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Add-on Therapy of Pitavastatin and Eicosapentaenoic Acid Improves Outcome of Peginterferon Plus Ribavirin Treatment for Chronic Hepatitis C

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Despite the use of pegylated-interferon (peg-IFN) plus ribavirin combination therapy, many patients infected with hepatitis C virus (HCV)-1b remain HCV-positive. To determine whether addition of pitavastatin and eicosapentaenoic acid (EPA) is beneficial, the "add-on" therapy option (add-on group) was compared retrospectively with unmodified peg-IFN/ribavirin therapy (standard group). Association of host- or virus-related factors with sustained virological response was assessed. In HCV replicon cells, the effects of pitavastatin and/or EPA on HCV replication and expression of innate-immunity- and lipid-metabolism-associated genes were investigated. In patients infected with HCV-1b, sustained virological response rates were significantly higher in the add-on than standard group. In both groups, sustained virological response rates were significantly higher in patients with genotype TT of IL-28B (rs8099917) than in those with non-TT genotype. Among the patients with non-TT genotype, sustained virological response rates were markedly higher in the add-on than standard group. By multivariate analysis, genome variation of IL28B but not add-on therapy remained as a predictive factor of sustained virological response. In replicon cells, pitavastatin and EPA suppressed HCV replication. Activation of innate immunity was obvious in pitavastatin-treated cells and EPA suppressed the expression of sterol regulatory element binding protein-1c and low-density lipoprotein receptor. Addition of pitavastatin and EPA to peg-IFN/ribavirin treatment improved sustained virological response in patients infected with HCV-1b. Genotype variation of IL-28B is a strong predictive factor in add-on therapy. ***J. Med. Virol. 85:250–260, 2013.***

KEY WORDS: cholesterol; hepatitis C virus; IL28B; replicon system

Abbreviations: EPA, eicosapentaenoic acid; HCV, hepatitis C virus; HMGR, HMG-CoA reductase; IRF3, IFN regulatory factor 3; ISG15, IFN-stimulated gene 15; ITPA, inosine triphosphatase; LDLR, low-density lipoprotein receptor; MAVS, mitochondrial antiviral signaling; NPC1L1, Niemann-Pick C1 like 1; OR, odds ratio; PCR, polymerase chain reaction; peg-IFN, pegylated-interferon; PUFA, polyunsaturated fatty acid; RIG-I, retinoic acid inducible gene I; SNP, single nucleotide polymorphism; SREBP, sterol regulatory element binding protein; TRAF6, TNF receptor associated factor 6.

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INTRODUCTION

Nearly, 170 million people are infected with hepatitis C virus (HCV) worldwide and natural history studies show that 5–20% of patients develop cirrhosis after approximately 20 years of infection [Alter, 2005]. Currently, pegylated-interferon (peg-IFN) plus ribavirin combination therapy has become the standard care for chronic hepatitis C because it achieves high rates of sustained virological response [Aghemo et al., 2009]. However, in patients infected with genotype 1b HCV (HCV-1b), at most, 50% of individuals achieve a sustained virological response following combination therapy, and HCV-1b in high viral loads (>5.0 log IU/ml) accounts for >70% of patients with HCV infection in Japan [Kumada et al., 2006]. The response to IFN-based treatment is influenced by virus-related factors including viral load and genotypes; host-related factors, such as sex, age, insulin resistance, staging of the disease and responses to previous antiviral therapies; as well as therapeutic factors, such as dose and duration of treatment [Shiffman, 2002; Backus et al., 2007; Kanwal et al., 2007; Bortoletto et al., 2010]. In addition, as a critical genetic factor for governing the outcomes of peg-IFN plus ribavirin combination therapy, genome variation of IL28B and inosine triphosphatase (ITPA) have been identified recently. At the spot of rs8099917 in the IL28B region, patients infected with HCV-1b with the major variation type (TT) show markedly higher sustained virological response rates than those with the minor variation type (TG + GG) [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Hayes et al., 2011]. Single nucleotide polymorphism (SNP) variation of the ITPA gene at rs1127354 is associated with anemia as an adverse effect during peg-IFN plus ribavirin combination therapy [Fellay et al., 2010; Azakami et al., 2011; Suzuki et al., 2011; Thompson et al., 2011]. In patients who have rs1127354 genotype CC (major type), ribavirin-induced anemia is more frequent and forces a reduction in dose of ribavirin, which worsens the therapeutic outcome. Alternatively, viral amino acid substitutions at core 70 and 91 are significant predictors of treatment outcome. In particular, a point mutation of core 70 from Arg to Gln is significantly associated with non-sustained virological response in patients infected with HCV-1b [Akuta et al., 2005, 2007; El-Shamy et al., 2012].

Investigation of patients treated by peg-IFN plus ribavirin combination therapy has indicated that serum cholesterol and statin use predict virological response to therapy [Harrison et al., 2010]. Recent studies have shown that virological response is improved by addition of fluvastatin or pitavastatin to peg-IFN and ribavirin treatment [Bader et al., 2008; Sezaki et al., 2009; Shimada et al., 2012]. Statins were associated with a reduced risk of hepatocellular carcinoma in a large cohort of patients with diabetes [El-Serag et al., 2009]. In other studies, it has been demonstrated that polyunsaturated fatty acids (PUFAs) inhibit HCV replication by a mechanism that is independent of their roles in regulating lipogenesis [Leu et al., 2004; Kapadia and Chisari, 2005; Huang et al., 2007]. Takaki et al. [2007] have reported that eicosapentaenoic acid (EPA), a type of n-3 PUFA, allows maintenance of the original ribavirin dose in chronic hepatitis C patients during peg-IFN plus ribavirin combination therapy. However, the effects of these lipid modulators on chronic hepatitis C patients with intractable IL-28B allele remain unknown.

As a result of this experimental and therapeutic evidence, a new antiviral strategy to improve treatment outcome for chronic hepatitis C was designed, that is, addition of pitavastatin and EPA to peg-IFN plus ribavirin combination therapy (add-on therapy). The validity of the add-on therapy was evaluated by comparing its effect on the final outcome (i.e., sustained virological response) with that of unmodified peg-IFN plus ribavirin combination therapy (standard therapy), and pretreatment predictors of virological response were investigated. Additionally, the antiviral effect of pitavastatin and/or EPA was estimated in HCV replicon cells.

MATERIALS AND METHODS

Study Patients

In Kyushu Medical Center, a standard protocol in Japan (subcutaneous peg-IFNα2a [180 μg] or peg-IFNα2b [median dose of 1.5 μg/kg, range 1.3–1.7] weekly, along with oral ribavirin daily for 48 weeks) was adopted for chronic hepatitis C patients from 2005 to 2008. The dose of ribavirin was adjusted according to body weight: 600 mg for patients weighing <60 kg, 800 mg for those weighing 60–80 kg, and 800 mg for those weighing >80 kg. From 2008, oral pitavastatin (2 mg/day) and ethyl icosapentate (1,800 mg/day) have been added to the standard protocol (add-on protocol). It has been shown that statins contribute to improving the virological response [Bader et al., 2008; Sezaki et al., 2009]. The add-on protocol was expected to improve treatment, and was applied to all patients after 2008 in Kyushu Medical Center, but a randomized study could not be designed. In these protocols, 48- and 24-week regimens were applied to patients infected with HCV-1b and HCV-2, respectively. Patients who experienced previous therapy using peg-IFN were excluded. Patients with cirrhosis were not included. Because of the possibility that vitamin E and bile acids including ursodeoxycholic acid promote HCV replication [Chang and George, 2007; Yano et al., 2007; Scholtes et al., 2008; Nakamura et al., 2010], treatment with these agents was withdrawn at least 1 month before the initiation of antiviral treatment. The study protocol was approved by the Ethics Committee of the National Hospital Organization, and written informed consent was obtained from all patients. Finally, 238 patients (genotype 1b/2 = 176/62) who were treated with the standard protocol (standard group) and 162 patients (genotype 1b/2 = 101/61) who were treated with the add-on protocol

TABLE I. Profile and Baseline Characteristics of Patients Infected With HCV-1b

Number of patients	Standard group	Add-on group	*P*
Gender: M/F	91/85	46/55	NS
Age (years)	59.5 ± 10.2	57.2 ± 12.5	NS
Past history of IFN therapy: naive/unmodified IFN/unmodified IFN + RBV	147/21/8	77/18/6	NS
HCV RNA (log IU/ml)	5.73 ± 0.16	6.08 ± 0.64	0.001
IL-28B (rs8099917): TT/TG + GG/ND	39/18/119	69/29/3	NS
ITPA (rs1127354): CC/CA + AA/ND	43/14/119	70/27/4	NS
Staging: F_{0-1}/F_{2-3}/ND	15/47/114	27/53/21	NS
ALT (IU/l)	74.5 ± 58.3	62.4 ± 45.2	NS
GGT (IU/l)	55.8 ± 46.8	51.9 ± 45.4	NS
WBC (/μl)	4,859 ± 1,239	4,870 ± 1,395	NS
Hemoglobin (g/dl)	13.9 ± 1.3	13.7 ± 1.5	NS
Platelet (/μl)	16.3 ± 5.7	19.1 ± 6.5	0.006
% of patients treated with enough total doses of Peg-IFN[a]	61.1	75.7	NS
% of patients treated with enough total doses of RBV[b]	76.4	77.1	NS

IFN, interferon; RBV, ribavirin; ITPA, inosine triphosphatase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; WBC, white blood cell; Peg-IFN, pegylated-interferon; ND, not determined; NS, not significant.
[a]Enough total doses: >80% of planned doses.
[b]Enough total doses: >60% of planned doses.

(add-on group) were enrolled and retrospectively analyzed. The profile and baseline characteristics of patients infected with HCV-1b are shown in Table I. In all patients infected with HCV-1b or HCV-2, baseline HCV RNA levels in serum were ≥5.0 log IU/ml.

Laboratory Data

Hematological, biochemical and virological parameters were determined by the clinical laboratory at Kyushu Medical Center. Serum HCV RNA concentrations were determined by the COBAS TaqMan PCR HCV test (Roche Diagnostics, Tokyo, Japan). Sustained virological response was defined as undetectable HCV RNA at week 24 after completion of therapy. Genotyping for the IL28B (rs8099917) and ITPA (rs1127354) polymorphisms was performed by TaqMan® SNP Genotyping Assays (Applied Biosystems, Branchburg, NJ) that apply a polymerase chain reaction (PCR)-based restriction fragment length polymorphism assay. To determine amino acid polymorphism in HCV core protein, the PCR method with primers specific for polymorphism at core 70 was performed as described previously [Nakamoto et al., 2009].

Cell Lines and Treatment

The human-hepatoma-derived cell line, Huh7/Rep-Feo-1b, which stably expresses the HCV Rep-Feo replicon, was a kind gift from the Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University. The HCV subgenomic replicon plasmids, which contained NS3, NS4, NS5A, and NS5B, were derived from the HCV-N strain (genotype 1b), and the construct expressed a chimeric reporter protein of luciferase and neomycin phosphotransferase that allowed selection of cells and rapid measurement of the replication levels in stable replicon-expressing cells [Yokota et al., 2003; Tanabe et al., 2004; Toyoda et al., 2011]. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin in a humidified 37°C/5% CO_2 incubator. Pitavastatin (donated by Kowa Pharmaceutical Co, Tokyo, Japan) and EPA (Otsuka Pharmaceutical Co, Tokyo, Japan) were dissolved in 10% carboxyl methylcellulose and chloroform, respectively, and stored in stock solutions at a concentration of 10 and 20 M, respectively. According to previous reports and our pretests for inhibition rates of HCV replication and cytotoxicity [Ye et al., 2003; Leu et al., 2004; Kapadia and Chisari, 2005; Ikeda et al., 2006], Huh7/Rep-Feo-1b cells were treated with 20 μM EPA, 10 μM pitavastatin, or 20 μM EPA plus 10 μM pitavastatin for 48 hr. The concentrations of EPA and pitavastatin may have been reasonable because they were lower than the reported maximum blood concentration of EPA or pitavastatin in healthy adult men with usual daily doses. For control cells, the same volume of 10% carboxyl methylcellulose and chloroform used for treated cells was added to medium and incubated for 48 hr.

Cell Proliferation/Viability and Luciferase Assays

The proliferation and viability of cultured cells were checked by Cell Viability and Proliferation Assay Kit (Funakoshi, Tokyo, Japan). Luciferase activity assay was performed using the Bright-Glo Luciferase Assay System (Promega, Tokyo, Japan). According to the manufacturer's protocol, luciferase was extracted from control and treated cells, and luciferase activity was quantified by use of a luminometer.

Real-Time PCR

mRNA expression levels in Huh7/Rep-Feo-1b cells under EPA and/or pitavastatin treatment were

analyzed using real-time RT-PCR and compared with untreated Huh7/Rep-Feo-1b cells. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized from 1.0 μg RNA using GeneAmp™ RNA PCR (Applied Biosystems) with random hexamers. Real-time RT-PCR was performed using LightCycler-FastStart DNA Master SYBR Green 1 (Roche, Basel, Switzerland) according to the manufacturer's instructions. The reaction mixture (20 μl) contained LightCycler-FastStart DNA Master SYBR Green 1, 4 mM $MgCl_2$, 0.5 μM upstream and downstream PCR primers, and 2 μl first-strand cDNA as a template. To control for reaction variations, all PCR data were normalized against the expression of retinoblastoma binding protein 6 [Nakamuta et al., 2011]. The real-time RT-PCR primer sets in this study are listed in Table II.

Statistical Analysis

Statistical analysis was performed using JMP software (SAS Institute, Inc., Cary, NC). Differences between categorical variables were analyzed using Fisher's extract test or χ^2 test. Mann–Whitney U test was used for continuous variables. Multivariate analysis was used to identify factors independently associated with the achievement of sustained virological response. The odds ratio (OR) and 95% confidence intervals were also calculated. $P < 0.05$ was considered to be statistically significant.

RESULTS

Sustained Virological Response Rates in Patients Infected With HCV-1b and HCV-2

Peg-IFN and/or ribavirin were discontinued or their doses reduced, as required, upon reduction of hemoglobin levels, neutrophil counts or platelet counts, or the development of other adverse effects. Therefore, to evaluate therapeutic effects properly, sustained virological response rates were examined by intention to treat analysis. Within the enrolled patients, 62 and 61 patients infected with HCV-2 were included in the standard and add-on therapy groups, respectively. In these patients, no significant difference was found in sustained virological response rates between the standard and add-on therapy groups; 80.7% and 77.1%, respectively (Fig. 1A). Hence, all subsequent examinations were conducted on patients infected with HCV-1b.

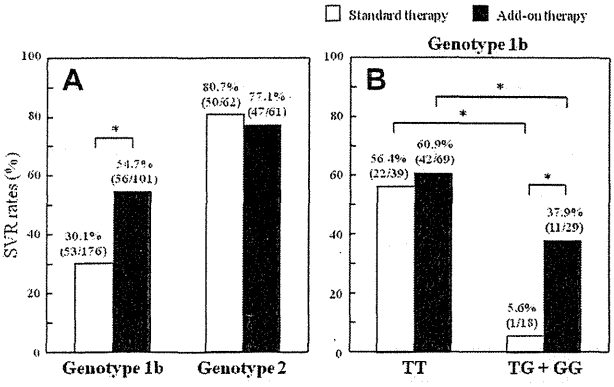

Fig. 1. Sustained virological response rates in chronic hepatitis C patients: comparison between standard and add-on therapy. **A**: Results for HCV genotype 1b and 2. **B**: Results for genome variation of IL28B (rs8099917); genotype TT and non-TT (TG + GG). Data for HCV-1b patients are shown. *$P < 0.01$.

In patients infected with HCV-1b, sustained virological response rates were significantly higher in the add-on than in the standard therapy group (54.7% vs. 30.1%, $P < 0.0001$; Fig. 1A), although background HCV RNA levels were significantly higher in the add-on therapy group (Table I). Platelet counts were higher in the add-on therapy group but those in the standard therapy group were still sufficient for IFN-based therapy. Of note, no significant difference was found between the standard and add-on therapy groups for the rate of patients in whom sufficient total doses of peg-IFN (>80% of planned doses) and ribavirin (>60% of planned doses) were administered (Table I).

TABLE II. Sequences of Primers Used for Real-Time PCR

Genes	Forward (5′ → 3′)	Reverse (5′ → 3′)
RIG-I	GGCCCACTGCCCCAGGTCAT	TCCCCAACACCAACCGAGGC
MAVS	CCCTCTGGCATCTCTTCAATACC	TTCGTCCGCGAGATCAACTA
IRF3	CCAGCTTGGACAATCCCACTC	GAAGGCTGTCACCTCGAACTC
TRAF6	GAGGTCTCCACCCGCTTTGA	TTGAGCAAGTGAGGGCAAGCTA
IFNβ1	GCGACACTGTTCGTGTTGTCA	CCAAGCAAGTTGTAGCTCATGGA
ISG15	GGGCTGGGACCTGACGGTGA	GGACAGCCAGACGCTGCTGG
HMGR	GCCTGGCTCGAAACATCTGAA	CTGACCTGGACTGGAAACGGATA
SREBP-1	GCTGTCCACAAAAGCAAATCTCT	GTCAGTGTGTCCTCCACCTCAGT
LDLR	CAACGGCTCAGACGAGCAAG	AGTCACAGACGAACTGCCGAGA
RBBP6	GCGACCTGCAGATCACCAA	TGCCATCGCTGGTTCAGTTC

RIG-I, retinoic acid inducible gene I; MAVS, mitochondrial antiviral signaling; IRF, interferon regulatory factor; TRAF, TNF receptor associated factor; IFN, interferon; ISG, interferon-stimulated gene; HMGR, HMG-CoA reductase; SREBP, sterol regulatory element binding protein; LDLR, LDL receptor; RBBP, retinoblastoma binding protein.

Effect of IL28B and ITPA Genotypes on Viral Response

According to genetic variation of IL28B gene (rs8099917), sustained virological response rates in patients infected with HCV-1b were determined (Fig. 1B). In both the standard and add-on therapy groups, sustained virological response rates were significantly higher in patients with the major type genome variation (TT) than in those with the minor type (TG + GG). In the latter, sustained virological response rates were markedly higher in the add-on than in the standard therapy group (37.9% vs. 5.6%, $P = 0.007$). In patients with the major type genome variation, addition of pitavastatin and EPA induced higher sustained virological response rates although no significant difference was found between the two treatment groups. In comparison between the major (CC) and minor (non-CC) types of ITPA (rs1127354), sustained virological response rates were comparable between the standard and add-on therapy groups (Fig. 2A). However, in the add-on group, the percentage of patients infected with HCV-1b who completed therapy without dose reduction of ribavirin was significantly higher among those with the minor type of ITPA than the major type (45.8% vs. 21.5%, $P = 0.004$; Fig. 2B).

Viral Kinetics With Add-on Therapy

Viral kinetics in patients infected with HCV-1b were examined in the add-on therapy group according to genome variation of the IL28B (rs8099917), and compared between the sustained virological response and non-sustained virological response groups. In patients with major variation type (TT), viral decline was significantly greater at all times (days 3–84) in the sustained virological response than in the non-sustained virological response group (Fig. 3A). However, in patients with minor variation type (TG + GG), viral kinetics were similar within the first 2 weeks of treatment in the sustained virological response and non-sustained virological response groups (Fig. 3B). Accordingly, sustained virological response was affected by the depth of early phase viral decline in patients with major variation but not in patients with minor variation. Viral kinetics in patients with minor type variation (TG ± GG) of IL-28B were compared between the standard therapy and add-on therapy

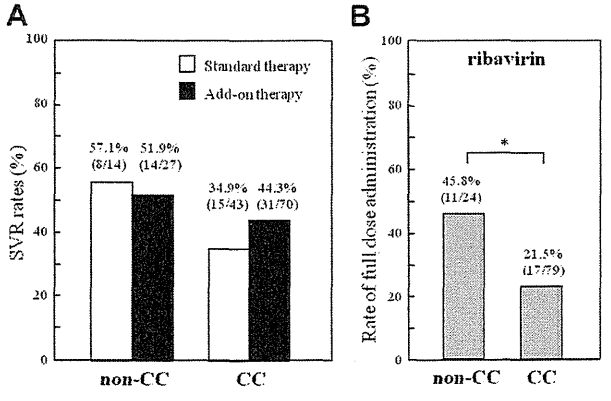

Fig. 2. Clinical data of patients infected with HCV-1b: comparison between genome variations of ITPA. **A**: Sustained virological response rates were compared between standard and add-on therapy. Results are presented for each genome variation of ITPA (rs1127354); genotype CC and non-CC. **B**: Numbers of patients in whom planned ribavirin doses were completed. Results in patients infected with HCV-1b treated with add-on therapy are shown in each genome variation of ITPA (rs1127354); genotype CC and non-CC. $^{*}P < 0.05$.

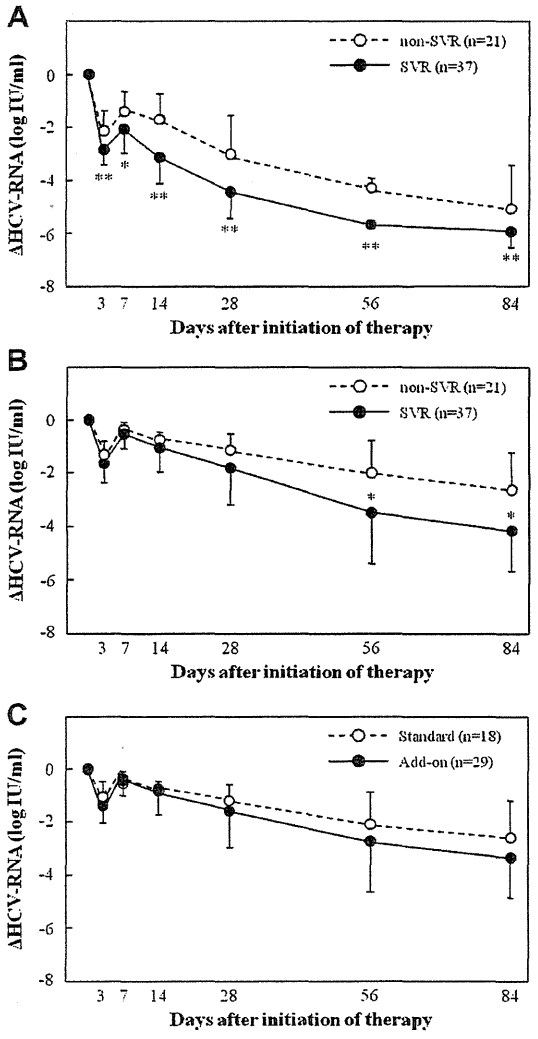

Fig. 3. Viral kinetics in patients infected with HCV-1b. **A**: Results in patients with major type variation (TT) of IL-28B: comparison between sustained virological response and non-sustained virological response groups. **B**: Results in patients with minor type variation (TG + GG) of IL-28B: comparison between sustained virological response and non-sustained virological response groups. **C**: Results in patients with minor type variation (TG + GG) of IL-28B: comparison between standard therapy and add-on therapy groups. $^{*}P < 0.05$, $^{**}P < 0.01$ (sustained virological response vs. non-sustained virological response).

groups (Fig. 3C). As a result, viral decline was somewhat greater after day 28 in the add-on therapy group but the difference was not significant.

Effect of Amino Acid Substitutions of HCV Core 70 on Viral Response

Add-on therapy was significantly more effective in patients with IL28B minor variation (TG + GG) compared with standard therapy, therefore, we investigated the association between HCV core 70 amino acid mutation and therapeutic outcome. In 27 patients infected with HCV-1b, who had minor variation of IL28B (TG + GG) and were treated with add-on therapy, core 70 amino acid mutation was determined. Sustained virological response was achieved in 10 patients and core 70 mutation (Gln) was found in 6 of the 10 patients (60%). Within the 17 non-sustained virological response patients, the mutation was identified in eight patients (47.1%). Accordingly, within these patients, the core 70 amino acid substitutions did not affect sustained virological response in the add-on therapy.

Predictive Factors Associated With Sustained Virological Response

Among the factors listed in Table III, predictive factors associated with sustained virological response were examined in patients infected with HCV-1b. Univariate analysis identified six parameters that correlated significantly with sustained virological response; age ($P = 0.0038$), fibrotic staging ($P = 0.0012$), γ-glutamyl transpeptidase ($P = 0.0009$), platelet count ($P = 0.0132$), genetic variation of IL28B ($P < 0.0001$) and add-on therapy ($P < 0.0001$; Table III). In multivariate analysis, significant contribution factors for sustained virological response were age (<60 years; OR 3.06, $P = 0.0221$), IL28B (genotype TT; OR 6.69, $P = 0.0019$) and staging (F_{0-1}; OR 5.71, $P = 0.0035$; Table IV). When IL28B was excluded from the factors in multivariate analysis, addition of pitavastatin and EPA (add-on therapy) was also selected as a significant contribution factor for sustained virological response (OR 2.13, $P = 0.0395$).

TABLE IV. Multivariate Analysis for Predictive Factors Associated With Sustained Virological Response

Factors	Category	95% confidence intervals	P
Age (years)	1. ≥60: 1.0		
	2. <60: 3.06	1.20–8.24	0.0221
IL-28B (rs8099917)	1. TG + GG: 1.0		
	2. TT: 6.69	2.17–24.66	0.0019
Staging	1. F_{2-3}: 1.0		
	2. F_{0-1}: 5.71	1.91–20.51	0.0035

Subgenomic HCV Replicon System

Suppression of HCV RNA replication by pitavastatin and/or EPA was examined in Huh7/Rep-Feo-1b cells by luciferase assay. The concentrations of pitavastatin and EPA for the following experiments were determined according to previous studies [Ye et al., 2003; Leu et al., 2004; Kapadia and Chisari, 2005; Ikeda et al., 2006] and our pilot study for cytotoxicity and luciferase assay (data not shown). Huh7/Rep-Feo-1b cells were incubated with or without 10 μM pitavastatin and/or 20 μM EPA for 48 hr. As a precondition, the proliferative activity and viability of pitavastatin- and/or EPA-treated cells were comparable with those of control cells (data not shown). As a result, luciferase activity was significantly suppressed in EPA- and/or pitavastatin-treated cells compared with the control cells (Fig. 4A). At these concentrations, the suppressive effect was more marked in pitavastatin-treated than EPA-treated cells.

TABLE III. Univariate Analysis Between Non-Sustained Virological Response and Sustained Virological Response Groups

Factors	Non-SVR	SVR	P
Gender (M/F)	82/86	55/54	NS
Age (years)	60.5 ± 10.6	55.7 ± 12.0	0.0038
Past history of IFN therapy: naive/unmodified IFN/unmodified IFN + RBV	136/24/8	88/15/6	NS
HCV RNA (log IU/ml)	6.03 ± 0.16	5.91 ± 0.55	NS
IL-28B (rs8099917) TT/TG + GG/ND	35/44/89	12/64/33	<0.0001
ITPA (rs1127354) CC/CA + AA/ND	59/20/89	54/21/34	NS
Staging (F_{0-1}/F_{2-3}/ND)	11/59/98	31/41/37	0.0012
Treatment add-on/standard	45/123	56/53	<0.0001
ALT (IU/l)	72.3 ± 57.7	63.9 ± 45.2	NS
GGT (IU/l)	65.3 ± 56.0	41.1 ± 27.1	0.0009
WBC (/μl)	4,935 ± 1,392	4,791 ± 1,254	NS
Hemoglobin (g/dl)	13.8 ± 1.4	13.8 ± 1.4	NS
Platelet (/μl)	16.7 ± 5.7	19.1 ± 6.6	0.0132
% of patients treated with enough total doses of Peg-IFN[a]	60.9	74.4	NS
% of patients treated with enough total doses of ribavirin[b]	71.9	80.8	NS

IFN, interferon; RBV, ribavirin; ITPA, inosine triphosphatase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; WBC, white blood cell; Peg-IFN, pegylated-interferon; ND, not determined; NS, not significant.
[a]Enough total dose: >80% of planned doses.
[b]Enough total doses: >60% of planned doses.

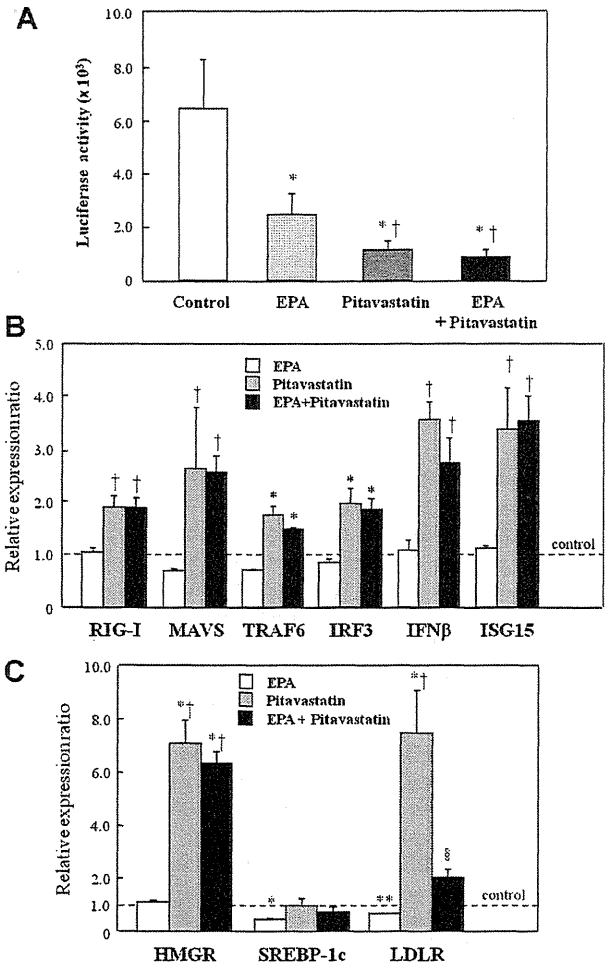

Fig. 4. Treatment of pitavastatin and/or EPA in HCV replicon cells. **A**: HCV replication was estimated by luciferase assay. Huh7/Rep-Feo-1b cells were treated with pitavastatin (10 μM) and/or EPA (20 μM) for 48 hr. $^{*}P < 0.01$ versus control, $^{\dagger}P < 0.01$ versus EPA. **B**: Expression levels of RIG-I, MAVS, TRAF6, IRF3, IFNβ, and ISG15 genes in Huh7/Rep-Feo-1b cells treated with pitavastatin (10 μM) and/or EPA (20 μM) for 48 hr. $^{*}P < 0.01$ versus control and EPA, $^{\dagger}P < 0.05$ versus control and EPA. **C**: Expression levels of HMGR, SREBP-1c and LDLR genes in Huh7/Rep-Feo-1b cells treated with pitavastatin (10 μM) and/or EPA (20 μM) for 48 hr. $^{*}P < 0.01$ versus control, $^{**}P < 0.05$ versus control, $^{\dagger}P < 0.01$ versus EPA, $^{\S}P < 0.01$ versus pitavastatin.

In Huh7/Rep-Feo-1b cells, the expression levels of innate-immunity-associated genes were examined after 48 hr treatment with 10 μM pitavastatin and/or 20 μM EPA. As shown in Figure 4B, retinoic acid inducible gene I (RIG-I), mitochondrial antiviral signaling (MAVS), TNF receptor associated factor 6 (TRAF6), IFN regulatory factor 3 (IRF3), IFNβ and IFN-stimulated gene 15 (ISG15) showed similar trend in expression. Accordingly, their expression was significantly increased by pitavastatin but not by EPA, and EPA did not show an additive effect with pitavastatin. With the same treatments, expression of lipid-metabolism-associated genes was analyzed (Fig. 4C). HMG-CoA reductase (HMGR) expression was significantly enhanced by pitavastatin but not by EPA. The sterol regulatory element binding protein 1c (SREBP-1c) expression was significantly suppressed by EPA but not by pitavastatin. Low-density lipoprotein receptor (LDLR) expression was significantly suppressed by EPA, whereas the expression was activated by pitavastatin, but the activation was lost in the presence of EPA.

DISCUSSION

For ethical reasons, standard therapy could not be selected after 2008; therefore, the present study was unable to eliminate some methodological issues that limit the interpretation and drawing of firm conclusions. For example, the percentage of patients receiving sufficient total dose of peg-IFN was lower in the historical standard group although the difference was not significant and, in order to prevent dose reduction, additional means might have been performed on the add-on group after 2008. However, in univariate and multivariate analyses, total dose of peg-IFN was not detected as a significant factor for sustained virological response. Nevertheless under these limitations, the presented clinical and in vitro studies indicate some sufficient trends in treatment response.

Previous studies on hepatic lipid metabolism have shown that, in the liver of patients with HCV infection, synthesis of cholesterol and fatty acids is still activated, regardless of overaccumulation of lipids [Kohjima et al., 2009; Nakamuta et al., 2009, 2011; Fujino et al., 2010]. This means that addition of pitavastatin and EPA to standard therapy is pathophysiologically reasonable for patients with chronic hepatitis C. Sustained virological response rates in patients infected with HCV-2 were sufficiently high and comparable between the standard and add-on therapy groups (Fig. 1A). Therefore, this study was focused on patients infected with HCV-1b with high virus load. This investigation of sustained virological response in patients treated with add-on or standard therapy had two clinically important findings.

First, add-on therapy led to significantly higher sustained virological response rates than did standard therapy (Fig. 1A). Although overall sustained virological response rate in this study was lower compared with the results from some other institutions, it may be because intention to treat analysis was used in this study and the ratio of IL28B minor (TG + GG) patients was higher in the standard and add-on groups. When sustained virological response rates were compared only in patients with IL28B major or in those with IL28B minor, the sustained virological response rates were not lower compared with those in other reports (data not shown). The suppressive effect against HCV replication by statins and EPA, and their synergistic action with IFN, has already been demonstrated in some HCV replicon systems [Ye

et al., 2003; Leu et al., 2004; Kapadia and Chisari, 2005; Huang et al., 2007; Ikeda and Kato, 2007]. In our investigation using the luciferase assay in Huh7/Rep-Feo-1b cells, a similar suppressive effect was seen with both pitavastatin and EPA treatments (Fig. 4A). It has been reported that the statins impede HCV replication through inhibition of host protein geranylgeranylation and FBL2 has been identified as a geranylgeranylated cellular protein required for HCV RNA replication [Wang et al., 2005; Nakamuta et al., 2011]. PUFAs, including EPA, inhibit HCV replication, although the precise mechanism is still unclear but may be independent of the route regulating lipogenesis [Leu et al., 2004; Kapadia and Chisari, 2005]. The synergistic and additive effect of EPA with pitavastatin was not significant in our luciferase assay; therefore, statins and EPA may act against cognate targets.

Second, the add-on therapy improved sustained virological response rates especially in patients with the minor type variation (TG + GG) of the IL28B gene (rs8099917), in whom sustained virological response is expected to be poor after standard therapy (Fig. 1B). Recent studies have revealed that SNPs within or adjacent to IL28B region provide a strong predictive value for the outcome of IFN-based therapy in patients infected with HCV-1b [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Hayes et al., 2011]. With add-on therapy, sustained virological response rates were significantly higher in patients with major type (TT) than minor type (TG + GG) variations, meaning that genome variation of IL28B (rs8099917) still governs the outcome even in add-on therapy. However, in the patients with minor type variation, the sustained virological response rate (37.9%) in the add-on group was markedly higher compared with that in the standard therapy group (Fig. 1B). This sustained virological response rate may be sufficiently high for clinical use of add-on therapy in patients infected with HCV-1b with minor type variation. From this point of view, the add-on therapy is a clinically valuable strategy for chronic hepatitis C. In the analysis of viral dynamics in patients with minor type variation of IL28B, no significant difference was found in viral decline within 84 days between the standard and add-on groups (Fig. 3C). Although there is still no evidence, in the add-on therapy, late phase viral decline (3 months after treatment initiation) may be more important for the achievement of sustained virological response in patients with minor type variation.

It has been emphasized that mutation of amino acids 70 and 91 in the core region of HCV-1b as a virus-related factor, as well as genome variation of IL28B gene as a host-related factor, greatly influences the outcome of IFN-based antiviral treatments. According to recent clinical studies in patients infected with HCV-1b, substitution of core 70 is assessed as a more influential factor affecting the outcome of peg-IFN plus ribavirin combination therapy, rather than that of core 91 [Akuta et al., 2007; Hayes et al., 2011; El-Shamy et al., 2012]. Even in the latest triple therapy with peg-IFN, ribavirin and a NS3/4A protease inhibitor, telaprevir, patients infected with HCV-1b with core 70 mutation were reported to be severely resistant to the therapy [Akuta et al., 2010]. In our assessment of patients with minor type IL28B variation at rs8099917 (TG + GG), mutation at core 70 was likely not to diminish the outcome of add-on therapy, although the number of patients examined was small (Fig. 4). Therefore, the lipid modulators, pitavastatin and EPA, may be expected to be more effective for patients infected with HCV-1b with core 70 mutation, compared with an NS3/4A protease inhibitor. However, for reliable assessment, further clinical data are needed from patients treated with add-on therapy.

As part of its pathogenic strategy, HCV interferes with the innate immune response of its host; mainly in the RIG-I/MAVS pathway [Breiman et al., 2005; Tasaka et al., 2007; Baril et al., 2009; Jouan et al., 2010; Lemon, 2010; Liu and Gale, 2010; Ekisioglu et al., 2011]. RIG-I undergoes a conformational change upon HCV RNA binding and interacts with MAVS, resulting in phosphorylation and nuclear translocation of IRF3, which leads to transcriptional activation and synthesis of IFNβ. IFNβ activates the Jak–STAT (Janus kinase–signal transducer and activator of transcription) signaling pathway and acts through the expression of ISGs. TRAF6 is recruited to the MAVS complex and is required for activation of nuclear factor-κB, which forms an enhanceosome on the IFNβ promoter in coordination with IRF3. In HCV-infected cells, NS3/4A protease cleaves MAVS, and the RIG-I/MAVS pathway is impeded. In the present study, the HCV replicon system was used to examine how pitavastatin and EPA influence the RIG-I/MAVS pathway, which plays an important role in the innate antiviral host response to HCV infection. The expression profile of innate-immunity-associated genes in pitavastatin- and/or EPA-treated Huh7/Rep-Feo-1b cells showed that only pitavastatin activated expression of the tested genes, RIG-I, MAVS, IRF3, TRAF6, IFNβ, and ISG15, similarly (Fig. 4B). EPA treatment did not increase expression levels of these genes. It is unclear whether the activation of these innate-immunity-associated factors directly contributes to elimination of HCV or whether inhibition of HCV replication by pitavastatin treatment directly leads to the activation of innate immunity through lowering NS3/4A protease expression.

Cholesterol, fatty acids, and lipid rafts have been demonstrated to be critical for efficient replication, infection and secretion of HCV [Simons and Ehehalt, 2002; Kushner et al., 2003]. For example, HCV replication was suppressed by inhibition of the liver X receptor α–SREBP-1c pathway [Kapadia and Chisari, 2005]. Therefore, negative modulation of lipid synthesis may be an antiviral step of statins and EPA. In pitavastatin treatment of HCV replicon cells, HMGR

and LDLR expression was enhanced in response to inhibition of cholesterol synthesis, whereas EPA decreased the expression of SREBP-1c and LDLR, by which fatty acid synthesis and cholesterol uptake might be lowered (Fig. 4C). Of note, although pitavastatin alone enhanced the expression of LDLR, the enhancement was abolished by addition of EPA (Fig. 4C). This effect of EPA indicates the clinical significance of the add-on therapy because LDLR is known to be an important cellular factor that is required for cell entry/infection of HCV. EPA addition is expected to accelerate the antiviral effect of peg-IFN, ribavirin and pitavastatin through repression of HCV entry/infection as well as HCV replication. It has recently been reported that HCV particles are enriched in cholesterol and virion cholesterol is involved in HCV cell entry, depending on Niemann-Pick C1-like 1 (NPC1L1), which is an HCV cell entry factor as well as a cellular cholesterol uptake receptor [Yamamoto et al., 2011; Sainz et al., 2012]. The NPC1L1 may be amenable to therapeutic intervention.

The analysis of viral dynamics during add-on therapy indicated that early phase viral decline within the first 2 weeks influenced the achievement of sustained virological response in patients with major type variation (TT) but not in those with minor type variation (TG + GG; Fig. 3A,B). It cannot be explained clearly why high sustained virological response rates were obtained in patients infected with HCV-1b with minor type variation, regardless of poorer viral decline with add-on therapy. Although there is still no evidence, in patients with minor type variation, statins and EPA may show their effect in a later phase, and the EPA effect of impeding HCV entry/infection through suppression of LDLR expression may contribute partly to the achievement of sustained virological response.

In univariate analysis, addition of pitavastatin and EPA, as well as genotype TT of IL28B at rs8099917, was positively associated with sustained virological response in peg-IFN plus ribavirin combination therapy (Table III). However, this association disappeared in a multivariate analysis, and IL28B variation remained as an independent factor. One of the major reasons may be that, compared with the addition of pitavastatin and EPA, TT variation of IL28B in the profile of individuals has overwhelming weight for governing the effect of peg-IFN plus ribavirin combination therapy. In our study, SNP variation of ITPA (rs1127354) did not influence treatment outcome but the planned dose of ribavirin was maintained well in patients with minor type variation (non-CC), as reported in previous studies (Fig. 2).

In conclusion, the lipid modulators, pitavastatin and EPA, could enhance the efficacy of peg-IFN plus ribavirin combination therapy through their synergistic antiviral effect, particularly in patients infected with HCV-1b with an intractable IL-28B allele. Although the research is still in the preliminary stages, there is a possibility that addition of pitavastatin and EPA may be effective for HCV-1b with core 70 mutation, and may increase sustained virological response rates in patients treated with triple therapy of peg-IFN, ribavirin, and telaprevir.

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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; FACL4, fatty acid-CoA ligase, long chain 4; HCV, hepatitis C virus; IFN, interferon; IKKε, IκB 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 IκB kinase ϵ (IKKϵ) kinases, which catalyze phosphorylation and activation of IFN regulatory factor-3 (IRF-3).[12] Activation of TBK1 and IKKϵ 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 IKKϵ-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/IKKϵ.

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 IKKϵ. 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 ΔRIG-I and RIG-IKA plasmids express constitutively active and inactive RIG-I, respectively.[5] Full-length Cardif (Cardif) and CARD-truncated Cardif (ΔCARD) 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 pIFNβ-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% CO_2.

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 μg/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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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 ΔRIG-I, Cardif, STING or poly(deoxyadenylic-deoxythymidylic) acid [poly(dA:dT)] (Invivogen). RIG-IKA, ΔCARD, 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 ± 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′-gctggcat ggtcatattacatcggatatc-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 ΔRIG-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 ΔRIG-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 ΔRIG-I, Cardif, or STING. NS3/4A also blocked production of pIRF-3 induced by ΔRIG-I or Cardif. Intriguingly, NS3/4A did not block STING-induced pIRF-3 production. These results demonstrate that both NS3/4A and

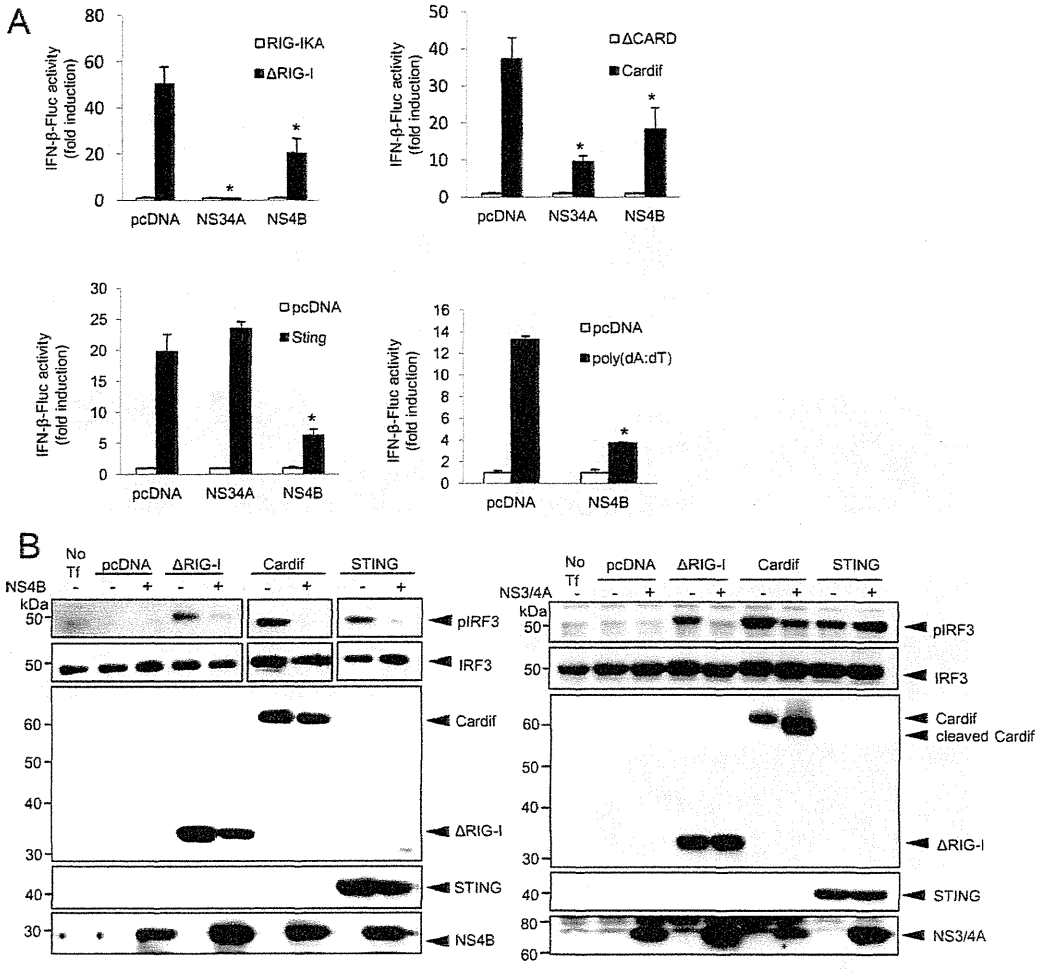

Fig. 1. NS4B suppressed IFN-β signaling mediated by RIG-I, Cardif, or STING. (A) Plasmids expressing ΔRIG-I, Cardif, or STING or poly(dA:dT) as well as NS3/4A or NS4B were cotransfected with pIFN-β-Fluc and pRL-CMV into HEK293T cells. After 24 hours, dual luciferase assays were performed. Plasmids expressing 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 performed in triplicate and error bars indicate mean ± 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 reports[33] (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 Barber[20] (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 mitochondria[34] and has been reported the site of Cardif-STING association.[24] Both NS4B and STING were adjacent to and partially colocalized

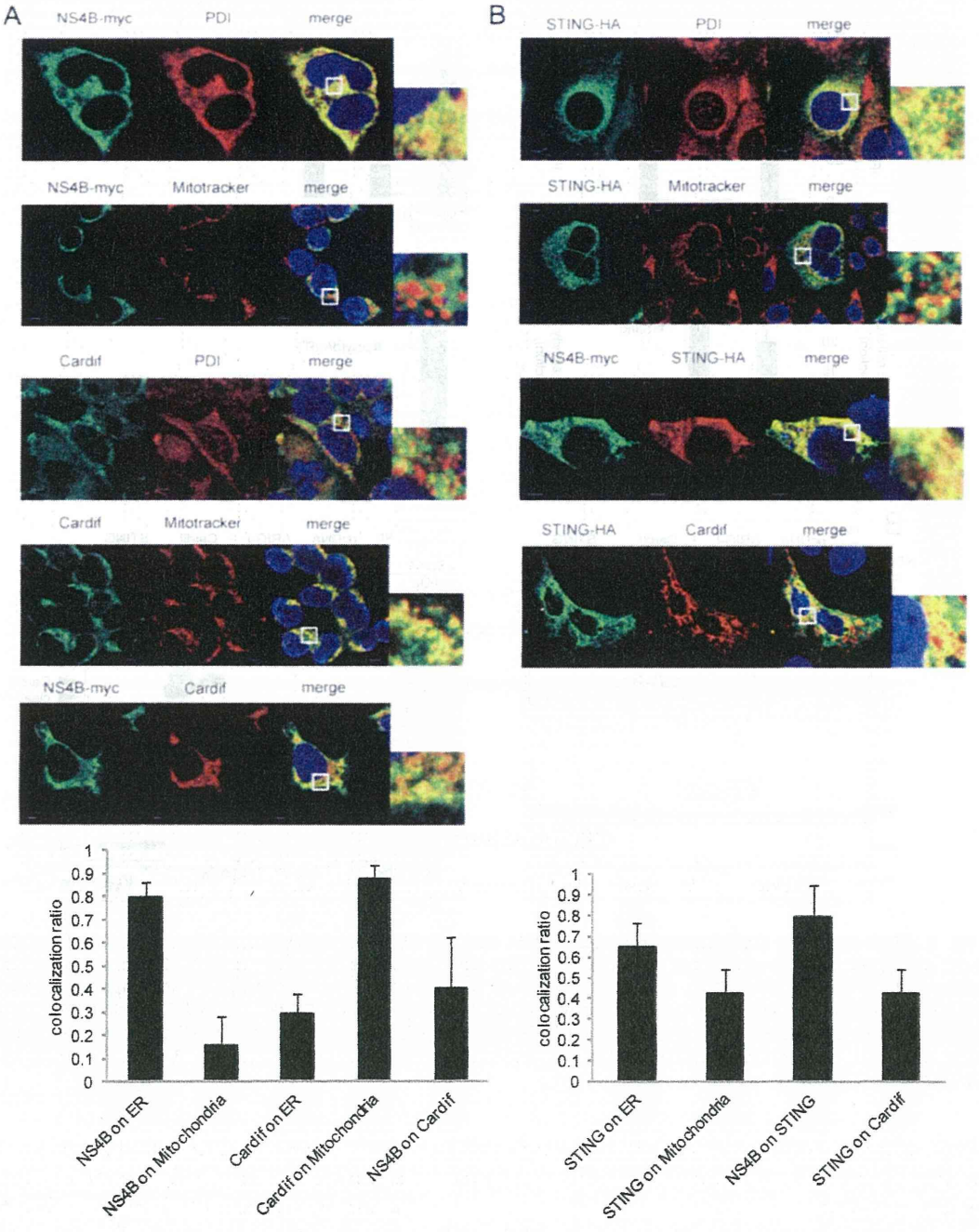

Fig. 2. Subcellular localization of NS4B, Cardif, and STING. (A-D) Subcellular localization of NS4B, Cardif, and STING in 293T (A,C) and Huh7 (B,D) cells. (A,C) NS4B-myc (first, second, and fifth panels of A and third panel of C) was transfected, and 24 hours later the cells were fixed and immunostained 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 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 the mean + SD.

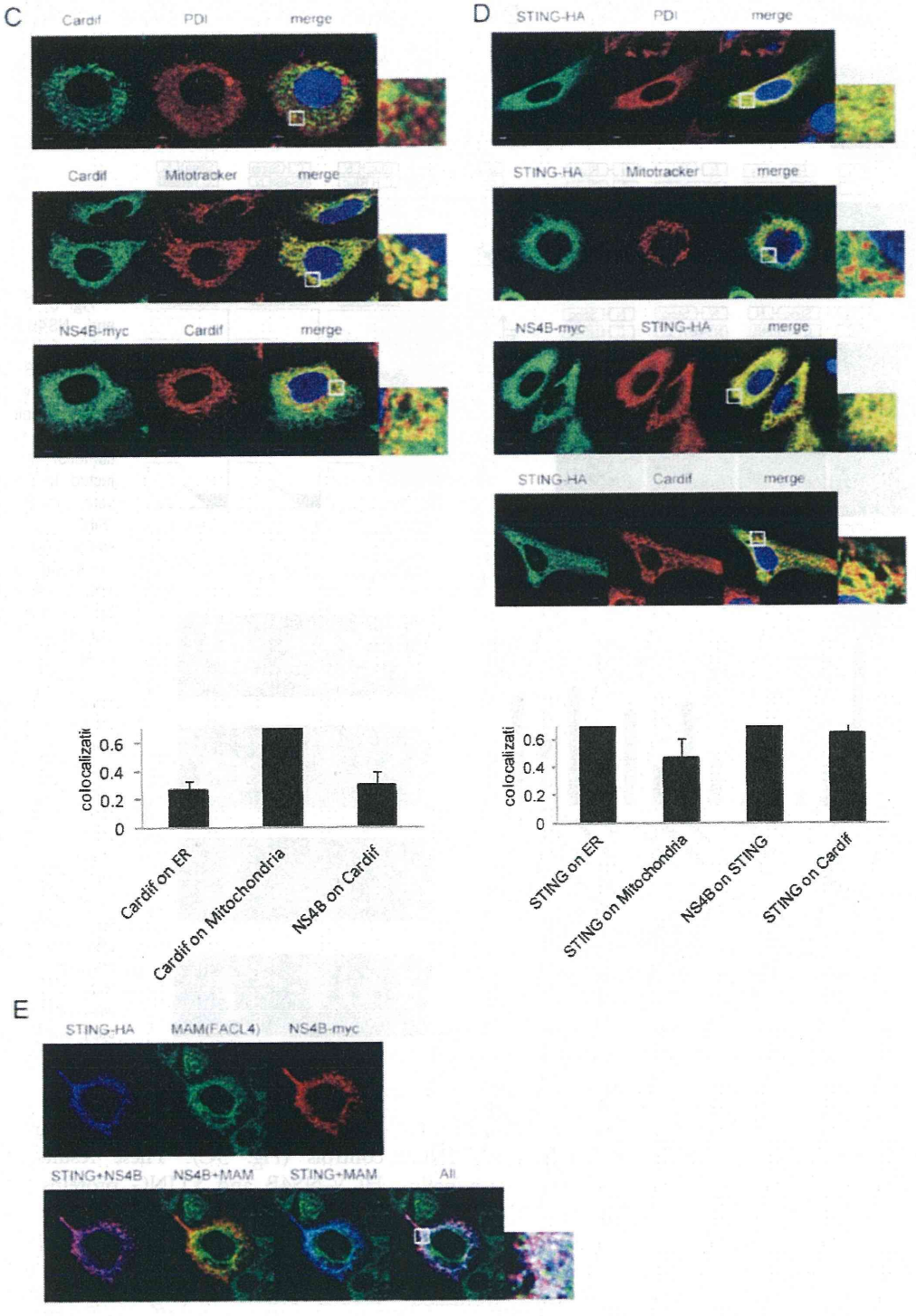

Fig. 2. Continued

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

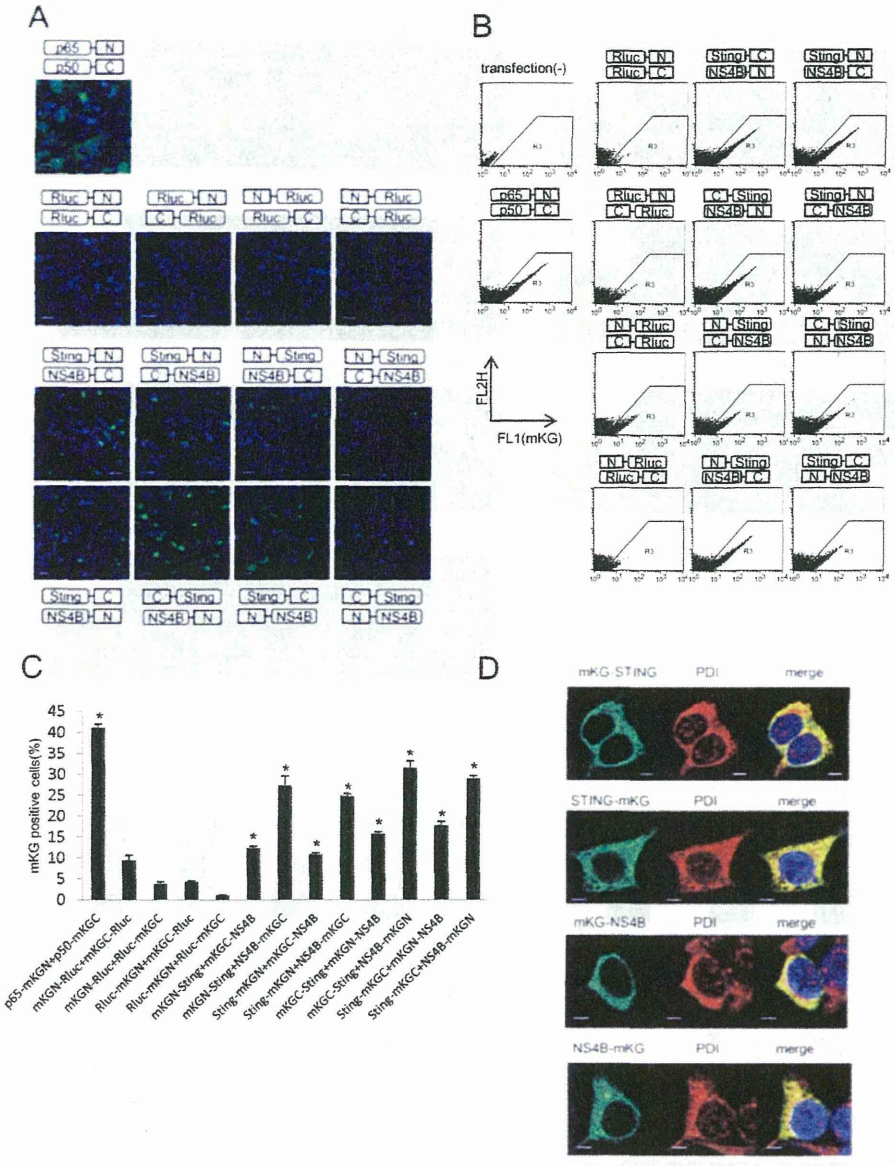

Fig. 3. BiFC assays of STING and NS4B. The complementary pairs of N- or C-terminally mKG-fused NS4B and STING expression plasmids were cotransfected in HEK293T cells. After 24 hours, the cells were fixed and observed by confocal microscopy (A) or subjected to flow cytometry to measure mKG-emitted fluorescence (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 ± SD. Scale bars indicate 10 μm (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 μm.

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