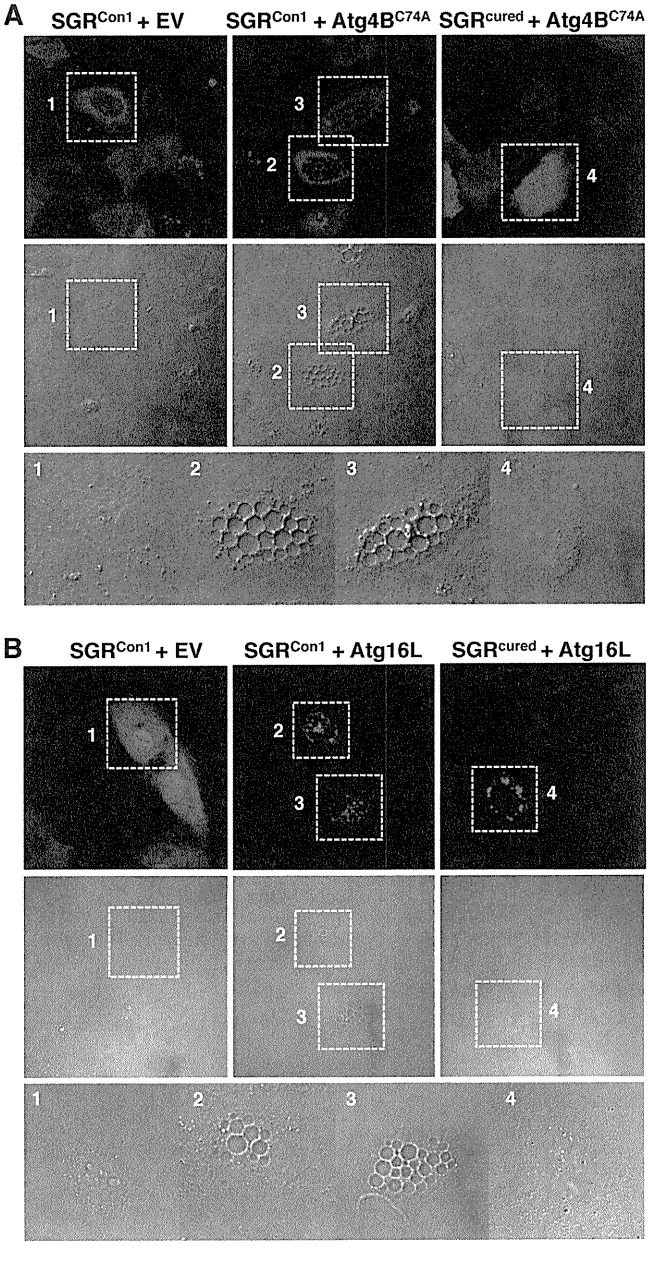

FIG. 6. Inhibition of autophagosome formation induces severe cytoplasmic vacuolations leading to cell death in the HCV replicon cells. (A) SGR^{Con1} and SGR^{cured} cells transfected with pStrawberry-$Atg4B^{C74A}$ or empty vector pStrawberry (EV) were fixed at 48 h posttransfection and examined by fluorescence microscopy. The boxed areas in the phase-contrast images are magnified. (B) SGR^{Con1} and SGR^{cured} cells transfected with pEGFP-Atg16L or EV were examined by fluorescence microscopy at 48 h posttransfection. The boxed areas in the phase-contrast images are magnified. (C) SGR^{cured}, SGR^{Con1}, and SGR^{cured} cells infected with JFH1 virus were transfected with pStrawberry-$Atg4B^{C74A}$, and the number of vacuole-positive cells in each of nine fields of view was counted at 48 h posttransfection. (D) SGR^{cured}, SGR^{Con1}, and SGR^{cured} cells infected with JFH1 virus were transfected with pStrawberry-$Atg4B^{C74A}$ (black bars) or EV (white bars), and cell viability was determined at 6 days posttransfection by using CellTiter-Glo (Promega) according to the manufacturer's protocol. The asterisks indicate significant differences ($P < 0.05$) versus the control value. The data shown are representative of three independent experiments.

was not observed in the cured cells infected with HCVcc, an infectious HCV strain derived from strain JFH1 (Fig. 5D, bottom). Collectively, these results suggest that the dysfunction of lysosomal acidification contributes to the impairment of autophagy in the HCV replicon cells of strain Con1.

Autophagy induced in cells replicating HCV is required for cell survival. Finally, we examined the pathological significance of autophagy during HCV replication. Atg4B is known as an LC3-processing protease, and overexpression of its protease-inactive mutant ($Atg4B^{C74A}$) results in inhibition of the autophagosome formation (7). To our surprise, severe cytoplasmic vacuolation was observed in the SGR^{Con1} cells expressing $Atg4B^{C74A}$ (Fig. 6A). These vacuolations were also observed in the SGR^{Con1} cells by the expression of Atg16L (Fig. 6B), a molecule that is an essential component of the autophagy complex and that, if expressed in excess amounts, can disrupt the autophagosome formation (8). Expression of $Atg4B^{C74A}$ induced a higher level of vacuole formation in the Con1 replicon cells than in cells infected with JFH1 virus but not in the cured cells (Fig. 6C). Along with these vacuolations, cell viability was significantly decreased by the expression of $Atg4B^{C74A}$ in SGR^{Con1} cells and slightly in JFH1 virus-infected cells (Fig. 6D). These results suggest that autophagy induced by the RNA replication of HCV is required for host cell survival.

DISCUSSION

In the present study, we demonstrated that two genotypes of HCV induce autophagy, whereas intact autophagy flux is required for the host cell to survive. The cell death characterized by cytoplasmic vacuolation that was induced in the HCV replicon cells by the inhibition of the autophagosome formation is similar to type III programmed cell death, which is distinguishable from apoptosis and autophagic cell death (4). Type III programmed cell death has been observed in the neurodegenerative diseases caused by the deposit of cytotoxic protein aggregates (15).

We previously reported that HCV hijacks chaperone complexes, which regulates quality control of proteins into the membranous web for circumventing unfolded protein response during efficient genome replication (53); in other words, the replication of HCV exacerbates the generation of proteins associated with cytotoxicity. In the experiments using a chimpanzee model, HCV of genotype 1 was successfully used to reproduce acute and chronic hepatitis similar to that in the human patients (3, 57), and transgenic mice expressing viral proteins of HCV of genotype 1b have been shown to develop

Sjögren syndrome, insulin resistance, hepatic steatosis, and hepatocellular carcinoma (27, 28). In contrast, HCVcc, based on the genotype 2a strain JFH1 isolated from a patient with fulminant hepatitis C (33, 56), was unable to establish chronic infection in chimpanzees (56) or to induce cell damage and inflammation in chimeric mice xenotransplanted with human hepatocytes (17). These results imply that the onset of HCV pathogenesis could be dependent not only upon an amount but also on a property of deposited proteins, and they might explain the aggravated vacuolations under the inhibition of autophagosome formation in strain Con1 compared to that in strain JFH1. Interestingly, the overexpression of $Atg4B^{C74A}$ or Atg16L causes eccentric cell death in the Con1 replicon cells in which autophagy flux is already disturbed. Thus, we speculated that the quarantine of undefined abnormalities endowed with high cytotoxicity by the engulfing of the autophagic membrane might be sufficient for the amelioration of HCV-induced degeneration. The autophagosomal dysfunction observed in the Con1 replicon cells may suggest that a replicant of strain Con1 was more sensitive to the lysosomal vacuolation than that of strain JFH1. Because a limitation of our study was that we were unable to use infectious HCV of other strains, it is still unclear whether the autophagic degradation can be impaired only in the replicon of HCV strain Con1 or genotype 1.

We also demonstrated that HCV replication of strain Con1 but not that of strain JFH1 facilitates the secretion of pro-CTSB. It has been well established that the secretion of pro-CTSB is enhanced in several types of tumors (26, 50). The secretion of CTSB, like the secretion of matrix metalloproteases, is a marker of the progression of the proteolytic degradation of the extracellular matrix, which plays an important part in cancer invasion and metastasis. Since infection with HCV of genotype 1 is clinically considered a risk factor for the development of hepatocellular carcinoma (14, 51), the enhanced secretion of pro-CTSB by the replication of genotype 1 strains might synergistically promote infiltration of hepatocellular carcinoma.

As shown elsewhere (see movies in the supplemental material), although most degradations of the autophagosome were impaired due to a dislocalization of a V-ATPase subunit, some autophagic degradation was achieved in the SGR^{Con1} cells similar to that in the starved Huh7 cells. Moreover, the stagnated autophagy flux was rescued by the treatment of alpha interferon accompanied by elimination of HCV (Fig. 1C and D). Interestingly, we observed neither a significant impairment of lysosomal degradation nor the intracellular activity of cathepsins in the replicon cells of HCV strain Con1 (data not shown). Therefore, there might be a specific dysfunction within the autolysosome during the replication of HCV strain Con1. Detailed studies are needed to elucidate how HCV strain Con1 disturbs the sorting of V-ATPase.

A close relationship between autophagy and the immune system has been gradually unveiled (47). Autophagy assists not only in the direct elimination of pathogens by hydrolytic degradation but also in antigen processing in antigen-presenting cells such as macrophage and dendritic cells (DC) for presentation by major histocompatibility complex (MHC) I and II (11). Moreover, autophagy plays important roles in T lymphocyte homeostasis (44). As such, in some instances, interruptions of autophagy can allow microorganisms to escape from the host immune system. Indeed, the immune response against herpes simplex virus was suppressed by blocking the autophagy (6). With regard to HCV, functionally impaired DC dysfunctions marked by poor DC maturation, impaired antigen presentation, and attenuated cytokine production have been reported in tissue culture models and chronic hepatitis C patients (1, 22, 46). In addition, reduction of cell surface expression of MHC-I in HCV genotype 1b replicon cells has been reported (55). We confirmed that levels of cell surface expression of MHC-I in the replicon cells of genotype 1b, but not of genotype 2a, were reduced in comparison with those in the cured cells (data not shown). Hence it might be feasible to speculate that the replication of HCV RNA of genotype 1 induces an incomplete autophagy for attenuating antigen presentation to establish persistent infection. In contrast, autophagy is known to serve as a negative regulator of innate immunity (21, 54). A recent report demonstrated that autophagy induced by infection with strain JFH1 or dengue virus attenuates innate immunity to promote viral replication (23), indicating that an HCV genotype 2a strain may facilitate autophagy to evade innate immunity.

In this study, we demonstrated that HCV utilizes autophagy to circumvent the cell death induced by vacuole formation for its survival. This unique strategy of HCV propagation may provide new clues to the virus-host interaction and, ultimately, to the pathogenesis of infection by various genotypes of HCV.

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Involvement of cyclophilin B in the replication of Japanese encephalitis virus

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ABSTRACT

Japanese encephalitis virus (JEV) is a mosquito-borne RNA virus that belongs to the *Flaviviridae* family. In this study, we have examined the effect of cyclosporin A (CsA) on the propagation of JEV. CsA exhibited potent anti-JEV activity in various mammalian cell lines through the inhibition of CypB. The propagation of JEV was impaired in the CypB-knockdown cells and this reduction was cancelled by the expression of wild-type but not of peptidylprolyl *cis-trans* isomerase (PPIase)-deficient CypB, indicating that PPIase activity of CypB is critical for JEV propagation. Infection of pseudotype viruses bearing JEV envelope proteins was not impaired by the knockdown of CypB, suggesting that CypB participates in the replication but not in the entry of JEV. CypB was colocalized and immunoprecipitated with JEV NS4A in infected cells. These results suggest that CypB plays a crucial role in the replication of JEV through an interaction with NS4A.

Introduction

The genus *Flavivirus* within the family *Flaviviridae* comprises over 70 viruses, many of which are predominantly arthropodborne viruses, such as Japanese encephalitis virus (JEV), West Nile virus (WNV), Murray Valley encephalitis virus, dengue virus (DENV), yellow fever virus (YFV), and tick-borne encephalitis virus. JEV is one of the most important flaviviruses in the medical and veterinary fields and exists in a zoonotic transmission cycle among mosquitoes, pigs, and birds mostly in Eastern and Southeast Asia. This virus spreads to dead-end hosts, including humans, through the bite of JEV-infected mosquitoes, and around 30,000–50,000 cases and up to 15,000 deaths are reported annually (Ghosh and Basu, 2009; Mackenzie et al., 2004; Solomon et al., 2003). JEV has a single-stranded positive-sense RNA genome of approximately 11 kb, which is capped at the 5′ end but lacks a 3′ polyadenine tail. The genome RNA is translated into a single large polyprotein at the endoplasmic reticulum (ER) membrane, then cleaved by the host- and virus-encoded proteases into three structural proteins, the capsid, precursor membrane (prM), and envelope (E) proteins, and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Sumiyoshi et al., 1987).

Flavivirus infection causes extensive rearrangement of cellular membranes to form two distinct membrane structures called the vesicle packet and convoluted membrane (Mackenzie et al., 1996; Miller and Krijnse-Locker, 2008). Whereas the vesicle packet is believed to contain the replication complex in which viral RNA synthesis takes place, the convoluted membrane is the putative site for viral polyprotein processing (Mackenzie et al., 1999). A recent tomography study clarified that the ER, convoluted membrane, and outer membrane of the vesicle packet were connected together to form a continuous membrane, with the vesicle packet being observed as an invagination of the ER with NS proteins and viral RNA, suggesting that viral replication occurred on the surface of the ER (Welsch et al., 2009). The structures of the convoluted membrane can be observed by infection with the WNV strain Kunjin virus or expression of the DENV NS4A protein alone (Miller et al., 2007; Roosendaal et al., 2006). Previous studies have indicated that NS4A localizes to both the vesicle packet and convoluted membrane and interacts with NS1, indicating that NS4A plays an important role as an integral scaffold of the replication complex (Lindenbach and Rice, 1999; Mackenzie et al., 1998).

In addition to NS proteins, flavivirus RNA replication is known to be regulated by several host factors, such as eEF1A, TIA/TIAR, HMGCR, and cyclophilin (Cyp) A (Davis et al., 2007; Emara and Brinton, 2007; Mackenzie et al., 2007; Qing et al., 2009). RNAi screening has identified various host factors involved in the replication of RNA viruses, including the hepatitis C virus (HCV), human immunodeficiency virus (HIV), and influenza A virus (Karlas et al., 2010; Konig et al., 2010, 2008; Tai et al., 2009). Host factors essential for viral replication might be an ideal target for antiviral development because the frequency of appearance of resistant viruses is lower by this method than when using antivirals targeted to the viral proteins.

In this study, we identified CypB as a host factor involved in the propagation of JEV. CypB is a member of the Cyp family, is ubiquitously expressed in most cells, and predominantly resides in the ER through the ER retention signal sequence in the C-terminus (Price et al., 1994,

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1991; Wang and Heitman, 2005). CypB participates in various biological functions, such as chaperone activities, immunosuppression, transcriptional regulation, apoptosis, and viral propagation (Allain et al., 1996; Kim et al., 2008; Rycyzyn and Clevenger, 2002; Watanabe et al., 2010; Watashi et al., 2005; Zhang and Herscovitz, 2003). Cyclosporin A (CsA), an inhibitor for Cyps, significantly impaired the propagation of JEV. Knockdown of CypB reduced the RNA replication in the JEV replicon cells, whereas it exhibited no effect on the infection of a pseudotype virus bearing JEV envelope proteins. Furthermore, CypB was colocalized and immunoprecipitated with the JEV NS4A protein. Collectively, these results suggest that CypB plays a crucial role in the propagation of JEV through its interaction with NS4A.

Results

CsA suppresses the production of JEV by inhibiting Cyps

CsA is an immunosuppressive agent widely used in the management of organ transplantation. In addition to this activity, it has been reported that CsA has potent antiviral effects against HCV (Chatterji et al., 2009; Kaul et al., 2009; Watashi et al., 2005; Yang et al., 2008), HIV (Franke et al., 1994; Thali et al., 1994), measles virus (MV) (Watanabe et al., 2010), influenza A virus (Liu et al., 2009), vesicular stomatitis virus (VSV) (Bose et al., 2003), and vaccinia virus (VV) (Castro et al., 2003; Damaso and Moussatche, 1998). To examine the possibility that CsA has an antiviral effect on JEV, mammalian cell lines including Huh7, BHK, and N18 cells were treated with various concentrations of CsA followed by infection with JEV. At 48 h post-infection, cells were subjected to immunoblotting. The level of expression of JEV NS1 was significantly decreased by treatment with CsA in a dose-dependent manner in all the cell lines examined (Fig. 1A). Furthermore, infectious particle production in the culture supernatant was also reduced by the treatment with CsA under the conditions employed without exhibiting any serious cytotoxic effect (Fig. 1B).

CsA exhibits three distinct inhibitory activities on, respectively, the calcineurin NF-AT signaling pathway, the peptidylprolyl *cis-trans* isomerase (PPIase) activity of Cyps, and the transport activity of p-glycoprotein (Silverman et al., 1997). To determine the antiviral activity of CsA, we used CsA derivatives and FK506, an immunosuppressant structurally different from CsA. cyclosporin D (CsD) has almost no effect on the calcineurin pathway (Sadeg et al., 1993) and cyclosporin H (CsH) has a specific inhibitory activity on the p-glycoprotein (Silverman et al., 1997). FK506 also inhibits the calcineurin NF-AT signaling pathway (Almawi and Melemedjian, 2000). Huh7 cells were infected with JEV and treated with various concentrations of the compounds at 1 h post-infection. The cells and culture supernatants were harvested at 48 h after treatment and the expression of JEV NS1 and infectivity were determined, respectively (Fig. 2). Treatment with CsA and CsD reduced the expression of the NS1 and the production of JEV in a dose-dependent manner, whereas CsH and FK506 exhibited almost no effect on the propagation of JEV (Fig. 2). These results suggest that CsA inhibits JEV propagation through the inhibition of Cyps, but not through the inhibition of calcineurin and p-glycoprotein.

CypB participates in the propagation of JEV

Cyps possessing the PPIase activity are highly conserved and ubiquitously expressed in both prokaryotic and eukaryotic cells (Wang and Heitman, 2005). Next, to determine whether the particular Cyp isoform participates in the propagation of JEV, short interference RNAs (siRNAs) targeted to CypA, CypB, or CypC were transfected into Huh7 cells and the expression of each Cyp was determined by immunoblotting or real-time PCR at 24 h post-transfection. CypA and CypB were specifically decreased by the transfection of the siRNAs (Fig. 3A). Although CypC could not be detected by immunoblotting due to the lack of a specific antibody in our laboratory, CypC mRNA was decreased by approximately 90% upon transfection with siRNA targeted

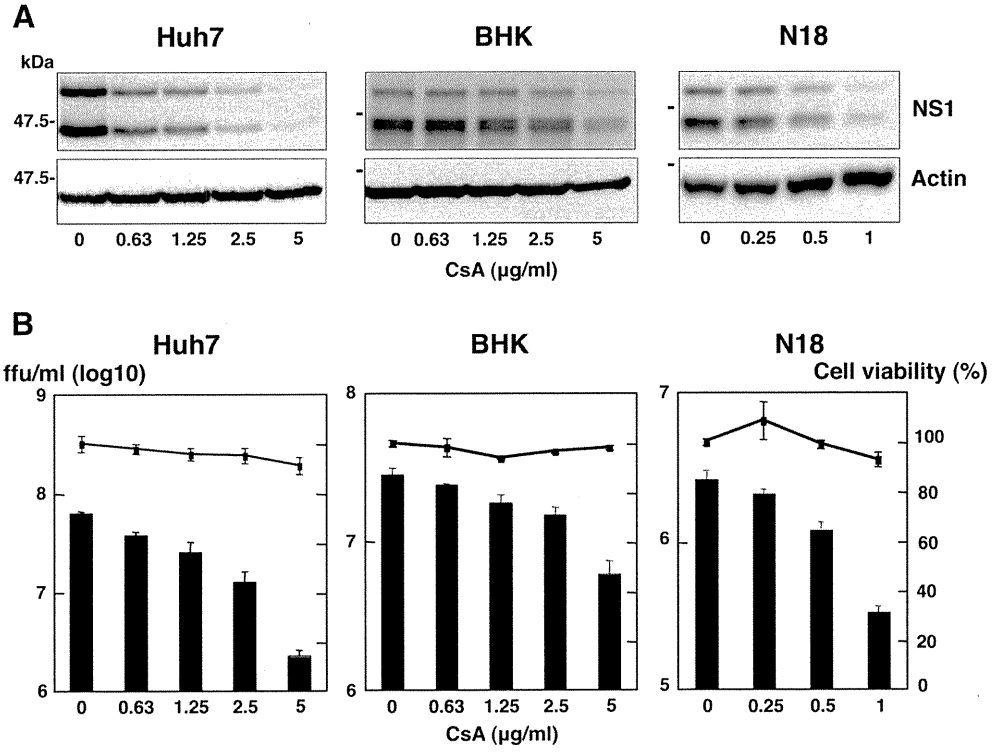


Fig. 1. Effect of CsA on the propagation of JEV in mammalian cells. (A) JEV was inoculated at an MOI of 0.1 (Huh7 and BHK cells) or 10 (N18 cells) and incubated for 1 h. Cells were washed with 10% FBS DMEM and treated with the indicated concentrations of CsA in 10% FBS DMEM for 48 h. The propagation of JEV was assessed by the expression of NS1. NS1 and actin were detected by immunoblotting. (B) The production of infectious JEV in the culture supernatant at 48 h post-infection was determined in Vero cells by a focus-forming assay. Cell viability was determined at 48 h post-incubation of CsA. The results are representative of three independent assays, with the error bars indicating the standard deviations.

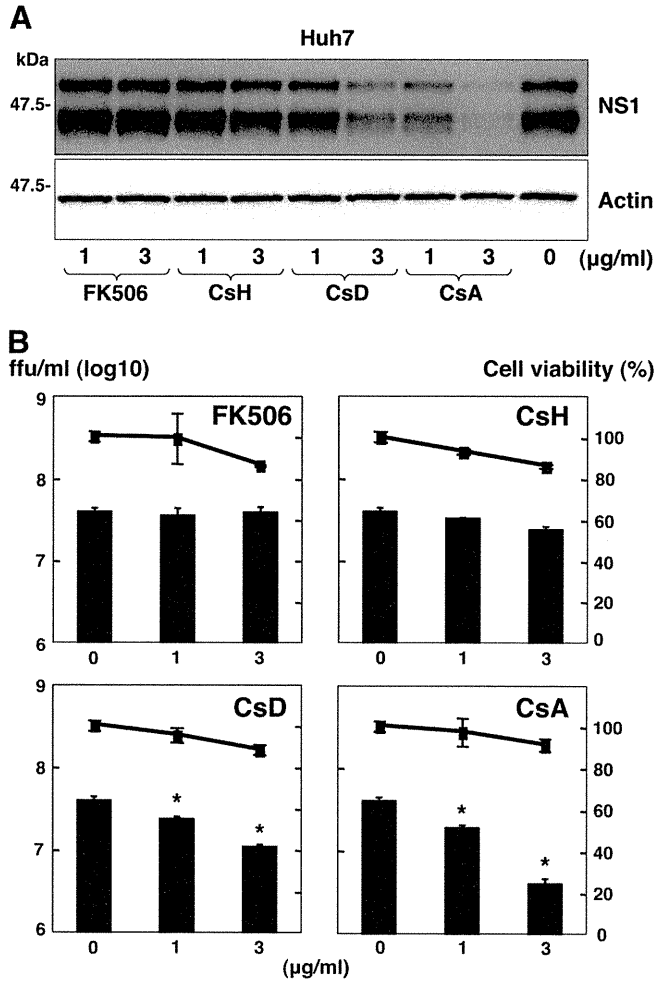


Fig. 2. CsA inhibits JEV propagation through the inhibition of Cyps. Huh7 cells were infected with JEV at an MOI of 0.1 for 1 h and then treated with 10% FBS DMEM containing the indicated concentrations of CsA, CsD, CsH, or FK506 for 48 h. The propagation of JEV was evaluated by immunoblotting (A) and focus-forming assay (B). The results are representative of three independent assays, with the error bars indicating the standard deviations. Asterisks indicate significant differences (*$P<0.01$).

to CypC compared to the level in the cells transfected with the control siRNA (Fig. 3B). JEV was inoculated into cells transfected with the siRNA at 48 h post-transfection and the cells and culture supernatants were harvested at 48 h post-infection. Expression of JEV NS1 was most effectively decreased by the knockdown of CypB, followed by CypC, and knockdown of CypA resulted in a marginal reduction of NS1 expression compared to the control siRNA (Fig. 3C). Furthermore, the production of JEV was also effectively suppressed in cells with knockdown of CypB, followed by those with knockdown of CypC and CypA (Fig. 3D). These results suggest that CypB plays an important role in the propagation of JEV. To further confirm the effect CypB on the propagation of JEV, we established stable knockdown cell lines expressing a short hairpin RNA (shRNA) targeted to CypB. Consistent with the data from transient knockdown experiments, both expression of NS1 and virus production were significantly reduced in the CypB-knockdown cell lines (Bose et al., 2003; Castro et al., 2003) in accordance with the reduction of CypB (Fig. 4A and B). There was no significant difference in cell growth among the cell lines (Fig. 4C).

PPIase activity of CypB is crucial for the propagation of JEV

The PPIase activity of Cyps is suggested to catalyze the proper folding of certain proteins (Andreotti, 2003; Wang and Heitman, 2005). It has been demonstrated that PPIase activity of Cyps is required for HCV replication (Chatterji et al., 2009; Kaul et al., 2009; Watashi et al., 2005). To examine the effect of the PPIase activity of CypB on the propagation of JEV, we constructed an expression plasmid encoding a PPIase-defective CypB in which the Arg^{62} was replaced with Ala, because the Arg^{62} in CypB has been shown to be critical for PPIase catalytic activity (Carpentier et al., 1999). Each of the expression plasmids encoding the FLAG-tagged wild- or Ala^{62}-CypB carrying the silent mutations resistant to the siRNA was introduced into the stable CypB-knockdown cell line (Bose et al., 2003) and cultured for a week in the presence of neomycin. Although expression of both endogenous and exogenous CypB was detected at a similar level (Fig. 4D), JEV production was partially rescued by introducing the wild-CypB but not the Ala^{62}-CypB (Fig. 4E). These results indicate that the PPIase activity of CypB is crucial for the propagation of JEV.

CypB participates in the replication but not in the entry of JEV

To further examine the effect of CsA on the JEV life cycle, we generated a subgenomic replicon of JEV to assess the effect of CsA on the JEV RNA replication (Fig. 5A). The replicon cells treated with CsA for 6 days exhibited a significant reduction of NS1 expression compared to the non-treated cells (Fig. 5B). The replicon RNA transcribed from the pJErepIRESpuro was transfected into the stable CypB-knockdown (#4) or control cell lines and incubated for 3 weeks in the presence of puromycin. A few colony formation was detected in the CypB-knockdown cell line, in contrast to the abundant colony formation in the control cell line (Fig. 5C). These results suggest that CypB is required for the efficient replication of JEV.

Next, to examine the impact of CypB on the entry of JEV, we generated pseudotype VSVs bearing envelope proteins of JEV (JEVpv) or VSV (VSVpv). Because these viruses possess the luciferase gene, the infectivity can be assessed by the luciferase activity (Tani et al., 2010). Huh7 cells pretreated with various concentrations of CsA were infected with JEVpv or VSVpv, and the infectivity was assessed by the expression of luciferase. There was no significant effect of CsA on the infection of either pseudotype virus (Fig. 5D). Similarly, no effect was observed on the infection of the pseudotype viruses in the CypB-knockdown cell lines (Fig. 5E). Collectively, these results clearly indicate that CypB participates in the replication but not in the entry of JEV.

CypB interacts with the JEV NS4A protein

Many viruses have been shown to utilize Cyps through the interaction with their viral proteins. For example, HCV recruits CypA and CypB to enhance viral RNA replication through the interaction with NS5A and NS5B, respectively (Chatterji et al., 2009; Kaul et al., 2009; Watashi et al., 2005; Yang et al., 2008). To determine whether the JEV proteins interact with CypB, we prepared expression plasmids encoding each of the JEV nonstructural proteins involved in the viral RNA replication. FLAG-tagged CypB was co-expressed with each of the HA-tagged JEV nonstructural proteins in 293T cells and immunoprecipitated with anti-HA antibody. The precipitates were subjected to immunoblotting by using either anti-FLAG or anti-HA antibodies. CypB was co-precipitated with the JEV NS4A protein but not with other proteins (Fig. 6A). Furthermore, interaction of CypB with NS4A was reduced in the immunoprecipitation analysis in the presence of CsA (Fig. 6B). To gain more insight into the interaction between CypB and NS4A, the intracellular localization of these proteins was examined by confocal microscopy. Huh7 cells were transfected with an expression plasmid encoding HA-tagged NS4A or an empty vector and fixed at 48 h post-transfection. Endogenous CypB was detected in the perinuclear region together with NS4A protein. In addition, NS4A colocalized with ER marker protein, calnexin (Fig. 6C). These results suggest that NS4A protein interacts with CypB at the replication complex localized in the ER.

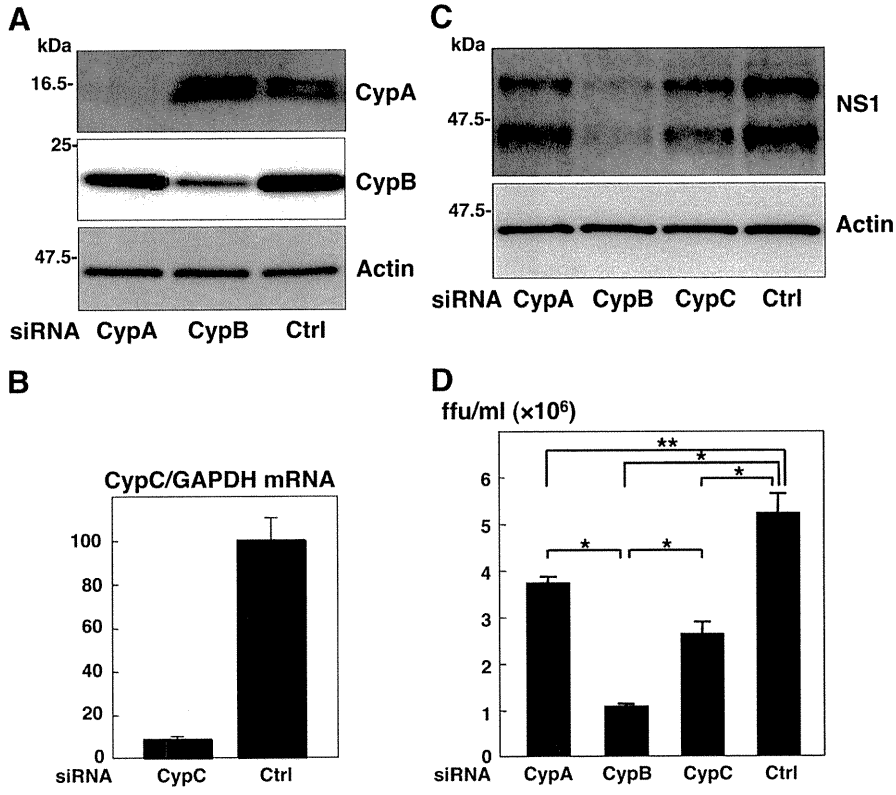


Fig. 3. CypB plays an important role in the propagation of JEV. (A) Knockdown of endogenous CypA and CypB by siRNA. Huh7 cells were transfected with 35 nM of siRNA targeted to CypA, CypB, or a non-specific control. Cell lysates after 96 h post-transfection were analyzed for expression of CypA, CypB, or actin by immunoblotting. (B) Huh7 cells transfected with 35 nM of siRNA targeted to CypC or a non-specific control were harvested at 24 h post-transfection. CypC mRNA levels were determined by quantitative real-time PCR. The level of CypC mRNA was normalized to the amount of GAPDH mRNA and expressed as a percentage of the control value. (C, D) Huh7 cells were transfected with siRNA targeted to CypA, CypB, or CypC and infected with JEV at an MOI of 0.1 at 48 h post-transfection. The propagation of JEV was determined by immunoblotting (C) and focus-forming assay (D). The results are representative of three independent assays, with the error bars indicating the standard deviations. Asterisks indicate significant differences ($^{*}P<0.01$; $^{**}P<0.05$).

Discussion

In this study, we have shown that CsA inhibits the replication of JEV through the inhibition of the PPIase activity of Cyps. A previous study showed that CsA does not induce interferon in Huh7 cells (Nakagawa et al., 2005), suggesting that the antiviral activity of CsA on the propagation of JEV relies on the inhibition of Cyps. Cyps are highly conserved PPIases that catalyze the *cis-trans* isomerization of peptide bonds to facilitate certain protein foldings (Andreotti, 2003; Wang and Heitman, 2005) and are involved in the correct folding of host and viral proteins. Among the Cyp isoforms, CypA and CypB are the most abundantly expressed in cells and play key roles in the propagation of various viruses. CypA is incorporated into HIV, influenza A virus, VSV, and VV to regulate their replication (Bose et al., 2003; Castro et al., 2003; Damaso and Moussatche, 1998; Franke et al., 1994; Liu et al., 2009; Thali et al., 1994). CypB is incorporated into MV particles to facilitate an efficient infection (Watanabe et al., 2010). Both CypA and CypB have been shown to serve as host factors involved in the replication of HCV through the interaction with NS5A and NS5B (Chatterji et al., 2009; Kaul et al., 2009; Watashi et al., 2005; Yang et al., 2008).

Recently, Qing et al. reported that CypA plays an important role in the replication of flaviviruses such as WNV, YFV, and DENV. The PPIase activity of CypA was shown to be crucial for the efficient replication of the viruses, indicating that CypA acts as a molecular chaperone for the viral and host proteins required for an effective RNA replication (Qing et al., 2009). Indeed, knockdown of CypA suppressed the JEV propagation in this study, but that of CypB exhibited more potent impairment of the JEV propagation, suggesting that CypB plays a crucial role in the propagation of JEV. However, we could not exclude the possibility of the involvement of other Cyps in the replication of JEV. Multiple Cyps have been shown to involved in the life cycle of HCV (Gaither et al., 2010; Nakagawa et al., 2005) and the knockdown experiment of Cyps in this study suggests that not only CypB, but also CypC and CypA are involved in the propagation of JEV. At least 16 Cyps have been shown to participate in various cellular functions in humans (Wang and Heitman, 2005), and therefore, further studies to clarify the precise function of these Cyps in the life cycle of the flaviviruses are needed.

In addition to Cyps, flavivirus recruits several host chaperones for an efficient propagation. HSP70 and HSP90 have been identified as comprising the DENV receptor complex in human cell lines. These chaperones presumably facilitate the viral envelope dimer–trimer transition after the binding of the envelope protein to the cellular receptor (Reyes-Del Valle et al., 2005). Moreover, inhibition of the interaction between the ER chaperone calnexin and JEV glycoproteins has been suggested to affect the folding of viral proteins, leading to a reduction in the mortality rate in a mouse model of lethal infection (Wu et al., 2002). It has been reported that ER chaperones including BiP, calnexin, and calreticulin interact with the DENV envelope protein, and that knockdown of these chaperones decreased viral production (Limjindaporn et al., 2009). In addition, BiP was shown to be upregulated in cells infected with DENV to facilitate viral production (Wati et al., 2009), and BiP and calreticulin have been associated with CypB (Zhang and Herscovitz, 2003). Therefore, these ER resident chaperones are considered to play important roles in the flavivirus replication through the proper folding of the viral and host proteins making up the viral RNA replication complex.

Lack of recovery of JEV propagation in the CypB-knockdown cell lines by the expression of the PPIase-deficient CypB mutant suggests that PPIase activity is crucial for the JEV production. Although the PPIase activity of CypA has been shown to be required for flavivirus replication

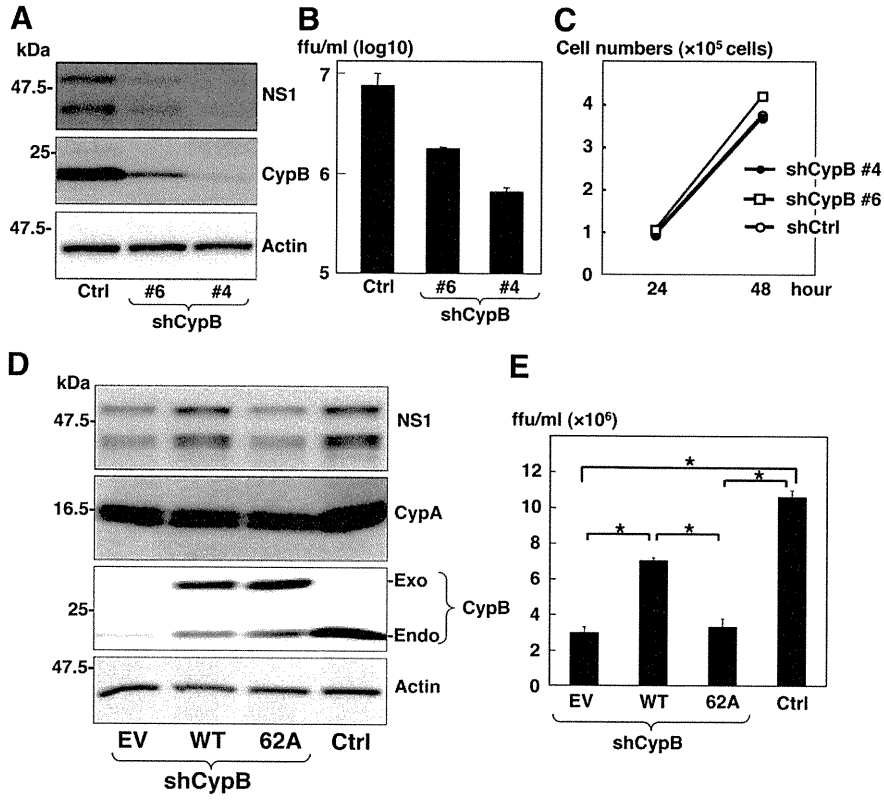


Fig. 4. PPIase activity of CypB is crucial for the propagation of JEV. Huh7 cell lines expressing shRNA targeted to CypB or the control were infected with JEV at an MOI of 0.1 for 1 h and cultured in 10% FBS DMEM for 48 h. The expressions of NS1, CypB, and actin were detected by immunoblotting (A). The propagation of JEV was determined by focus-forming assay (B). Growth kinetics of the stable CypB-knockdown cell lines were determined by the method of trypan blue dye exclusion (C). The stably knocked-down cell lines were transfected with the siRNA-resistant FLAG-tagged wild- or Ala^{62}-CypB, or empty vector and cultured for 1 week in the presence of 1 μg/ml puromycin. The remaining cells were infected with JEV at an MOI of 1. The expressions of NS1, CypA, endogenous and exogenous CypBs, and actin were detected by immunoblotting (D). Virus production in the culture supernatant at 36 h post-infection was determined by a focus-forming assay (E). The results are representative of three independent assays, with the error bars indicating the standard deviations. Asterisks indicate significant differences ($^*P<0.01$).

through the interaction with the NS5 polymerase (Qing et al., 2009), CypB was colocalized and specifically co-immunoprecipitated with JEV NS4A. CypA is abundantly expressed in the cytoplasm of mammalian cells (Galigniana et al., 2004) and NS5 is predominantly detected on the cytoplasmic side of the ER (Zhang et al., 1992). Thus, it is conceivable that an interaction between CypA and NS5 occurs on the cytoplasmic side of the ER. On the other hand, CypB is localized in the ER lumen and targeted to the secretory pathway via its ER signal sequence (Price et al., 1994, 1991). NS4A is predicted to be a three-transmembrane protein with its C-terminal end localized in the ER lumen (Miller et al., 2007). Therefore, it is plausible that CypB interacts with NS4A within the ER lumen and confers proper folding to form the RNA replication complex of JEV. Expression of DENV NS4A alone has been shown to induce rearrangement of the cytoplasmic membrane to form the convoluted membrane required for viral replication (Roosendaal et al., 2006). It might be feasible to speculate that JEV NS4A undergoes conformational change through the interaction with CypB and induces formation of the convoluted membrane in the ER essential for genome replication of JEV. It was reported that HCV NS5A from CsA resistant mutant exhibits an enhanced interaction with CypB and NS5B facilitates a stronger binding of the mutant NS5A to endogenous CypB than wild-type in cell culture (Fernandes et al., 2010). Study of the molecular mechanism underlying the CsA resistant of JEV may shed light on the complex interaction among Cyps and viral proteins.

In conclusion, we have demonstrated that CsA suppresses the propagation of JEV by inhibiting the interaction between CypB and NS4A, which is required for viral RNA replication. Further studies are needed to elucidate the precise molecular mechanism underlying the involvement of cellular Cyps in the efficient propagation of JEV. Three inhibitors of the PPIase activity of Cyps, DEBIO-025, SCY635, and NIM811, are currently under clinical trial for the treatment of hepatitis C patients (Puyang et al., 2010). The PPIase inhibitor may be an attractive therapeutic target for the treatment of patients infected with not only HCV but also other flaviviruses.

Materials and methods

Plasmids

The human CypB gene was amplified from the total cDNA of Huh7 by PCR using *LA taq* (Takara Bio Inc., Shiga, Japan) and cloned into pcDNA3.1 and pCAGPM (Mori et al., 2007). The plasmids encoding the NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 of the JEV AT31 strain were generated by PCR and cloned into pCAGPM. The pSilencer-CypB, carrying an shRNA targeted to CypB under the control of the U6 promoter, was constructed by cloning of the oligonucleotide pair 5′-GATCCGGTGGAGAGCACCAAGACATTCAAGAGATGTCTTGGTGCTCTC-CACCTTTTTTGGAAA-3′-5′-AGCTTTTCCAAAAAAGGTGGAGAGCACCAA-GACATCTCTTGAATGTCTTGGTGCTCTCCACCG-3′ between the *Bam*HI and *Hin*dIII sites of pSilencer 2.1-U6 hygro (Ambion, Austin, TX). A plasmid coding a mutant CypB resistant to shRNA was prepared by insertion of four silent mutations (the nucleotides at positions 543, 549, 555, and 561 were changed from G to A, G to A, C to G, and A to C, respectively) into CypB cDNA by the method of splicing by overlap extension (Ho et al., 1989). The pSilencer negative-control plasmid (Ambion) has no homology to any human gene. The pJErep plasmid was kindly provided by Dr. Konishi (Kobe University, Kobe, Japan). A puromycin-resistant gene under the internal ribosomal entry site (IRES) of encephalomyocarditis virus was inserted into pJErep and designated as pJErepIRESpuro.

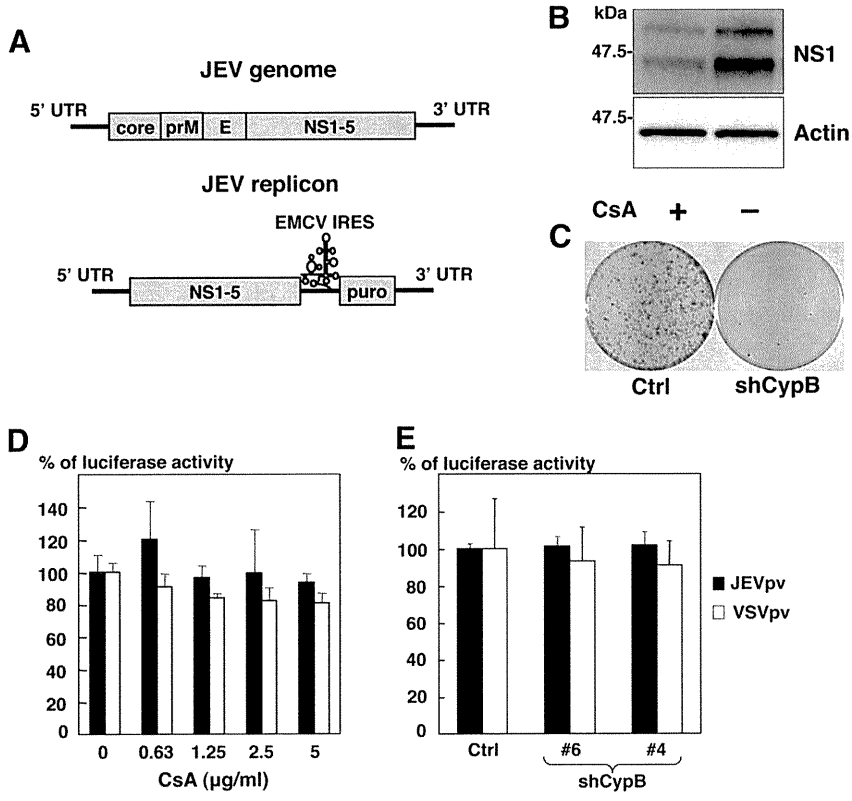


Fig. 5. CypB participates in the replication but not in the entry of JEV. (A) Schematic representations of the JEV genome and its subgenomic replicon. (B) JEV replicon cells were treated with CsA (1 μg/ml) for 6 days, and the expressions of NS1 and actin were detected by immunoblotting. (C) The stable CypB-knockdown and control cell lines were electroporated with the JEV replicon RNA and cultured for 3 weeks in the presence of 1 μg/ml of puromycin. The remaining cells were fixed with 4% paraformaldehyde and stained with crystal violet. (D) Huh7 cells treated with the indicated concentrations of CsA for 1 h were infected with the pseudotype viruses, JEVpv and VSVpv, and luciferase activities were determined at 24 h post-infection. (E) The stable CypB-knockdown and control cell lines were incubated with the pseudotype viruses, and the luciferase activities were determined. The results shown are representative of three independent assays, with error bars indicating standard deviations.

Cells and viruses

All cell lines were cultured at 37 °C under the condition of a humidified atmosphere and 5% CO_2. The human embryonic kidney cell line, 293T, African green monkey kidney cell line, Vero, hepatocellular carcinoma cell line, Huh7, mouse neural cell line, N18, and baby hamster kidney cell line, BHK, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, non-essential amino acid (Sigma), and 10% fetal bovine serum (FBS). The mosquito C6/36 cell line (*Aedes albopictus*) was cultured at 27 °C and maintained in modified Eagle's medium (MEM) (Sigma). Huh7 cells were transfected with pSilencer-CypB or control plasmid and drug-resistant clones were selected by treatment with hygromycin B (Wako, Tokyo, Japan) at a final concentration of 50 μg/ml. Huh7 cells were electroporated with *in vitro*-transcribed RNA from pJErepIRESpuro and drug-resistant clones were selected by treatment with puromycin (InvivoGen, San Diego, CA) at a final concentration of 1 μg/ml. Wild-type JEV strain AT31 was used as described previously (Tani et al., 2010). The wild-type JEV was amplified on C6/36 cells and stored at −80 °C. Pseudotype VSVs bearing JEV PrM and E proteins (JEVpv) and VSVG (VSVpv) were produced in 293T cells transfected with pCAG105E and pCAGVSVG, respectively, as described previously (Tani et al., 2010). The infectivities of JEV and the pseudotype VSVs were assessed by both a focus-forming assay and luciferase activity as described previously (Tani et al., 2010). Cell viability was determined by using CellTiter-Glo (Promega Corporation, Madison, WI) according to the manufacturer's protocol.

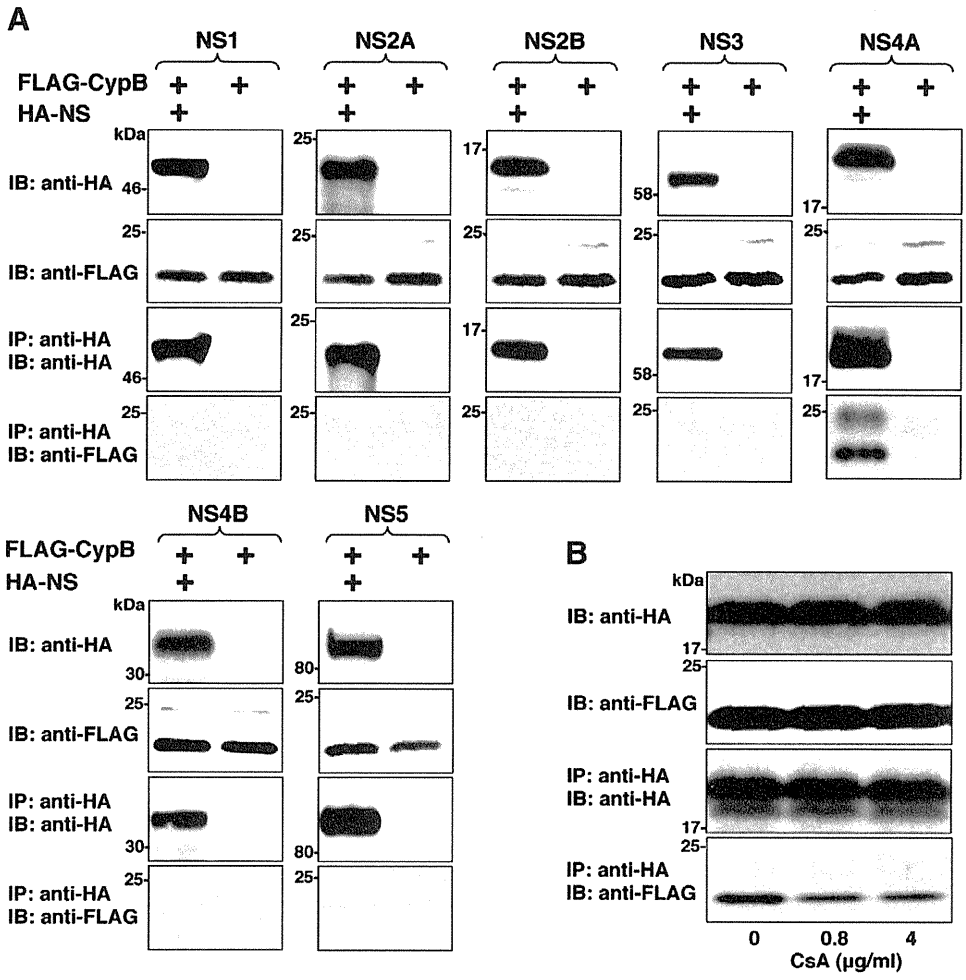
Reagents and antibodies

CsA and FK506 were purchased from Sigma, and CsD and CsH from Eton Bioscience Inc. (San Diego, CA). Mouse monoclonal antibodies to tags of HA and FLAG and β-actin were previously described (Taguwa et al., 2009). Rabbit polyclonal antibodies to CypA and CypB were purchased from Upstate Cell Signaling (Lake Placid, NY) and Affinity BioReagents (Golden, CO), respectively. Rabbit polyclonal antibody to calnexin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to JEV NS1 protein (34A1) was kindly provided by Dr. Yasui.

Transfection, immunoblotting, and immunoprecipitation

Transfection and immunoprecipitation were carried out as described previously (Taguwa et al., 2009). Immunoprecipitates boiled in loading buffer were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene

Fig. 6. NS4A protein recruits CypB to the replication complex in the JEV-infected cells. (A) FLAG-tagged CypB was co-expressed with HA-tagged NS1, NS2A, NS2B, NS3, NS4A, NS4B, or NS5 in 293T cells and immunoprecipitated with anti-HA antibody. The immunoprecipitates were subjected to immunoblotting by using either anti-FLAG or anti-HA antibody. (B) FLAG-tagged CypB was co-expressed with HA-tagged NS4A in 293T cells. The cell lysates obtained after lysis with the buffer containing CsA were immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were subjected to immunoblotting by using either anti-FLAG or anti-HA antibody. (C) Huh7 cells transfected with an expression plasmid encoding HA-tagged NS4A or empty vector were fixed at 48 h post-transfection, permeabilized, and stained with the appropriate antibodies to HA (green), calnexin (red), and CypB (red). Cell nuclei were stained with DAPI (blue). Intracellular localization of CypB and NS4A was examined by confocal microscopy.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NS1
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IB: anti-HA
IP: anti-HA
IB: anti-FLAG
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NS5
B
IB: anti-HA
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IB: anti-HA
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0
0.8
4
CsA (μg/ml)
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NS4A
CypB
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Empty
Calnexin

difluoride membranes (Millipore, Bedford, MA) and were reacted with the appropriate antibodies. The immune complexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

Gene silencing by siRNA

The siRNAs against CypA and CypB were 5′-AAGCATACGGGTCCTGGCATC-3′ and 5′-AAGGTGGAGAGCACCAAGACA-3′, respectively (QIAGEN, Tokyo, Japan). FlexTube siRNAs against CypC and the negative control were purchased from QIAGEN. The cells were grown on 6-well plates and transfected with 35 nM siRNA by using Dharmafect (Dharmacon, Buckinghamshire, UK) according to the manufacturer's protocol. The transfected cells were incubated in DMEM supplemented with 10% FBS.

Quantitative RT-PCR

RNA was determined by the method described previously (Taguwa et al., 2009). The total RNA was prepared from cells by using an RNeasy mini kit (QIAGEN). First-strand cDNA was synthesized using an RNA LA PCR™ *in vitro* cloning kit (Takara Bio Inc.) and random primers. Each cDNA was determined by Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Fluorescent signals were analyzed by an ABI PRISM 7000 (Applied Biosystems, Tokyo, Japan).

In vitro transcription and RNA transfection

Plasmid pJErepIRESpuro linearized at the *Swa* I site was transcribed *in vitro* using an mMESSAGE mMACHINE (Ambion) according to the manufacturer's protocol. The *in vitro*-transcribed RNA was introduced into Huh7 cells at 5 million cells/0.5 ml by electroporation at 270 V and 960 μF using Gene Pulser™ (Bio-rad, Hercules, CA).

Colony formation assay

Colony formation was determined as previously described (Taguwa et al., 2009). Briefly, *in vitro*-transcribed RNA was electroporated into Huh7 cells and plated on DMEM containing 10% FBS and non-essential amino acids. The medium was replaced with fresh DMEM containing 10% FBS, non-essential amino acids, and 1 μg/ml puromycin at 24 h post-transfection. The remaining colonies were fixed with 4% paraformaldehyde (PFA) and stained with crystal violet at 3 weeks after electroporation.

Indirect immunofluorescence assay

Cells cultured on glass slides were fixed with 4% PFA in phosphate buffered saline (PBS) at room temperature for 30 min. After washing three times with PBS, the cells were permeabilized for 20 min at room temperature with PBS containing 0.25% saponin and blocked with phosphate buffer containing 2% BSA for 1 h at room temperature. The cells were incubated with blocking buffer containing mouse anti-HA or rabbit anti-CypB at room temperature for 1 h, then washed three times with PBS and incubated with blocking buffer containing AF488-conjugated anti-mouse IgG and AF594-conjugated anti-rabbit IgG at room temperature for 1 h. Cell nuclei were stained blue with DAPI. Finally, the cells were washed three times with PBS and observed a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

Statistical analysis

Results are expressed as the means ± standard deviation. The significance of differences between the means was determined by Student's *t*-test.

Acknowledgments

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Association of Gene Expression Involving Innate Immunity and Genetic Variation in Interleukin 28B With Antiviral Response

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Innate immunity plays an important role in host antiviral response to hepatitis C viral (HCV) infection. Recently, single nucleotide polymorphisms (SNPs) of *IL28B* and host response to peginterferon α (PEG-IFNα) and ribavirin (RBV) were shown to be strongly associated. We aimed to determine the gene expression involving innate immunity in *IL28B* genotypes and elucidate its relation to response to antiviral treatment. We genotyped *IL28B* SNPs (rs8099917 and rs12979860) in 88 chronic hepatitis C patients treated with PEG-IFNα-2b/RBV and quantified expressions of viral sensors (*RIG-I, MDA5*, and *LGP2*), adaptor molecule (*IPS-1*), related ubiquitin E3-ligase (*RNF125*), modulators (*ISG15* and *USP18*), and *IL28* (*IFNλ*). Both *IL28B* SNPs were 100% identical; 54 patients possessed rs8099917 TT/rs12979860 CC (*IL28B* major patients) and 34 possessed rs8099917 TG/rs12979860 CT (*IL28B* minor patients). Hepatic expressions of viral sensors and modulators in *IL28B* minor patients were significantly up-regulated compared with that in *IL28B* major patients (≈3.3-fold, $P < 0.001$). However, expression of *IPS-1* was significantly lower in *IL28B* minor patients (1.2-fold, $P = 0.028$). Expressions of viral sensors and modulators were significantly higher in nonvirological responders (NVR) than that in others despite stratification by *IL28B* genotype (≈2.6-fold, $P < 0.001$). Multivariate and ROC analyses indicated that higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio were independent factors for NVR. IPS-1 down-regulation in *IL28B* minor patients was confirmed by western blotting, and the extent of IPS-1 protein cleavage was associated with the variable treatment response. *Conclusion:* Gene expression involving innate immunity is strongly associated with *IL28B* genotype and response to PEG-IFNα/RBV. Both *IL28B* minor allele and higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio are independent factors for NVR. (HEPATOLOGY 2012;55:20-29)

Infection with hepatitis C virus (HCV) is a common cause of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma in many patients.[1] Pegylated interferon α (PEG-IFNα) and ribavirin (RBV) combination therapy has been used to treat chronic hepatitis C (CH-C) to alter the natural course of this disease. However, 20% patients are nonvirological responders (NVR) whose HCV-RNA does not become negative during the 48 weeks of PEG-IFNα/RBV combination therapy.[2] In a recent genome-wide association study, single nucleotide polymorphisms (SNPs) located near interleukin 28B

Abbreviations: CH-C, chronic hepatitis C; γ-GTP, γ-glutamyl transpeptidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HMBS, hydroxymethylbilane synthase; IL28, interleukin 28; IPS-1, IFNβ promoter stimulator 1; ISG15, interferon-stimulated gene 15; MDA5, melanoma differentiation associated gene 5; NVR, nonvirological responders; PEG-IFNα, pegylated interferonα; SNP, single nucleotide polymorphism; RIG-I, retinoic acid-inducible gene I; RBV, ribavirin; RNF125, ring-finger protein 125; ROC, receiver operator characteristic; SVR, sustained viral responder; TVR, transient virological responder; USP18, ubiquitin-specific protease 18; VR, virological responder.

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(*IL28B*) that encodes for type III IFNλ3 were shown to be strongly associated with a virological response to PEG-IFNα/RBV combination therapy.[3-5] In particular, the rs8099917 TG and GG genotypes were shown to be strongly associated with a null virological response to PEG-IFNα/RBV.[3] However, mechanisms involving resistance to PEG-IFNα/RBV have not been completely elucidated.

The innate immune system has an essential role in host antiviral defense against HCV infection.[6] The retinoic acid-inducible gene I (RIG-I), a cytoplasmic RNA helicase, and related melanoma differentiation associated gene 5 (MDA5) play essential roles in initiating the host antiviral response by detecting intracellular viral RNA.[7,8] The IFNβ promoter stimulator 1 (IPS-1)—also called the caspase-recruiting domain adaptor inducing IFNβ, mitochondrial antiviral signaling protein, or virus-induced signaling adaptor—is an adaptor molecule. IPS-1 connects RIG-I sensing to downstream signaling, resulting in *IFNβ* gene activation.[9-12] RIG-I sensing of incoming viral RNA has been shown to be modified by LGP2,[8,13] a helicase related to RIG-I and MDA5 lacking caspase-recruiting domain. The ubiquitin ligase ring-finger protein 125 (RNF125) has been shown to conjugate ubiquitin to RIG-I, MDA5, and IPS-1 and this suppresses the functions of these proteins.[14] Further, these molecules are ISGylated by the IFN-stimulated gene 15 (ISG15), a ubiquitin-like protein,[15] and ISG15 is specifically removed from ISGylated protein by ubiquitin-specific protease 18 (USP18) to regulate the RIG-I/IPS-1 system.[16,17] Moreover, the NS3/4A protease of HCV specifically cleaves IPS-1 as part of its immune-evasion strategy.[9,18] Therefore, the RIG-I/IPS-1 system and its regulatory systems have essential roles in the innate antiviral response.

Recently, we demonstrated that baseline intrahepatic gene expression levels of the RIG-I/IPS-1 system were prognostic biomarkers of the final virological outcome in CH-C patients who were treated with PEG-IFNα/RBV combination therapy.[19] We found that up-regulation of *RIG-I* and *ISG15* and a higher expression ratio of *RIG-I/IPS-1* could predict NVR for subsequent treatment with PEG-IFNα/RBV combination therapy.[19] However, association of gene expression involving innate immunity and genetic variation of *IL28B* has not yet been elucidated. Hence, the aim of this study was to determine gene expression involving the innate immune system in different genetic variations of *IL28B* and elucidate the relation of gene expression to final virological outcome of PEG-IFNα/RBV combination therapy in CH-C patients.

Patients and Methods

Patients. Among histologically proven CH-C patients admitted at the Musashino Red Cross Hospital, 88 patients with HCV genotype 1b and a high viral load (>5 log IU/mL by TaqMan HCV assay; Roche Molecular Diagnostics, Tokyo, Japan) were included in the present study (Table 1). Patients with decompensated liver cirrhosis, autoimmune hepatitis, or alcoholic liver injury were excluded. No patient had tested positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody or had received immunomodulatory therapy before enrollment. Forty-two patients had been enrolled in a previous study that determined hepatic gene expression involving innate immunity.[19] Written informed consent was obtained from all patients and the study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Treatment Protocol. The patients were administered subcutaneous injections of PEG-IFNα-2b (PegIntron, MSD, Whitehouse Station, NJ) at a dose of 1.5 μg kg^{-1} week^{-1} for 48 weeks. RBV (Rebetol, MSD) was administered concomitantly over this treatment period, administered orally twice daily at 600 mg/day for patients who weighed less than 60 kg and 800 mg/day for patients who weighed between 60-80 kg. The dose of PEG-IFNα-2b was reduced to 0.75 μg kg^{-1} week^{-1} when either neutrophil count was less than 750/mm^3 or platelet count was less than 80 $\times$ 10^3/mm^3. The dose of RBV was reduced to 600 mg/day when the hemoglobin concentration decreased to 10 g/dL. More than 80% adherence was achieved in all patients.

Measurement of Hepatic Gene Expression. Liver biopsy was performed immediately before initiating

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

Table 1. Patient Characteristics and *IL28B* Genotype

	IL28B Major*	*IL28B* Minor†	*P*-value‡
Patients, n	54	34	
Age (SD), year	58.8 (10.0)	59.1 (10.3)	0.918§
Sex, n (%)			0.051‖
Male	13 (24.1)	15 (44.1)	
Female	41 (75.9)	19 (55.9)	
BMI (SD), kg/m^2	22.7 (3.5)	23.5 (3.6)	0.193§
ALT (SD), IU/L	61.3 (50.7)	62.4 (44.7)	0.962§
γ-GTP (SD), IU/L	36.7 (25.9)	57.3 (52.4)	0.010§
LDL-cholesterol (SD), mg/dL	103.3 (29.8)	91.8 (26.9)	0.067§
Hemoglobin (SD), g/dL	14.1 (1.4)	14.4 (1.3)	0.186§
Platelet count (SD), $\times 10^3$/μL	161 (6.4)	163 (4.4)	0.489§
Fibrosis stage, n (%)			0.532‖
F1, 2	38 (70.4)	26 (76.5)	
F3, 4	16 (29.6)	8 (23.5)	
Viral load (SD), $\times 10^{63}$ IU/mL	1.7 (1.4)	1.9 (2.0)	0.788§
%HCV core 70 & 91 a.a. double mutation¶	8.9	43.5	0.001‖
%ISDR wild**	43.5	51.7	0.486‖
Viral response, n (%)			<0.001‖
SVR	17 (31.5)	13 (38.2)	
TVR	26 (48.1)	3 (8.8)	
NVR	11 (20.4)	18 (52.9)	

Unless otherwise indicated, data are given as mean (SD).

*rs8099917 TT and rs12979860 CC.

†rs8099917 TG and rs12979860 CT.

BMI, body mass index; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; SVR, sustained virological response; TVR, transient virological response; NVR, nonvirological response.

‡Comparison between *IL28B* major and minor genotypes.

§Mann-Whitney *U* test.

‖Chi-square test.

¶HCV core mutation was determined in 68 patients.

**ISDR was determined in 75 patients.

the therapy. After extraction of total RNA from liver biopsy specimens, the messenger RNA (mRNA) expression of the positive and negative cytoplasmic viral sensor (*RIG-I, MDA5*, and *LGP2*), the adaptor molecule (*IPS-1*), the related ubiquitin E3-ligase (*RNF125*), the modulators of these molecules (*ISG15* and *USP18*), and *IFNλ* (*IL28A/B*) was quantified by real-time quantitative polymerase chain reaction (PCR) using target gene-specific primers. In brief, total RNA was extracted by the acid-guanidinium-phenol-chloroform method using Isogen reagent (Nippon Gene, Toyama, Japan) from the liver biopsy specimen, which was 0.2-0.4 cm in length and 13G in diameter. Complementary DNA (cDNA) was transcribed from 2 μg of total RNA template in a 140-μL reaction mixture using the SYBR RT-PCR Kit (Takara Bio, Otsu, Japan) with random hexamer. Real-time quantitative PCR was performed using Smart Cycler version II (Takara Bio) with the SYBR RT-PCR Kit (Takara Bio) according to the manufacturer's instructions. Assays were performed in duplicate and the expression levels of target genes were normalized to the expressions of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene and hydroxymethylbilane synthase (*HMBS*), an enzyme that is stable in the liver, as quantified using real-time quantitative PCR as internal controls. For accurate normalization, a set of two housekeeping genes was used in the present study. Sequences of the primer sets were as follows: *RIG-I*, 5′-AAAGCATGCA TGGTGTTCCAGA-3′, 5′-TCATTCGTGCATGCTC ACTGATAA-3′; *MDA5*, 5′-ACATAACAGCAACATG GGCAGTG-3′, 5′-TTTGGTAAGGCCTGAGCTGG AG-3′; *LGP2*, 5′-ACAGCCTTGCAAACAGTACAAC CTC-3′, 5′-GTCCCAAATTTCCGGCTCAAC-3′; *IPS-1*, 5′-GGTGCCATCCAAAGTGCCTACTA-3′, 5′-CAGC ACGCCAGGCTTACTCA-3′; *RNF125*, 5′-AGGGCA CATATTCGGACTTGTCA-3′, 5′-CGGGTATTAAAC GGCAAAGTGG-3′; *ISG15*, 5′-AGCGAACTCATCT TTGCCAGTACA-3′, 5′-CAGCTCTGACACCGACA TGGA-3′; *USP18*, 5′-TGGTTCTGCTTCAATGACT CCAATA-3′, 5′-TTTGGGCATTTCCATTAGCACT C-3′; *IFNλ*: 5′-CAGCTGCAGGTGAGGGA-3′, 5′-G GTGGCCTCCAGAACCTT-3′; *GAPDH*, 5′-GCACC GTCAAGGCTGAGAAC-3′, 5′-ATGGTGGTGAAGA CGCCAGT-3′; *HMBS*, 5′-AAGCGGAGCCATGTCT GGTAAC-3′, 5′-GTACCCACGCGAATCACTCTCA-3′.

Genotyping for* IL28B *(rs8099917 and rs12979860) Polymorphism. Genetic polymorphism in a tagged SNP located near the *IL28B* gene (rs8099917 and rs12979860) was determined by direct sequencing of PCR-amplified DNA. In brief, after extraction from whole blood samples, genomic DNA was amplified by PCR. Sequences of the primer sets were: rs8099917, 5′-ATCCTCCTCTCATCCCTCA TC-3′, 5′-GGTATCAACCCCACCTCAAAT-3′; rs129 79860, 5′-GGACGAGAGGGCGTTAGAG-3′, 5′-AG GGACCGCTACGTAAGTCAC-3′.

Both strands of the PCR products were sequenced by the dye terminator method using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Chiba, Japan); nucleotide sequences were determined by a capillary DNA sequencer ABI3730xl (Applied Biosystems). Homozygosity (rs8099917 GG and rs12979860 TT) or heterozygosity (rs8099917 TG and rs12979860 CT) of the minor sequence was defined as having the *IL28B* minor allele, whereas homozygosity for the major sequence (rs8099917 TT and rs12979860 CC) was defined as having the *IL28B* major allele.

Western Blotting. Western blotting was performed using samples from 14 patients (six from *IL28B* major patients and eight from *IL28B* minor patients) as described.[19] In brief, liver biopsy specimens of

approximately 10 mg were homogenized in 100 μL of Complete Lysis-M (Roche Applied Science, Penzberg, Germany). Next, 30 μg of protein was separated by NuPAGE 4%-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and blotted on polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-RIG-I (Cell Signaling Technology, Danvers, MA) or anti-IPS-1 (Enzo Life Science, Farmingdale, NY), followed by anti-β-actin (Sigma Aldrich, St. Louis, MO). After immunoblotting with horseradish peroxidase-conjugated secondary antibody, signals were detected by chemiluminescence (BM Chemiluminescence Blotting Substrate, Roche Applied Science, Mannheim, Germany). Optical densitometry was performed using ImageJ software (NIH, Bethesda, MD). Naive Huh7 cells were used for a positive control for full-length IPS-1, and cells transfected with HCV-1b subgenomic replicon[20] were used for a positive control for cleaved IPS-1.

Definitions of Response to Therapy. A patient negative for serum HCV-RNA during the first 6 months after completing PEG-IFNα-2b/RBV combination therapy was defined as a sustained viral responder (SVR), and a patient for whom HCV-RNA became negative at the end of therapy and reappeared after completion of therapy was defined as a transient virological responder (TVR). A patient for whom HCV-RNA became negative at the end of therapy (SVR + TVR) was defined as a virological responder (VR). A patient whose HCV-RNA did not become negative during the course of therapy was defined as an NVR. HCV-RNA was determined by TaqMan HCV assay (Roche Molecular Diagnostics).

Statistical Analysis. Categorical data were compared using the chi-square test and Fisher's exact test. Distributions of continuous variables were analyzed by the Mann-Whitney U test for two groups. All tests of significance were two-tailed and $P < 0.05$ was considered statistically significant.

Results

Patient Characteristics and* IL28B *Genotype. Table 1 shows patient characteristics according to *IL28B* genotype. SNPs at rs8099917 and rs12979860 were 100% identical; 54 patients were identified as having the major alleles (rs8099917 TT/rs12979860 CC; *IL28B* major patients) and the remaining 34 had the minor alleles (rs8099917 TG/rs12979860 CT; *IL28B* minor patients). Patients having a minor homozygote (rs8099917 GG or rs12979860 TT) were not found in this study, which is consistent with a recent report of the rarity of a minor homozygote in Japanese patients.[3] *IL28B* minor patients were significantly associated with a higher γ-glutamyl transpeptidase (γ-GTP) level and higher frequency of mutations at amino acid positions 70 and 91 of the HCV core region (glutamine or histidine mutation at amino acid position 70; methionine mutation at amino acid position 91). NVR rate was significantly higher in *IL28B* minor patients than in *IL28B* major patients.

Gene Expression Involving Innate Immunity and* IFNλ *in the Liver. Hepatic expression levels of cytoplasmic viral sensors (*RIG-I, MDA5*, and *LGP2*) were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). Similarly, expressions of *ISG15* and *USP18* were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). In contrast, the hepatic expression of the adaptor molecule (*IPS-1*) was significantly lower in *IL28B* minor patients than that in *IL28B* major patients (Fig. 1). Hepatic expression of *RNF125* was similar among *IL28B* genotypes (Fig. 1). *IFNλ (IL28A/B)* expression was higher in *IL28B* minor patients, but not statistically significant (Fig. 1). Because expression of *RIG-I* and *IPS-1* were negatively correlated, the expression ratio of *RIG-I/IPS-1* in *IL28B* minor patients was significantly higher than in *IL28B* major patients (Fig. 1).

Next, to assess the relationship between baseline hepatic gene expression and treatment efficacy, we compared levels of gene expression involving innate immunity and *IFNλ* based on the final virological response (Fig. 2). Overall, hepatic expressions of cytoplasmic viral sensors and the ISG15/USP18 system in NVR patients were significantly higher than those in VR patients. In a similar but opposite manner, hepatic expressions of *IPS-1* and *RNF125* in NVR patients were significantly lower than that in VR patients, and the expression of *IFNδ* was higher in NVR patients, but the differences were not statistically significant. Expression ratio of *RIG-I/IPS-1* was significantly higher in NVR patients than that in VR patients.

Because hepatic expressions of the RIG-I/IPS-1 and ISG15/USP18 systems were significantly related both to *IL28B* minor and NVR patients, *RIG-I* and *ISG15* expression levels and the *RIG-I/IPS-1* ratio between VR and NVR patients were further stratified by *IL28B* genotype (Fig. 3). Even in the subgroup of *IL28B* minor patients, the expressions of *RIG-I* and *ISG15* were significantly higher in NVR patients than those in VR patients. Similar tendencies were observed in a subgroup of *IL28B* major patients, in whom the *RIG-I/IPS-1* expression ratio was significantly higher in

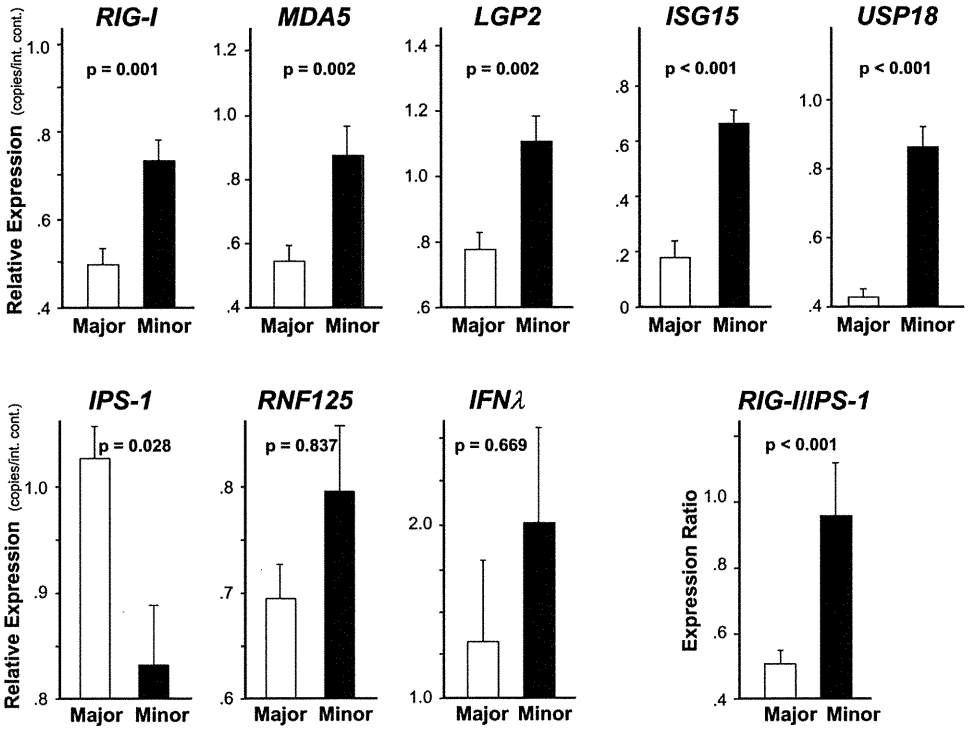

Fig. 1. Comparison of hepatic gene expression levels between *IL28B* major (rs8099917 TT/rs12979860 CC, n = 54) and *IL28B* minor patients (rs8099917 TG/rs12979860 CT, n = 34). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and expression ratio of the *RIG-I/IPS-1* are shown. Error bars indicate standard error. The *P*-values were determined by the Mann-Whitney *U* test.

NVR patients than in VR patients. However, in patients of the same virological response subgroup, *RIG-I* and *ISG15* expression levels and *RIG-I/IPS-1* ratio were higher in *IL28B* minor patients, and the difference in *ISG15* expression in subgroup of VR and NVR patients and that in *RIG-I/IPS-1* ratio in subgroup of VR patients was statistically significant between *IL28B* genotypes (Fig. 3).

Receiver Operator Characteristic (ROC) Analysis. To determine the usefulness of these gene quantifications and *IL28B* genotyping as predictors of NVR, an ROC analysis was conducted (Fig. 4A). The area under the ROC curve for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio was 0.712, 0.782, and 0.732, respectively, suggesting that quantification of these gene transcripts is useful for

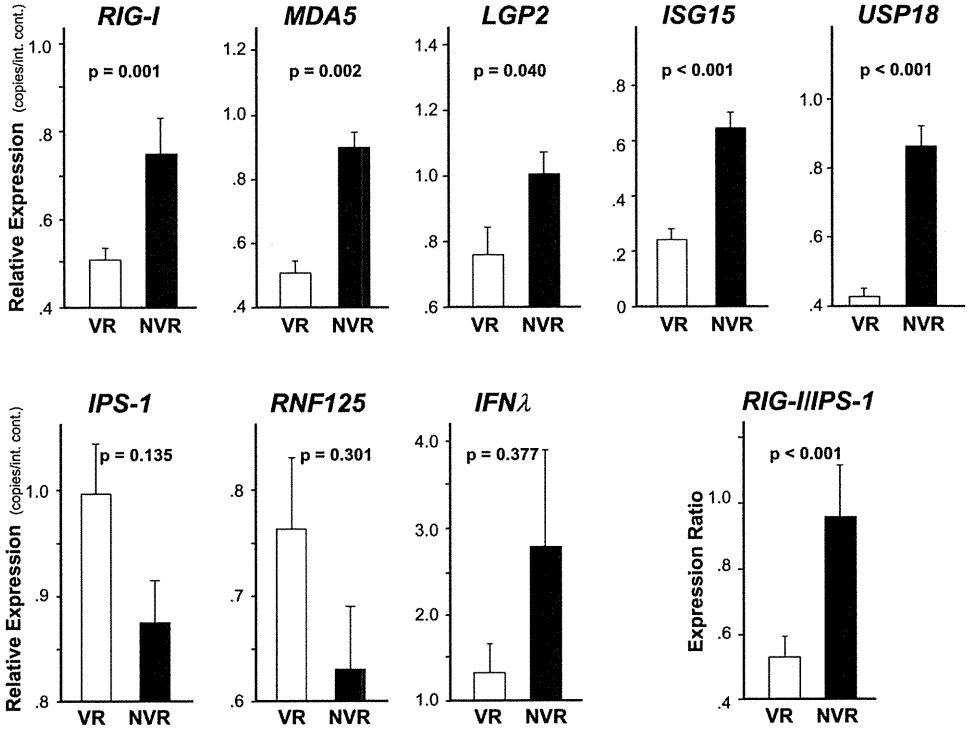

Fig. 2. Comparison of hepatic gene expression levels between virological responders (VR, n = 60) and nonvirological responders (NVR, n = 28). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The *P*-values were determined by the Mann-Whitney *U* test.

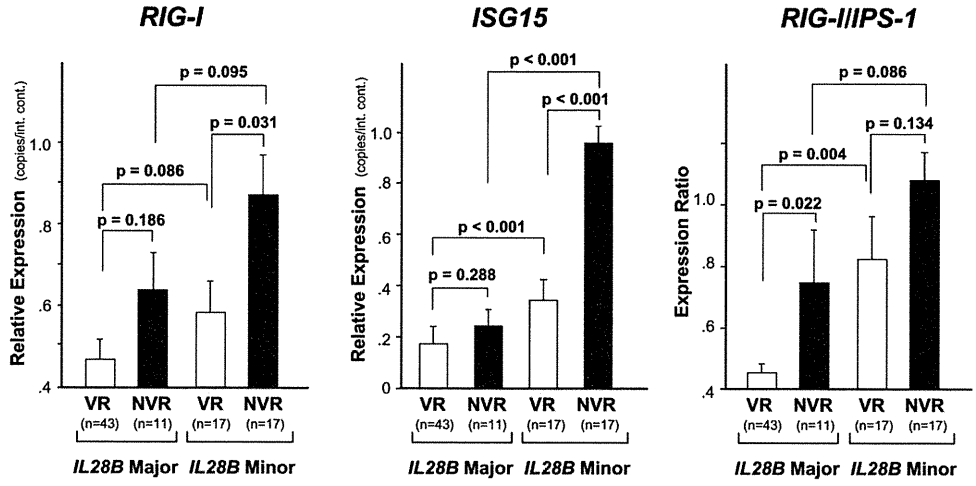

Fig. 3. Comparison of hepatic gene expression levels between virological responders (VR) and nonvirological responders (NVR) in subgroups of the *IL28B* genotype (*IL28B* Major, rs8099917 TT/rs12979860 CC; *IL28B* Minor, rs8099917 TG/rs12979860 CT). Expressions of *RIG-I* and *ISG15* as well as the *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The numbers of patients in each subgroup are shown in the bottom of the figure.

prediction of NVR (Table 2). The area under the ROC curve for *IL28B* genotype was 0.662, which was lower compared with that for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio.

When we stratified the patients by the cutoff value for *RIG-I* and *ISG15* expressions and *RIGI/IPS-1* ratio, no statistically significant difference was found in NVR rates among *IL28B* genotypes within the same subgroup (Fig. 4B).

Factors Associated with NVR. In univariate analysis, age, platelet counts, double mutation at amino acid positions 70 and 91 of the HCV core region, *IL28B* minor allele, and hepatic expressions of *RIG-I, MDA5, LGP2, ISG15*, and *USP18*, and *RIG-I/IPS-1* ratio were significantly

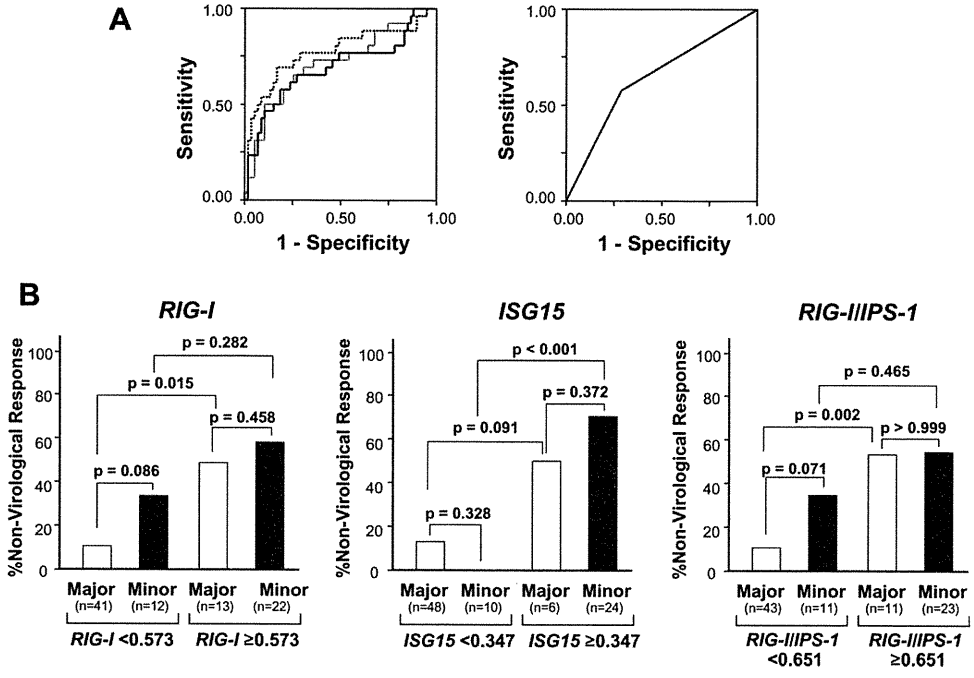

Fig. 4. (A) Receiver operator characteristics (ROC) curve for prediction of nonvirological response. ROC curves were generated to compare *RIG-I* (black line), *ISG15* (dotted line), and *RIG-I/IPS-1* ratio (gray line) (all in the left panel), and *IL28B* genotype (in the right panel). (B) Nonvirological response rate in *IL28B* major (rs8099917 TT/rs12979860 CC) and minor patients (rs8099917 TG/rs12979860 CT) in subgroups divided by the cutoff value of *RIG-I* and *ISG15* expression and the *RIG-I/ISG15* ratio determined by ROC analysis. Cutoff values of *RIG-I* and *ISG15* expression are expressed as expression copy number normalized to the expression of an internal control. The numbers of patients in each subgroup are shown in the bottom of the figure.

Table 2. Area Under the ROC Curves, Sensitivity, Specificity, and Negative as Well as Positive Predictive Values of Nonvirological Responses

Variables	AUC	95% CI	Cutoff	Sensitivity	Specificity	NPV	PPV
RIG-I (copies/int. control)	0.712	0.584-0.840	0.573	0.679	0.733	0.830	0.543
ISG15 (copies/int. control)	0.782	0.666-0.899	0.347	0.714	0.833	0.862	0.667
RIG-I/IPS-1 (copies/int. control)	0.732	0.611-0.852	0.651	0.679	0.750	0.833	0.559
IL28B genotype	0.662	0.537-0.787	TG*/CT†	0.607	0.717	0.796	0.500

AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.
*Genotype at rs8099917.
†Genotype at rs12979860.

associated with NVR (Table 3). Among these, multivariate analysis identified old age, HCV core double mutant, and higher hepatic expressions of *RIG-I* and *ISG15* as factors independently associated with NVR (Table 3).

IPS-1 and RIG-I Protein Expression in the Liver. Western blotting revealed that full-length and cleaved IPS-1 were variably present in all the samples from CH-C patients (Fig. 5A). Similar to mRNA

Table 3. Factors Associated with Nonvirological Response

	Univariate Analysis		Multivariate Analysis*	
Factors	Risk Ratio (95% CI)	*P*-value	Risk Ratio (95% CI)	*P*-value
Age (by every 10 year)	1.84 (1.10-3.14)	0.027	3.76 (1.19-11.7)	0.023
Sex				
Male	1			
Female	1.62 (0.59-4.42)	0.350		
BMI (by every 5 kg/m^2)	0.87 (0.46-1.65)	0.672		
Fibrosis stage				
F1/F2	1			
F3/F4	1.82 (0.69-4.85)	0.228		
Degree of steatosis				
<10%	1			
≥10%	1.46 (0.43-5.03)	0.544		
Albumin (by every 1 g/dL)	0.41 (0.11-1.56)	0.190		
AST (by every 40 IU/L)	0.89 (0.53-1.56)	0.681		
ALT (by every 40 IU/L)	0.85 (0.57-1.32)	0.481		
γ-GTP (by every 40 IU/L)	1.32 (0.82-2.07)	0.235		
Fasting blood sugar (by every 100 mg/dL)	1.35 (0.74-2.45)	0.340		
Hemoglobin (by every 1 g/dL)	0.93 (0.67-1.31)	0.683		
Platelet counts (by every 10^4/μL)	0.90 (0.82-0.99)	0.037	0.92 (0.78-1.08)	0.296
HCV load (by every 100 KIU/mL)	1.00 (1.00-1.00)	0.688		
Core 70 & 91 double mutation				
Wild	1		1	
Mutant	3.92 (1.14-13.5)	0.030	11.1 (1.40-88.7)	0.023
ISDR				
Nonwildtype	1			
Wildtype	1.38 (0.13-3.61)	0.513		
IL28B genotype				
Major allele†	1		1	
Minor allele‡	3.91 (1.52-10.0)	0.005	1.53 (0.20-11.9)	0.684
Hepatic gene expression (by every 0.1 copy/*int. control*)				
RIG-I	1.28 (1.10-1.50)	0.002	1.53 (1.07-2.22)	0.021
MDA5	1.53 (1.12-2.00)	0.001		
LGP2	1.34 (1.04-1.74)	0.026		
IPS-1	0.90 (0.78-1.04)	0.143		
RNF125	0.93 (0.83-1.04)	0.204		
ISG15	1.37 (1.16-1.62)	<0.001	1.28 (1.04-1.58)	0.021
USP18	1.67 (1.27-2.20)	<0.001		
IFNλ	1.02 (0.99-1.05)	0.170		
RIG-I/IPS-1 ratio (by every 0.1)	1.21 (1.07-1.36)	0.002		

Risk ratios for nonvirological response were calculated by the logistic regression analysis. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, IFN sensitivity determining region.
*Multivariate analysis was performed with factors significantly associated with nonvirological response by univariate analysis except for *MDA5*, *LGP2*, *USP18*, and *RIG-I/IPS-1* ratio, which were significantly correlated with *RIG-I* and *ISG15*.
†rs8099917 TT and rs12979860 CC.
‡rs8099917 TG and rs12979860 CT.

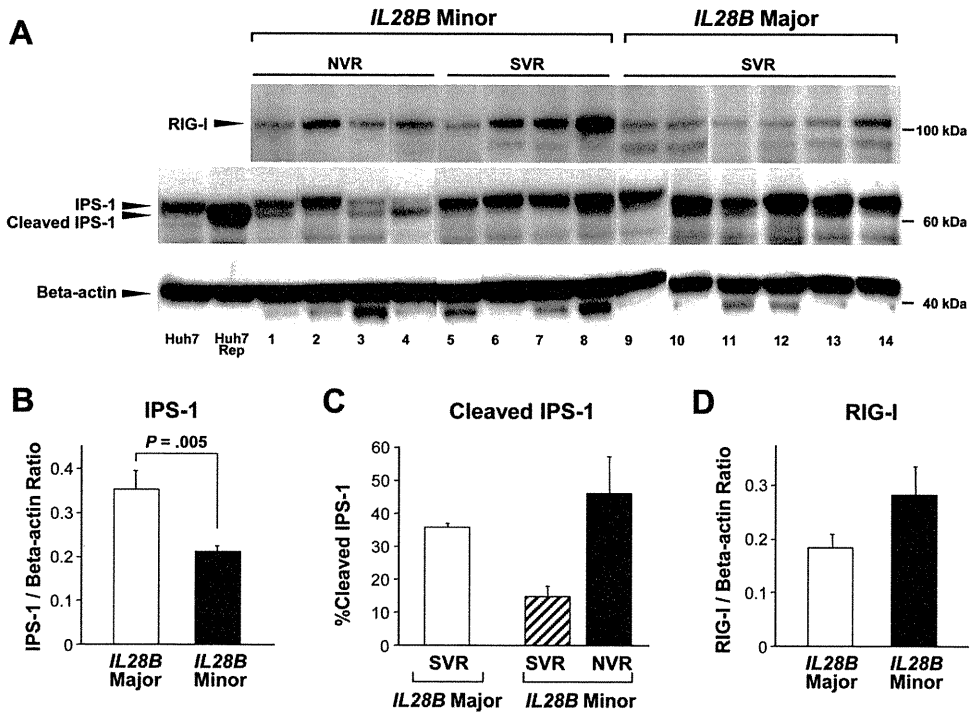

Fig. 5. (A) Western blotting for IPS-1 and RIG-I protein expression levels. Eight lanes contain samples from *IL28B* minor patients (lanes 1-8) and six lanes contain samples from *IL28B* major patients (lanes 9-14). Four lanes contain samples from nonvirological responders (NVR, lanes 1-4) and 10 lanes contain samples from sustained virological responders (SVR, lanes 5-14). Specific bands for RIG-I, full-length IPS-1, cleaved IPS-1, and β-actin are indicated by arrows. Naive Huh7 cells were used for a positive control for full-length IPS-1 (lane Huh7), and cells transfected with HCV-1b subgenomic replicon (Reference #20) were used for a positive control for cleaved IPS-1 (lane Huh7 Rep). (B) Total IPS-1 protein expression levels normalized to β-actin according to *IL28B* genotype. Error bars indicate standard error. *P*-value was determined by Mann-Whitney *U* test. (C) Percentage of cleaved IPS-1 products in total IPS-1 protein according to treatment responses stratified by *IL28B* genotype. Error bars indicate standard error. (D) RIG-I protein expression levels normalized to β-actin according to *IL28B* genotype. Error bars indicate standard error.

expression, total hepatic IPS-1 protein expression was significantly lower in *IL28B* minor patients than in *IL28B* major patients (Fig. 5B). With regard to *IL28B* minor patients, the percentage of cleaved IPS-1 protein in total IPS-1 in SVR was lower than that in NVR (Fig. 5C). In contrast to IPS-1 protein expression, hepatic RIG-I protein expression was higher in *IL28B* minor patients than that in *IL28B* major patients (Fig. 5D).

Discussion

In the present study we found that the baseline expression levels of intrahepatic viral sensors and related regulatory molecules were significantly associated with the genetic variation of *IL28B* and final virological outcome in CH-C patients treated with PEG-IFNα/RBV combination therapy. Although the relationship between the *IL28B* minor allele and NVR in PEG-IFNα/RBV combination therapy is evident, mechanisms responsible for this association remain unknown. *In vitro* studies have suggested that cytoplasmic viral sensors, such as RIG-I and MDA5, play a pivotal role in the regulation of IFN production and augment IFN production through an amplification circuit.[7,8] Our results indicate that expressions of *RIG-I* and *MDA5* and a related amplification system may be up-regulated by endogenous IFN at a higher baseline level in *IL28B* minor patients. However, HCV elimination by subsequent exogenous IFN is insufficient in these patients, as reported,[19] suggesting that *IL28B* minor patients may have adopted a different equilibrium in their innate immune response to HCV. Our data are further supported by recent reports of an association between intrahepatic levels of IFN-stimulated gene expression and PEG-IFNα/RBV response as well as with *IL28B* genotype.[21-23]

In contrast to cytoplasmic viral sensor (*RIG-I, MDA5*, and *LGP2*) and modulator (*ISG15* and *USP18*) expression, the adaptor molecule (*IPS-1*) expression was significantly lower in *IL28B* minor patients. Moreover, western blotting further confirmed IPS-1 protein downregulation in *IL28B* minor patients by revealing decreased protein levels. Because IPS-1 is one of the main target molecules of HCV evasion,[9,18]