitis, and over 50% of those diagnosed with alcoholic hepatitis, in fact suffered from liver disease caused by HCV. Currently, there are an estimated 170 million carriers worldwide, and 1.5–2 million in Japan. Even in healthy adults, once an HCV infection occurs, only approximately 30% resolve completely in the acute phase. HCV

infection is prolonged in approximately 70% of cases, causing chronic hepatitis. Once an HCV infection has become chronic, spontaneous elimination of the virus is rare (0.2% annual rate), and persistent inflammation can induce fibrosis, progressing to cirrhosis or hepatocellular carcinoma (HCC).² Interferon (IFN) therapy commenced in 1986, when Hoofnagle *et al.* administered human recombinant IFN- α to patients with non-A non-B hepatitis, confirming normalization of transaminase levels.³ IFN therapy has been used in the general clinical setting in Western countries since 1991, and in Japan since 1992. Since that time, with the development of the polymerase chain reaction (PCR) method, a revolutionary new technology for viral detection, quiescence of hepatitis has been confirmed in patients in whom HCV RNA was eradicated by IFN therapy;⁴ furthermore, inhibition of progression of liver disease and hepatocellular carcinogenesis has been demonstrated in these patients.^{5–8}

The aim of treatment of chronic hepatitis C is to improve the long-term prognosis of chronic liver disease (CLD) associated with persistent HCV infection; in other words, to prevent mortality associated with HCC and CLD. Sustained virological response (SVR) rates have improved with the standard therapy combining pegylated interferon (Peg-IFN) and ribavirin. SVR rates are no better than 40–50% in patients with genotype 1 infection who have high viral loads, however, so HCV cannot be eliminated in around half of these patients. In recent years, a number of new antiviral agents have been developed with the aims of increased therapeutic efficacy and decreased adverse reactions. In November 2011, the first generation protease inhibitor telaprevir became available for clinical use in patients with HCV genotype 1 infection and high viral loads. Triple therapy with telaprevir, Peg-IFN- α -2b and ribavirin has shown an increased antiviral effect, improving initial SVR rates to around 70% in treatment-naïve cases, but adverse reactions are also increased, including severe anemia and serious skin rashes.^{9–13} In Japan, trials are underway with triple therapy comprising a second generation protease inhibitor (TMC435,¹⁴ MK7009¹⁵ or BI-201335), Peg-IFN and ribavirin, as well as IFN-free oral antiviral therapy comprising a protease inhibitor and an NS5A inhibitor.¹⁶ Much is anticipated from the next generation direct antiviral agents (DAA), reported to have considerably fewer adverse reactions, and even greater antiviral effects, with SVR rates exceeding 80% in treatment-naïve cases.

Therapeutic guidelines for chronic hepatitis C should be formulated with the above-mentioned background

in mind, with careful consideration of the appropriateness of the presently available antiviral therapies for each individual patient.

Indications for antiviral therapy for HCV infection

In general, in patients with chronic hepatitis C, liver disease progresses gradually in association with elevation of alanine aminotransferase (ALT) levels, and the risk of developing cancer increases with the progression of fibrosis.⁸ Conversely, cancers are rarely seen arising from a normal liver with no inflammation or fibrosis. Accordingly, in general, antiviral therapy is indicated in all chronic hepatitis C patients with elevated ALT levels (ALT >30 IU/L), indicating hepatic inflammation, or a decreased platelet count (platelet count <150 000/ μ L), reflecting the degree of liver fibrosis. The indication for antiviral therapy should be individualized for patients with ALT \leq 30 IU/L and a platelet count \geq 150 000/ μ L, considering the risk of developing HCC is low.

Early viral eradication is required in the group at high risk of developing cancer. In patients with HCV infection, three factors have been identified as independent risk factors for hepatocellular carcinogenesis: (i) advanced age; (ii) advanced fibrosis; and (iii) male sex.^{5–7} Accordingly, the risk of developing cancer is particularly high in patients with multiple risk factors, and early introduction of antiviral therapy should be considered in this group.

Basic guidelines for treatment of chronic hepatitis C

In developing these guidelines, we formulated separate treatment plans according to the risk of developing cancer in different subgroups of patients with chronic hepatitis C, for elderly and non-elderly patients, and those with advanced fibrosis and mild fibrosis. Analyses of hepatocellular carcinogenesis in older patients with chronic hepatitis C show that the risk of cancer increases with increasing age, although the definition of “older age” varies, considered by some to be greater than 55, 60 or 65 years. In these guidelines, we have defined “elderly” as \geq 66 years old, based on Japanese clinical trials of telaprevir conducted with subjects aged \leq 65 years,¹¹ and the increased risk of HCC over the age of 65 years.¹⁷ Furthermore, although we have defined “advanced fibrosis” as a METAVIR score \geq F2, or platelet count of <150 000/ μ L, it should be kept in mind that the risk of cancer is particularly high in the

patient group with a METAVIR score \geq F3, or platelet count of $<120\,000/\mu\text{L}$.

For the group at high risk of developing HCC (elderly and advanced fibrosis), antiviral therapy should be commenced as soon as possible with due consideration to tolerability. Early commencement of antiviral therapy is also desirable in the medium-risk group (elderly or advanced fibrosis). However, some in the particularly high-risk group, elderly and/or with advanced fibrosis, are non-responders, so in order to avoid adverse reactions and the development of drug-resistant mutations, the treatment discontinuation criteria should be kept in mind during antiviral therapy. On the other hand, in the low-risk group comprising non-elderly patients without advanced fibrosis, early introduction of antiviral therapy is not always necessary. In some patients, it may be possible to await the introduction of the new generation antiviral agents, so the present indication for antiviral therapy should be decided after consideration of anticipated therapeutic effect, adverse reactions and the risk of HCC.

In any patient group, in case it is difficult with any presently available antiviral regimens to ensure viral eradication, and ALT levels are elevated (≥ 30 IU/L), patients should be administered long-term low-dose Peg-IFN or supportive therapy, for example, stronger neo-minophagen C (SNMC), ursodeoxycholic acid (UDCA). If an adequate therapeutic effect is not achieved, and iron overload is suspected, then the addition of, or changeover to, therapeutic phlebotomy should be considered. The aim of these therapies is to keep the ALT level ≤ 30 IU/L, maintaining it as low as possible. Strict control of the ALT level is particularly necessary in the group at high risk of developing HCC. Low-dose Peg-IFN therapy should be discontinued if no improvement is seen within 6 months in the ALT level (to ≤ 40 IU/L) or the α -fetoprotein (AFP) level (to ≤ 10 ng/mL).^{18,19}

Recommendations:

- 1 *In general, antiviral therapy is indicated in all chronic hepatitis C patients with elevated ALT levels (>30 IU/L) or a decreased platelet count ($<150\,000/\mu\text{L}$).*
- 2 *The indication for antiviral therapy should be individualized for patients with ALT levels ≤ 30 IU/L and a platelet count $\geq 150\,000/\mu\text{L}$, considering the risk of developing HCC is low.*
- 3 *For the group at high risk of developing HCC (elderly and advanced fibrosis), antiviral therapy should be commenced as soon as possible with due consideration to tolerability.*

- 4 *Following commencement of antiviral therapy in patients either elderly or with advanced fibrosis, in order to avoid adverse reactions and the development of drug-resistant mutations, the treatment discontinuation criteria, used for the early detection of non-responders, should be kept in mind during antiviral therapy.*
- 5 *In the low-risk group (non-elderly, non-advanced fibrosis), the present indication for antiviral therapy should be decided after consideration of anticipated therapeutic effect, adverse reactions and the risk of HCC.*
- 6 *If viral eradication is not achieved, long-term low-dose Peg-IFN or supportive therapy (SNMC or UDCA) should be administered with the aim of preventing progression of liver disease and preventing hepatocellular carcinogenesis. If an adequate therapeutic effect is not achieved, and iron overload is suspected, then the addition of, or changeover to, therapeutic phlebotomy should be considered.*
- 7 *Low-dose Peg-IFN therapy should be discontinued if no improvement is seen within 6 months in the ALT level (to ≤ 40 IU/L) or the AFP level (to ≤ 10 ng/mL).*

3. INTERFERON THERAPY

3.1 Interferon

THE α - AND β -types of IFN have been approved for use in the treatment of chronic hepatitis C. IFN- α preparations come in non-pegylated and pegylated forms, depending on whether polyethylene glycol (PEG) has been attached. The former comes in the form of natural human IFN- α and recombinant IFN- α -2b, and the latter as Peg-IFN- α -2a and Peg-IFN- α -2b. IFN- β preparations comprise natural non-pegylated-IFN- β .

IFN- α

Standard non-pegylated-IFN- α is unstable, with a plasma half-life of 3–8 h, and becomes undetectable after 24 h.²⁰ Administration at least three times per week is therefore required when treating chronic hepatitis C. Adverse reactions, including fever, chills and headache, are common with non-pegylated-IFN due to repeated rises and falls in the plasma levels. Of the non-pegylated IFNs, natural human IFN- α is approved for self-injection, and patients only need to attend hospital once every 2 weeks. Furthermore, patients can self-inject at night before retiring, better taking advantage of diurnal variations in plasma cortisol levels, and minimizing fever and other adverse reactions.^{21–23}

Peg-IFN- α

PEG is a water-soluble neutral molecule with no toxicity of itself. The number of ethylene oxide subunits determines the molecular weight. The aims of pegylating IFN are twofold: (i) to alter its *in vivo* pharmacodynamic properties; and (ii) protect the IFN molecule from recognition and elimination by the host immune defenses. Peg-IFN- α used in the treatment of chronic hepatitis C comes in the form of Peg-IFN- α -2a, with a 40-kD PEG branch chain covalently attached to IFN- α -2a, and Peg-IFN- α -2b, with a 12-kD PEG branch chain attached via a urethane bond to IFN- α -2b. They reach a maximum concentration (C_{max}) at 72–96 and 15–44 h after administration, respectively, and after a single dose maintain plasma levels within the therapeutic range for approximately 168 and 80 h, respectively.²⁴ As the molecular weight of PEG attached to IFN in this way increases, the intracorporeal retention time also increases, although the pharmacological effect decreases in inverse proportion. The IFN activity of Peg-IFN- α -2a is 7% that of non-pegylated-IFN- α -2a, whereas the IFN activity of Peg-IFN- α -2b is 28% that of non-pegylated-IFN- α -2b, with the latter more active. Accordingly, the actual antiviral effect is determined in a complex fashion by the balance between intracorporeal retention time and IFN activity, as well as the patient's body type and weight. Peg-IFN- α -2a is approved as monotherapy and in combination with ribavirin for national medical insurance coverage, whereas Peg-IFN- α -2b is approved in combination with ribavirin with or without telaprevir.

The two forms of Peg-IFN- α have different standard doses. The standard dosage regimen for Peg-IFN- α -2a is fixed at 180 μ g/week, and the dose of Peg-IFN- α -2b varies according to the patient's weight, the standard dosage regimen being 1.5 μ g/kg per week.

IFN- β

Interferon- β is a natural IFN that can be used in a non-pegylated form, and is approved as monotherapy and in combination with ribavirin for medical insurance coverage. It is administered at least three times per week as an i.v. injection or i.v. infusion. Although IFN- β binds to the same type I IFN receptor as IFN- α , and has a similar antiviral effect to IFN- α , their adverse reaction profiles differ. A retrospective study of natural human IFN- β + ribavirin combination therapy in the treatment of 40 cases with genotype 1b HCV infections reported fewer discontinuations due to adverse reactions, and only mild decreases in platelet counts.²⁵ Even patients with a history of discontinuing IFN- α therapy due

to depression tolerated IFN- β + ribavirin combination therapy well in terms of depressive symptoms and other adverse reactions.^{26–28} IFN therapy with natural human IFN- β is therefore recommended in patients in whom IFN- α therapy is not tolerated, for example, those with a history of depression.

Anti-IFN- α neutralizing antibodies were detected in 15% of non-responders to Peg-IFN- α + ribavirin therapy in one study.²⁹ Anti-IFN- α neutralizing antibodies do not block IFN- β activity, so a changeover to natural human IFN- β should be considered in cases of non-response to Peg-IFN- α + ribavirin due to these neutralizing antibodies.

Natural human IFN- β can be administered twice daily in divided doses, providing a more potent antiviral effect than once daily dosing as measured by the HCV dynamics.³⁰ Divided dosing IFN- β induction prior to Peg-IFN- α + ribavirin therapy has been trialed.³¹

Antiviral effects of IFN^{32–34}

IFN acts through binding to type I IFN receptors on the target cell membrane. Type I IFN receptors are common to IFN- α and IFN- β , and binding of either IFN type to the receptor causes activation of the tyrosine-protein kinase, Janus kinase 1 (JAK1). This induces phosphorylation of tyrosine residues in the intracellular domain of the receptor, resulting in phosphorylation and formation of dimer complexes of signal transducer and activator of transcription 1 (STAT1), which transmit signal to the cell nucleus. This in turn induces and upregulates expression of IFN-stimulated genes (ISG). The family of ISG includes a wide variety of antiviral and immunoregulatory genes, and the antiviral effects of IFN are thought to derive from proteins induced by ISG.

Adverse reactions

Adverse reactions to IFN therapy are experienced by almost all patients. The most common are influenza-like symptoms, such as general malaise, fever, headache and aching joints, and are reported by 60–95% of patients. Most influenza-like syndrome can be controlled with anti-inflammatory analgesic medication. Blood tests show leukopenia, with white blood cell counts <1000/mm³ seen in approximately 60% of patients. Serious infections associated with neutropenia are, however, considered rare.³⁵ White blood cell, neutrophil and platelet counts tend to decrease for the first 4 weeks of IFN therapy, then often remain stable without further decline. Neuropsychiatric symptoms such as depression and insomnia occur in 5–10% of patients, and are more