

IL-18 had a higher NVR rate, but this difference was not statistically significant (100% versus 50%; $P=0.063$).

Factors associated with an NVR to PEG-IFN and ribavirin therapy

All factors found to be associated with an NVR were evaluated for independence in multivariate analysis. Genotype TG or GG (OR 10.43, 95% CI 1.73–62.96; $P=0.011$), serum IL-10 levels ≥ 5.0 pg/ml (OR 1.21, 95% CI 1.03–1.41; $P=0.018$) and IL-12p40 levels ≥ 17.4 mg/dl (OR 0.84, 95% CI 0.72–0.97; $P=0.020$) were all independent predictive factors of an NVR.

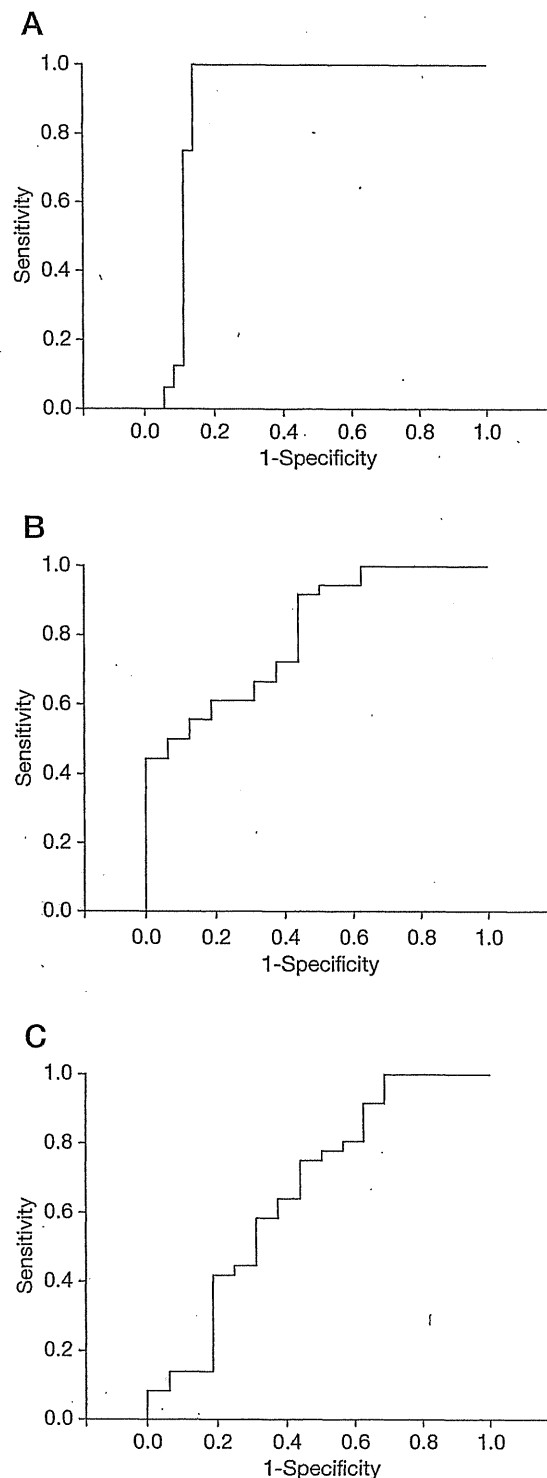
Discussion

This study examined the *IL28B* (rs8099917) genotype and serum levels of IL-10, IL-12p40 and IL-18 in patients with chronic hepatitis C to assess their predictive value in treatment outcome with PEG-IFN and ribavirin. The key findings were as follows: *IL28B* G-allele carriers were associated with an NVR to PEG-IFN and ribavirin therapy in patients infected with HCV genotype 1, consistent with recent findings; *IL28B* genotype was associated with baseline serum IL-10, IL-12p40 and IL-18 levels; in carriers of an *IL28B* G-allele, NVR rates were high (80–100%) and associated with increased IL-10 and decreased IL-12p40 and IL-18 levels, thus providing new predictive markers of an NVR in PEG-IFN and ribavirin therapy; and *IL28B* genotype, high serum IL-10 levels, and low serum IL-12p40 levels were all independent factors related to an NVR in multivariate analyses.

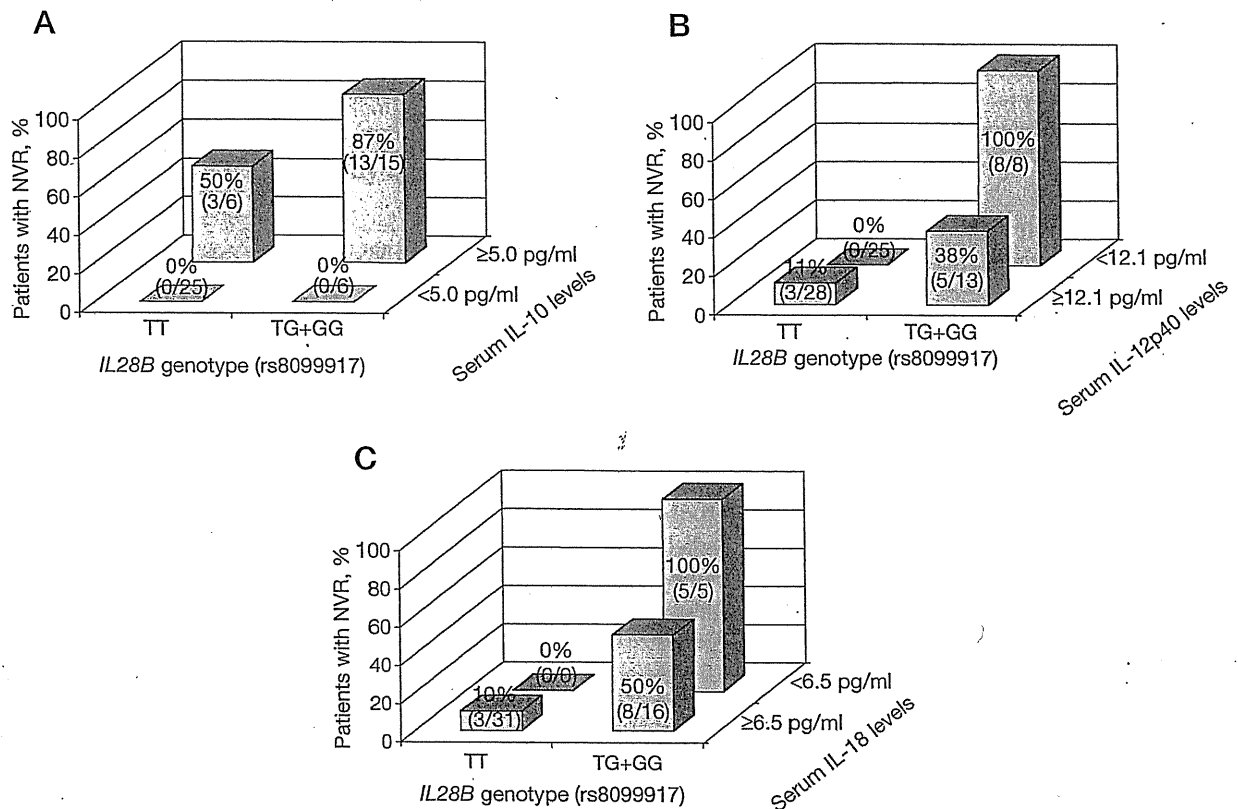
IL28B gene polymorphisms have recently been linked to the outcome of HCV infection during spontaneous and treatment-induced elimination of HCV [8–10,12]. In particular, carriage of a G-allele at the *IL28B* gene SNP (rs8099917) is associated with an NVR to PEG-IFN and ribavirin therapy in Japanese patients infected with HCV genotype 1 [10]. This finding was confirmed in our cohort with NVR rates of 62% with GT or GG genotypes versus 10% with TT genotypes ($P<0.001$). Therefore, detection of the *IL28B* genotype is a useful marker to predict the outcome of PEG-IFN and ribavirin therapy in patients with chronic hepatitis C. Data for *IL28B* SNP in healthy subjects were not available for this study.

IFN- λ produces an antiviral state by triggering a cascade through the JAK-STAT pathway that up-regulates IFN-stimulated genes. *IL28B* binds to a distinct receptor that may up-regulate a different set of IFN-stimulated genes [26,27]; the precise role of IFN- λ in controlling multiple viral infections, including HCV, is currently under way. Further studies are also needed on how SNPs affect the function of *IL28B* and other cytokines.

Figure 3. Receiver–operating characteristic curves for serum cytokine levels on treatment outcome



The areas under the curve for (A) interleukin (IL)-10, (B) IL-12p40 and (C) IL-18 were 0.89, 0.81 and 0.67, respectively. All areas under the curve values were significantly higher than a 0.50 non-predictive value ($P<0.01$ for all comparisons). IL-10 was predictive of a non-response. IL-12p40 and IL-18 were predictive of a virological response.

Figure 4. Non-virological response rate determined by serum cytokine levels and *IL28B* gene genotype

The prevalence of a non-virological response (NVR) in patients with high or low serum (A) interleukin (IL)-10, (B) IL-12p40 and (C) IL-18 levels according to *IL28B* genotype.

A strong association between high IL-10, low IL-12p40 and low IL-18 levels and an NVR to PEG-IFN and ribavirin therapy was found in this study, which is consistent with previous studies [22,28–30]. In ROC curve analyses, AUCs were high, especially for IL-10 (AUC=0.89) and IL-12p40 (AUC=0.81), confirming that these cytokines are strong predictive markers for an NVR. This study showed a strong correlation between the *IL28B* genotype and serum IL-10, IL-12p40 and IL-18 levels at baseline. Most strikingly, all patients who had low pretreatment IL-10 levels achieved a virological response regardless of *IL28B* genotype. By contrast, among patients with high IL-10 levels (≥5.0 pg/ml), NVR rates were 87% in *IL28B* G-allele carriers and 50% for the *IL28B* TT genotype. Additionally, all *IL28B* G-allele carriers showed an NVR when pretreatment serum IL-12p40 and IL-18 levels were <12.1 pg/ml and <6.5 pg/ml, respectively. It is unclear how serum IL-10, IL-12p40 and IL-18 are associated with an NVR to antiviral therapy in patients with chronic hepatitis C. Although IL-10 was originally described as a cytokine synthesis

inhibitory factor, recent studies have demonstrated that IL-10 produced by Th17 cells restrains the pathological effects of Th17 [31]. Production of IL-12p40 is directed towards the elimination of intracellular pathogens and viruses because IL-12p40 is a proinflammatory cytokine that promotes the differentiation of Th1 cells, suppresses Th2 function and amplifies the cytotoxicity of cytotoxic T-lymphocytes and natural killer cells [32]. Megjugorac *et al.* [33] reported that IL-29-treated plasmacytoid dendritic cells inhibiting production of IL-13, IFN- γ and IL-10 by allogeneic T-cells were consistent with a role for this cytokine in plasmacytoid dendritic cell maturation and activation. Very recently, another report has been published demonstrating that IL-29 enhances IL-12p40 by macrophages and that IL-29 pretreatment primes the activation of macrophages induced by IFN- γ [34]. However, the association between IL-28B and such cytokines has not been studied. To explain this relationship, further studies are needed to clarify whether a direct or indirect interaction exists between pretreatment levels of these cytokines and *IL28B* genotype.

Although other predictive factors of PEG-IFN and ribavirin therapy have been reported, including core amino acid 70 and 91 and ISDR mutations [6,7], no such significant associations were found here, possibly because of our study population size, which indicates that other factors may be more significant in predicting treatment outcome. However, multivariate analysis confirmed that *IL28B* G-allele, high IL-10 and low IL-12p40 levels were significant predictors of an NVR in patients with PEG-IFN and ribavirin therapy in this study. Hence, *IL28B* G-allele carriers combined with high IL-10 and/or low IL-12 may require alteration of treatment dose, duration, or regimen with a new antiviral drug.

In conclusion, serum IL-10, IL-12p40 and IL-18 levels are associated with *IL28B* genotype in patients with genotype 1 chronic hepatitis C. Pretreatment serum IL-10 and IL-12p40 levels with *IL28B* GT or GG genotypes are particularly useful for predicting an NVR to PEG-IFN and ribavirin therapy. The clinical significance of *IL28B* genotyping combined with baseline serum IL-10 and IL-12p40 levels to predict an NVR warrants further prospective validation.

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Disclosure statement

The authors declare no competing interests.

References

- Alter HJ, Purcell RH, Shih JW, *et al.* Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N Engl J Med* 1989; 321:1494–1500.
- Kiyosawa K, Sodeyama T, Tanaka E, *et al.* Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; 12:671–675.
- Umamura T, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *J Gastroenterol* 2009; 44 Suppl 19:102–107.
- Manns MP, McHutchison JG, Gordon SC, *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; 358:958–965.
- Fried MW, Shiffman ML, Reddy KR, *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347:975–982.
- Akuta N, Suzuki F, Sezaki H, *et al.* Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005; 48:372–380.
- Enomoto N, Sakuma I, Asahina Y, *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334:77–82.
- Ge D, Fellay J, Thompson AJ, *et al.* Genetic variation in *IL28B* predicts hepatitis C treatment-induced viral clearance. *Nature* 2009; 461:399–401.
- Suppiah V, Moldovan M, Ahlenstiel G, *et al.* *IL28B* is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009; 41:1100–1104.
- Tanaka Y, Nishida N, Sugiyama M, *et al.* Genome-wide association of *IL28B* with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; 41:1105–1109.
- Rauch A, Kutalik Z, Descombes P, *et al.* Genetic variation in *IL28B* is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology* 2010; 138:1338–1345.
- Thomas DL, Thio CL, Martin MP, *et al.* Genetic variation in *IL28B* and spontaneous clearance of hepatitis C virus. *Nature* 2009; 461:798–801.
- Donnelly RP, Sheikh F, Kolenko SV, Dickensheets H. The expanded family of class II cytokines that share the IL-10 receptor-2 (*IL-10R2*) chain. *J Leukoc Biol* 2004; 76:314–321.
- Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 1989; 170:2081–2095.
- Fiorentino DF, Zlotnik A, Vieira P, *et al.* IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 1991; 146:3444–3451.
- McGeachy MJ, Bak-Jensen KS, Chen Y, *et al.* TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol* 2007; 8:1390–1397.
- Stumhofer JS, Silver JS, Laurence A, *et al.* Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol* 2007; 8:1363–1371.
- Edwards-Smith CJ, Jonsson JR, Purdie DM, Bansal A, Shorthouse C, Powell EE. Interleukin-10 promoter polymorphism predicts initial response of chronic hepatitis C to interferon alfa. *Hepatology* 1999; 30:526–530.
- Yee LJ, Tang J, Gibson AW, Kimberly R, Van Leeuwen DJ, Kaslow RA. Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. *Hepatology* 2001; 33:708–712.
- Knapp S, Hennig BJ, Frodsham AJ, *et al.* Interleukin-10 promoter polymorphisms and the outcome of hepatitis C virus infection. *Immunogenetics* 2003; 55:362–369.
- Morgan TR, Lambrecht RW, Bonkovsky HL, *et al.* DNA polymorphisms and response to treatment in patients with chronic hepatitis C: results from the HALT-C trial. *J Hepatol* 2008; 49:548–556.
- Yoneda S, Umemura T, Katsuyama Y, *et al.* Association of serum cytokine levels with treatment response to pegylated interferon and ribavirin therapy in genotype 1 chronic hepatitis C patients. *J Infect Dis* 2011; 203:1087–1095.
- Umemura T, Wang RY, Schechterly C, Shih JW, Kiyosawa K, Alter HJ. Quantitative analysis of anti-hepatitis C virus antibody-secreting B cells in patients with chronic hepatitis C. *Hepatology* 2006; 43:91–99.
- Umemura T, Zen Y, Hamano H, Kawa S, Nakanuma Y, Kiyosawa K. Immunoglobulin G4-hepatopathy: association of immunoglobulin G4-bearing plasma cells in liver with autoimmune pancreatitis. *Hepatology* 2007; 46:463–471.
- Kato N, Hijikata M, Ootsuyama Y, *et al.* Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci U S A* 1990; 87:9524–9528.
- Honda M, Sakai A, Yamashita T, *et al.* Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. *Gastroenterology* 2010; 139:499–509.

27. Urban TJ, Thompson AJ, Bradrick SS, et al. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. *Hepatology* 2010; 52:1888–1896.
28. Rossol S, Marinos G, Carucci P, Singer MV, Williams R, Naoumov NV. Interleukin-12 induction of Th1 cytokines is important for viral clearance in chronic hepatitis B. *J Clin Invest* 1997; 99:3025–3033.
29. Quiroga JA, Martin J, Navas S, Carreno V. Induction of interleukin-12 production in chronic hepatitis C virus infection correlates with the hepatocellular damage. *J Infect Dis* 1998; 178:247–251.
30. Marín-Serrano E, Rodríguez-Ramos C, Díaz F, Martín-Herrera L, Giron-González JA. Modulation of the anti-inflammatory interleukin 10 and of proapoptotic IL-18 in patients with chronic hepatitis C treated with interferon alpha and ribavirin. *J Viral Hepat* 2006; 13:230–234.
31. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol* 2010; 10:170–181.
32. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 1995; 13:251–276.
33. Megjugorac NJ, Gallagher GE, Gallagher G. Modulation of human plasmacytoid DC function by IFN-lambda1 (IL-29). *J Leukoc Biol* 2009; 86:1359–1363.
34. Liu BS, Janssen HL, Boonstra A. IL-29 and IFN α differ in their ability to modulate IL-12 production by TLR-activated human macrophages, and exhibit differential regulation of the IFN γ receptor expression. *Blood* 2011; 117:2385–2395.

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Association of Serum Cytokine Levels With Treatment Response to Pegylated Interferon and Ribavirin Therapy in Genotype 1 Chronic Hepatitis C Patients

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Background. We sought to clarify the associations among serum cytokines, amino acid substitutions in the interferon sensitivity-determining region (ISDR) and core region, and treatment outcome of pegylated interferon and ribavirin therapy in genotype 1 hepatitis C virus (HCV)-infected patients.

Methods. We quantified a total of 8 serum cytokines before, during, and after treatment in 79 genotype 1 chronic HCV patients. Viral ISDR and core region variants were determined by direct sequencing.

Results. High levels of interleukin (IL)-12 and IL-18 and more than 2 mutations in the ISDR were associated with a sustained virological response (SVR). Conversely, high baseline IL-10 levels and glutamine at amino acid 70 of the HCV core protein (Gln70) were significantly associated with a nonresponse to treatment, and patients with Gln70 had significantly higher IL-10 levels. In multivariate analysis, low IL-10, high IL-12, and high IL-18 levels were independently associated with an SVR. These 3 cytokine levels were decreased from baseline levels 4 weeks into treatment and remained low in patients with an SVR.

Conclusion. Serum IL-10, IL-12, and IL-18 levels are predictive of the response to HCV treatment with pegylated interferon and ribavirin and are associated with amino acid substitutions in the ISDR and core region.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. More than half of patients with acute HCV infections develop chronic hepatitis, which leads to liver cirrhosis or hepatocellular carcinoma (HCC) in at least 20% of cases [1, 2]. HCC is ranked fourth in men and fifth in women as a cause of death from malignant neoplasms in Japan [3, 4]. Because approximately 70%–80% of Japanese HCC patients are infected with HCV, viral eradication is

important to decrease the incidence of HCC. Interferon-based therapy can reduce HCV to undetectable levels and improve prognosis. The primary aim of antiviral therapy in HCV patients is a sustained virological response (SVR), which is defined as undetectable serum HCV RNA 24 weeks after completion of therapy. Despite recent advances, however, approximately 50% of patients with genotype 1 HCV infection do not achieve an SVR by antiviral therapy [5, 6].

Cytokines play an important role in the pathogenesis, progression, and treatment outcome of HCV infection. Because the control of cytokine production is highly complex and the effects of cytokines are widespread throughout multiple regulatory networks, it would seem that screening for multiple biomarkers could best clarify the immunopathogenesis of the disease and predict responses to antiviral therapy. However, such analysis is difficult using enzyme-linked immunosorbent assay, which requires each biomarker be tested individually. In

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this study, we used a new broad-spectrum bead-based multiplex immunoassay to simultaneously test multiple factors in the sera of patients with chronic hepatitis C. Wan et al recently reported that some cytokines are elevated in non-SVR HCV patients using this bead system, but only 17 patients with genotype 1 were evaluated [7]. Thus, the association between multiple cytokines and treatment outcome are largely unknown.

The objective of this study was to determine which cytokines in patients with genotype 1 chronic hepatitis C relate to the clinical and virologic characteristics of hepatitis and how they affect the HCV response to pegylated interferon (PEG-IFN) and ribavirin therapy.

PATIENTS AND METHODS

Participants

We included 79 consecutive patients with genotype 1 chronic hepatitis C in this study. We based diagnosis of chronic hepatitis C on the following criteria, as reported previously [8]: 1) presence of serum HCV antibodies and detectable viral RNA; 2) absence of detectable hepatitis B surface antigen; and 3) exclusion of other causes of chronic liver disease. No patient had a history of or developed decompensated cirrhosis or hepatocellular carcinoma. The baseline characteristics of patients are shown in Table 1. We used a group of 26 healthy individuals with normal transaminase levels and negative serologic results for hepatitis B and hepatitis C as the control. All participants were negative for the antibody to the human immunodeficiency virus. The protocol of this study was approved by the ethics committee of the Shinshu University School of Medicine, and all patients provided written informed consent.

Laboratory Testing

We measured antibodies to HCV in serum samples via third-generation enzyme-linked immunosorbent assays (EIA-3; Abbott Laboratories). We determined serum levels of HCV RNA using the COBAS AMPLICOR assays (Roche Diagnostic

Systems), which amplify HCV RNA by reverse transcriptase-polymerase chain reaction. The lower limit of the assay was 50 IU/mL. We determined HCV genotypes using INNO-LiPA HCV II (Innogenetics). We found that all patients in our test cohort were infected with genotype 1b. We performed alanine aminotransferase (ALT), aspartate aminotransferase (AST), and other relevant biochemical tests using standard methods [9].

Antiviral Therapy

All patients received body weight-adjusted PEG-IFN α -2b (PegIntron, Schering-Plough K.K.; \leq 45 kg, 60 μ g/dose; 46–60 kg, 80 μ g/dose; 61–75 kg, 100 μ g/dose; 76–90 kg, 120 μ g/dose; \geq 91 kg, 150 μ g/dose), and ribavirin (Rebetol, Schering-Plough K.K.; \leq 60 kg, 600 mg/day; 61 kg–80 kg, 800 mg/day; \geq 81 kg, 1000 mg/day) for 48 weeks, as reported previously [10].

Definition of Viral Kinetic Response and Treatment Outcome

An early virological response (EVR) was defined as undetectable serum HCV RNA by 12 weeks of therapy. An SVR was classified as serum HCV RNA that was undetectable 24 weeks after completing therapy. Post-treatment relapse was defined as a re-appearance of serum HCV RNA after treatment in patients whose HCV RNA level was undetectable during or at the completion of therapy. A nonresponse was defined as a decrease in HCV RNA of <2 log copies/mL at week 12 and detectable HCV RNA during the treatment course.

Detection of Amino Acid Substitutions in the Core and NS5A Regions

We determined the sequence of 1–191 amino acids (aa) in the core protein of genotype 1b HCV, and we evaluated substitutions at aa70 of arginine (Arg70) or glutamine (Gln70) [11] with the use of HCV-J as a reference [12]. We also determined the sequence of 2209–2248 aa in the NS5A region of genotype 1b HCV containing the interferon sensitivity-determining region (ISDR), and the number of aa substitutions in the ISDR was defined as wild-type (0), intermediate-type (1), or mutant-type

Table 1. Demographic and Clinical Characteristics of Patients with Hepatitis C Virus Infection

Characteristics	All (n = 79)	SVR (n = 31)	Non-SVR (n = 48)	P
Median age, y (range)	60 (17–74)	56 (28–72)	61 (17–74)	0.08
Male, n (%)	40 (51)	23 (74)	17 (35)	0.001
Median values (range)				
ALT, IU/L (range)	54 (22–389)	53 (24–172)	61 (22–389)	0.25
AST, IU/L (range)	44 (20–288)	36 (21–133)	48 (20–288)	0.012
HCV RNA, 10 ⁵ IU/mL (range)	17 (1.1–51)	15 (1.1–50)	19 (2.2–51)	0.13
Substitutions				
Core aa 70(Arg70/Gln70)	47/28	22/6	25/22	0.028
ISDR of NS5A(wild/intermediate/mutant)	46/17/13	13/7/9	33/10/4	0.026

NOTE. HCV, hepatitis C virus; SVR, sustained virological response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; aa, amino acid; ISDR, interferon sensitivity-determining region.

(≥ 2) [13]. We determined all aa substitutions in the core region and ISDR by direct sequencing.

Detection of Cytokines

We quantified 8 cytokines (interleukin [IL]-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-18, and vascular endothelial growth factor [VEGF]) using Luminex Multiplex Cytokine Kits (Procarta Cytokine assay kit) for serum samples obtained before the start of treatment, 4 weeks after the start of treatment, and 24 weeks after treatment completion. All collected samples were immediately stored at -70°C and remained in storage until testing.

Statistical Analysis

We used the Mann–Whitney *U* test and Kruskal–Wallis test to analyze continuous variables where appropriate. We used the Friedman test to evaluate changes in serum cytokine levels over time. We used the Spearman rank correlations to evaluate the relationship between pairs of markers. We used the χ^2 test with the Yates correction for the analysis of categorical data. In cases where the number of participants was < 5 , we used the Fisher exact test. We considered a *P* value of $\leq .05$ statistically significant. To predict treatment outcome, cutoff points for continuous variables were decided by receiver-operating characteristic (ROC) curve analysis. Multivariate analysis was performed using a stepwise logistic regression model. Statistical analyses were performed using SPSS software version 18.0J.

RESULTS

Detection and Quantification of Serum Markers in Patients with Chronic Hepatitis C and Controls

Of the 79 patients receiving PEG-IFN and ribavirin therapy, 31 (39%) were sustained responders with accompanying normalization of ALT levels. Of the 48 patients without an SVR, 23 had a relapse and 25 did not respond to treatment. Patients with an SVR had a higher male ratio compared with patients without ($P = .001$) (Table 1). Before treatment, the median AST level in the SVR group was significantly lower than that in the non-SVR group (36 vs 48 IU/L; $P = .012$). Substitutions of aa 70 in the core region ($P = .028$) and in the ISDR ($P = .026$) were both significantly associated with treatment outcome.

Serum samples obtained prior to antiviral therapy were examined for the presence of 8 cytokines by multiplex assays. Of these, 6 could be reliably quantified in a large majority of samples. As shown in Figure 1, the median baseline serum concentrations of 4 cytokines [IL-10 (4.8 vs 4.3 pg/mL; $P = .032$), IL-12p40 (20.4 vs 8.5 pg/mL; $P < .001$), IL-12p70 (12.8 vs 1.0 pg/mL; $P < .001$), and IL-18 (21.9 vs 14.5 pg/mL; $P = .008$)] were significantly higher in patients with HCV infection than in healthy controls. Conversely, serum levels of IL-4 (7.3 vs 7.9 pg/mL; $P = .011$) and VEGF (57.5 vs 78.0 pg/mL; $P = .025$) were significantly lower in patients with HCV infection compared with those in controls.

Effects of Antiviral Therapy on Serum Cytokine Levels

The median baseline serum levels of 4 cytokines (IL-12p40 [24.1 vs 17.2 pg/mL; $P = .003$], IL-12p70 [15.9 vs 12.6 pg/mL; $P < .001$], IL-18 [27.9 vs 17.7 pg/mL; $P = .001$], and VEGF [93.0 vs 39.7 pg/mL; $P < .001$]) were significantly higher in patients who achieved an SVR than in those who did not (Figure 2). In contrast, SVR patients showed significantly lower baseline IL-10 concentrations (4.1 pg/mL) than non-SVR patients (7.3 pg/mL; $P = .002$).

Significantly higher baseline levels of 3 cytokines (IL-4 [7.8 vs 7.0 pg/mL; $P = .001$], IL-12p40 [24.1 vs 14.6 pg/mL; $P < .001$], and VEGF [65.5 vs 43.0 pg/mL; $P = .025$]) were observed in patients with a virological response compared with levels in those without. Conversely, IL-10 levels (4.3 vs 7.9 pg/mL; $P < .001$) were significantly lower in virological responders compared with that in nonresponders.

Several demographic (age and sex) and clinical (ALT level, AST level, and viral load) findings were examined for their correlation with serum cytokines in patients with HCV infection, but no significant associations were observed. However, serum IL-12p40 levels were significantly correlated with serum IL-18 ($P = .004$, $r = 0.325$) (Figure 3A) and VEGF ($P = .024$, $r = 0.253$) (Figure 3B). There was also a significant correlation between IL-18 and VEGF ($P < .001$, $r = 0.394$) (Figure 3C).

Prediction of Treatment Outcome in Patients with Chronic Hepatitis C

We performed ROC curve analyses to determine the optimal cutoff values for serum cytokines in predicting treatment outcome for genotype 1 HCV-infected patients. We obtained the ROC curve for serum IL-10 via calculations using the values obtained from 25 nonresponders and 54 patients with a virological response. The ROC curves for serum IL-12p40, IL-18, and VEGF were obtained from 31 patients who achieved an SVR and 48 non-SVR patients. We selected optimal cutoff point values based on the cytokine level at which accuracy was maximal. The optimal cutoff value, sensitivity, specificity, positive predictive value, negative predictive value, and calculated area under the curve (AUC) for the 4 cytokines are listed in Table 2. The AUC values were consistently high and ranged between .70 (IL-12p40) and .86 (IL-10).

In addition, ROC curves for serum IL-10, IL-12p40, IL-18, and VEGF at 4 weeks after the start of treatment were obtained (Table 2). The AUCs for these 4 cytokines (.62–.86) were also high, but lower than those at baseline.

Correlation Between Core Region and Interferon Sensitivity–Determining Region Amino Acid Substitutions and Cytokine Production.

Because core region and ISDR substitutions have been associated with treatment outcome both in this study and elsewhere, we analyzed whether substitutions in these regions were correlated with baseline serum cytokine concentrations as

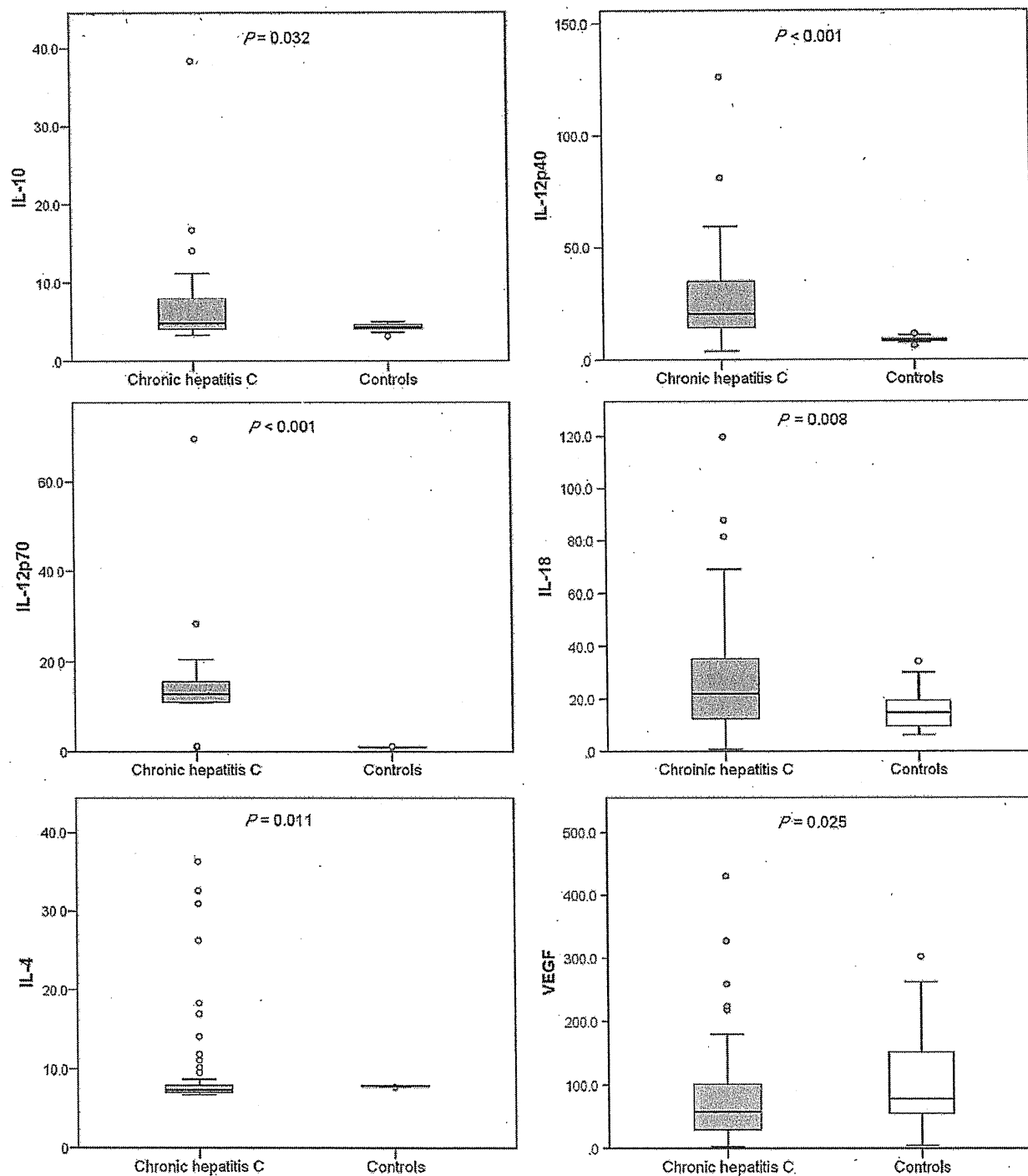


Figure 1. Detection of Serum Cytokines in Patients with HCV Infection and Healthy Subjects. Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. Serum IL-10, IL-12p40, IL-12p70, IL-18, IL-4, and VEGF levels were detected in 79 patients with HCV infection and 26 controls. **NOTE.** HCV, hepatitis C virus; IL, interleukin; VEGF, vascular endothelial growth factor.

well. Before treatment, median IL-10 levels in patients with Gln70 (7.5 pg/mL) were significantly higher than those in patients with Arg70 (4.3 pg/mL; $P = .045$). The prevalence of higher serum IL-10 (≥ 5.0 pg/mL at baseline) was significantly

greater in the nonresponse group than in the response group (25 of 25 patients [100%] vs 11 of 50 [22%]; $P < .001$). The frequencies of the combination of higher IL-10 and HCV with and without core Gln70 were 14 of 25 patients (56%) and 3 of 50

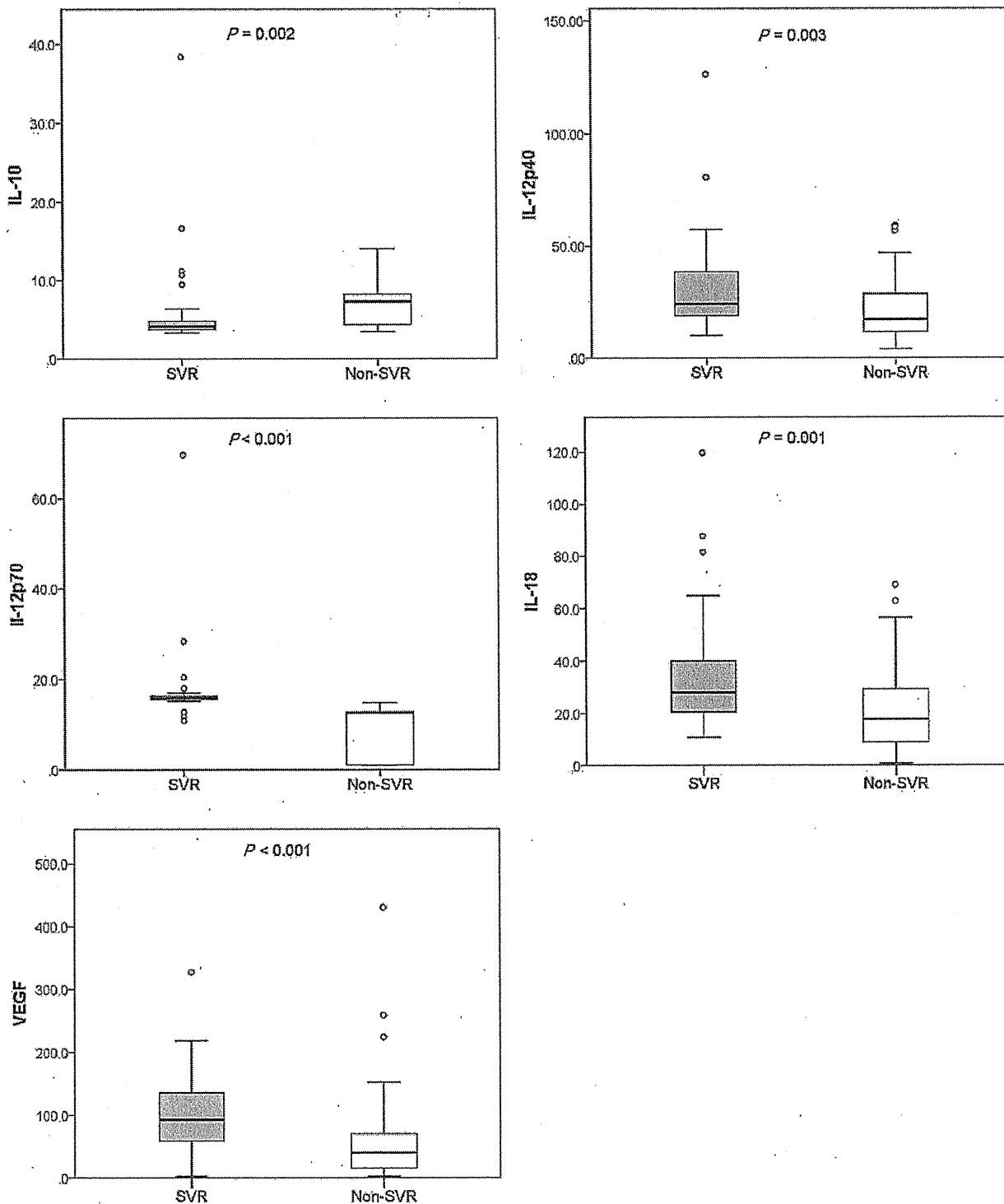


Figure 2. Serum Cytokines Related to Antiviral Therapy Outcome. Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. (A) Serum IL-10, IL-12p40, IL-12p70, IL-18, and VEGF were detected in 31 patients who achieved a sustained virological response and 48 patients who did not. **NOTE.** SVR, sustained virological response; IL, interleukin; VEGF, vascular endothelial growth factor.

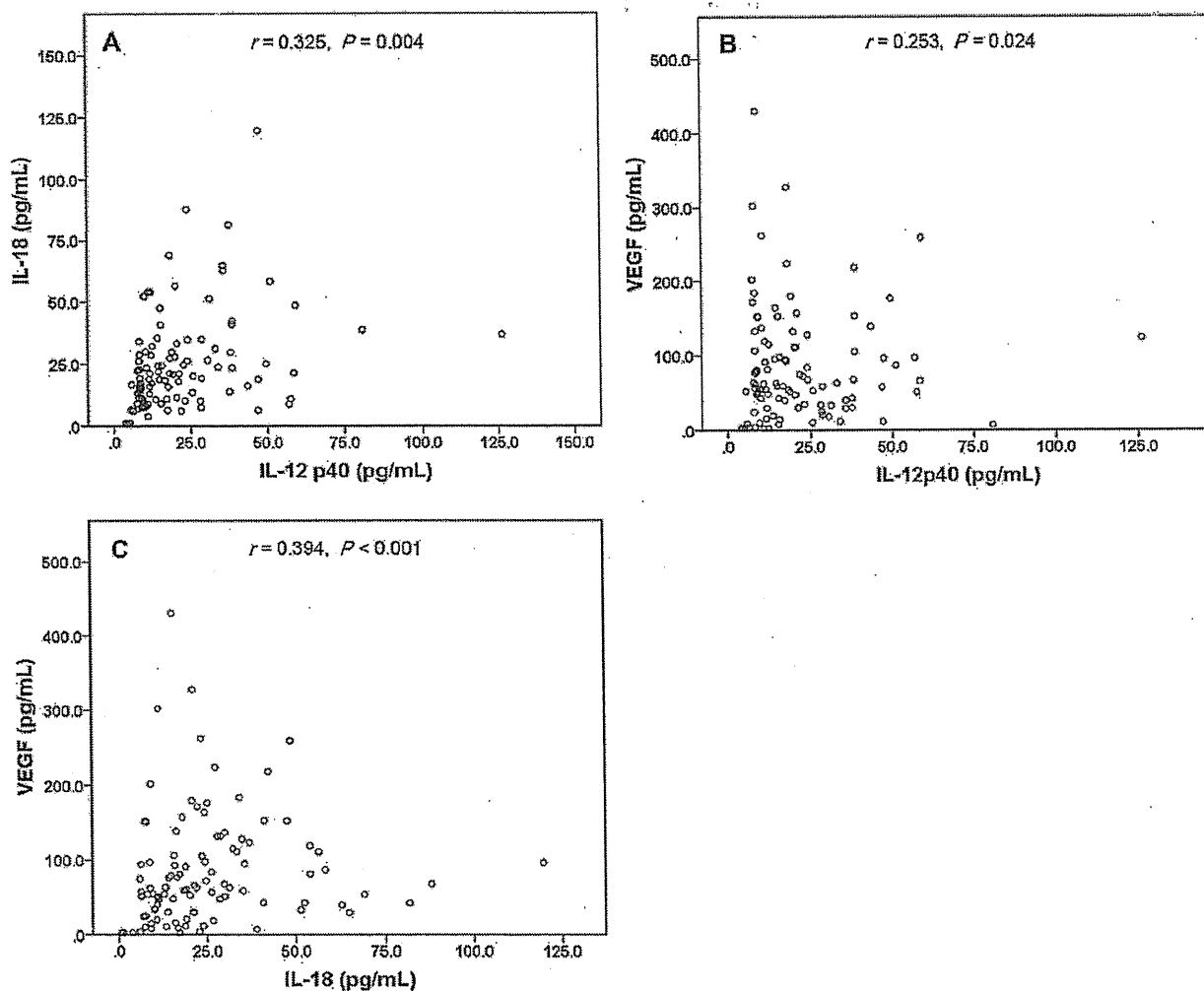


Figure 3. Correlation Between Serum Cytokines in 79 Patients with HCV Infection. (A–B) Serum IL-12p40 was significantly correlated with the level of (A) IL-18 ($r = .325$; $P = .004$) and (B) VEGF ($r = .253$; $P = .024$). (C) Serum IL-18 was correlated with the level of VEGF ($r = .394$; $P < .001$). **NOTE.** HCV, hepatitis C virus; IL, interleukin; VEGF, vascular endothelial growth factor.

Table 2. Optimal Cutoff Value, Sensitivity, Specificity, Area Under The Curve, and Predictive Values of Serum IL-10, IL-12p40, IL-18, and VEGF at Baseline and After 4 Weeks of Treatment in 79 Patients with Chronic Hepatitis C

Cytokine	Collection Time	Cutoff Value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	AUC (95% CI)	PPV (%)	NPV (%)
IL-10	baseline	5.0	100 (86–100)	80 (67–89)	.86 (.84–.98)	69	100
	4 wk	6.8	82 (69–91)	100 (86–100)	.86 (.78–.95)	100	71
IL-12p40	baseline	17.4	81 (63–93)	52 (37–67)	.70 (.59–.82)	52	81
	4 wk	21.3	81 (63–93)	60 (45–74)	.69 (.57–.81)	57	83
IL-18	baseline	15.4	97 (83–100)	46 (31–61)	.72 (.61–.83)	54	96
	4 wk	24.6	87 (70–96)	42 (28–57)	.62 (.50–.75)	49	83
VEGF	baseline	57.6	77 (59–90)	69 (54–81)	.74 (.63–.86)	62	83
	4 wk	62.6	74 (55–88)	67 (52–80)	.70 (.58–.82)	59	80

NOTE. CI, confidence interval; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; IL, interleukin

All AUC values were significantly higher than a 0.50 nonpredictive value ($P < .01$ for all comparisons). Cutoff values were determined by constructing receiver operating characteristic curves and are expressed as pg/mL. IL-10 is predictive of a nonresponse. IL-12p40, IL-18, and VEGF are predictive of a sustained virological response.

Table 3. Multivariate Analysis of Factors Independently Associated with a Sustained Virological Response to Pegylated Interferon and Ribavirin Therapy in Patients Infected with Hepatitis C Virus Genotype 1

Factors	OR	95% CI	P
Gender: male	10.932	2.178–54.780	.004
AST \geq 40 IU/L	.946	.906–.989	.013
IL-10 \geq 5.0 pg/mL	.823	.704–.962	.014
IL-12p40 \geq 17.4 pg/mL	1.071	1.009–1.137	.024
IL-18 \geq 15.4 pg/mL	1.085	1.024–1.150	.006

NOTE. OR, odds ratio; CI, confidence interval; AST, aspartate aminotransferase; IL, interleukin.

Only variables that achieved statistical significance ($P < .05$) in multivariate logistic regression analysis are shown.

patients (6%), respectively, which was statistically significant ($P < .001$).

Serum levels of IL-12p70 were significantly correlated with the number of substitutions in the ISDR (Kruskal–Wallis; $P = .027$). In addition, median baseline serum IL-12p70 levels were significantly higher in patients with mutant-type ISDR than in those with wild or intermediate types (15.6 vs 12.7 pg/mL; $P = .009$).

Factors Independently Associated with a Sustained Virological Response

We evaluated several factors found in association with an SVR from PEG-IFN and ribavirin therapy for their independence by multivariate analysis (Table 3). Male (odds ratio 10.93 [95% confidence interval 2.18–54.87], $P = .004$), AST \geq 40 IU/L (.95 [.91–.99], $P = .013$), IL-10 \geq 5.0 pg/mL (.82 [.70–.96], $P = .014$), IL-12p40 \geq 17.4 pg/mL (1.07 [1.01–1.14], $P = .024$), and IL-18 \geq 15.4 pg/mL (1.09 [1.02–1.15], $P = .006$) were independent risk factors related to an SVR. Conversely, core region or ISDR substitutions were not significant independent associations in this study.

Serum Cytokine Changes During and After Treatment

We next measured cytokine levels 4 weeks after the initiation of therapy and 6 months after its completion (Table 4). The levels of IL-10 ($P < .001$, Friedman test), IL-12p40 ($P = .008$), and

IL-18 ($P < .001$) were significantly decreased in samples collected from patients who achieved an SVR. The reduction in serum cytokine levels from baseline to 4 weeks of treatment was determined and compared between SVR and non-SVR groups, and showed that the ratio of IL-10 had a significant negative association with both an EVR ($P = .024$) and an SVR ($P = .001$).

DISCUSSION

In this study, we measured the levels of 8 cytokines in patients with genotype 1 chronic hepatitis C and analyzed their association with the outcome of PEG-IFN and ribavirin therapy using a newly developed bead-array multiplex system. Serum IL-10, IL-12p40, IL-12p70, and IL-18 were higher in patients with HCV infection than in healthy participants. In addition, cytokines IL-10, IL-12p40, and IL-18 all decreased during treatment and remained low in patients with an SVR. These findings suggest that cytokines may in fact compromise host immune responses to the virus.

A strong association between high baseline serum IL-10 and a nonresponse to PEG-IFN and ribavirin therapy was found in our cohort, which is consistent with previous studies [7, 14, 15]. We found achievement of an EVR or SVR to be diminished in patients who had a lower IL-10 ratio between baseline and 4 weeks of treatment. In addition, using ROC curve analysis, we found sensitivity, specificity, and AUC were all high for IL-10, suggesting that serum IL-10 values at baseline and 4 weeks of treatment are predictive markers for treatment nonresponse (Table 2). Although humoral immunity is said to play a minor role in recovery from HCV infection and B-cell immunity is strongest in those with persistent infection [8, 16], a strong natural killer cell-mediated and Th1 cell-mediated immune response seems to be a key factor in protection from HCV infection. IL-10 was originally described as a cytokine synthesis inhibitory factor [17, 18], but recent studies have demonstrated that IL-10 produced by Th17 cells restrains the pathologic effects of Th17 [19, 20]. Furthermore, there is strong evidence of a substantial genetic component to IL-10 production [21, 22]; the –1082 G/G genotype is known to be related to increased IL-

Table 4. Serum Cytokine Levels Changes During and After Treatment of Pegylated Interferon Plus Ribavirin

Cytokines	Treatment Outcome	Baseline	Week 4	Week 72	P
IL-10	SVR	4.1 (3.3–25.4)	3.7 (3.1–19.9)	3.5 (2.9–9.0)	< .001
	Non-SVR	7.3 (3.7–10.8)	7.5 (3.9–8.8)	7.4 (3.9–10.9)	0.962
IL-12p40	SVR	24.1 (11.3–99.0)	22.1 (11.6–75.2)	18.4 (7.8–76.5)	0.008
	Non-SVR	17.2 (4.6–57.9)	19.2 (8.1–50.1)	21.6 (5.8–77.0)	0.281
IL-18	SVR	27.9 (13.8–100.6)	25.1 (13.2–95.2)	23.3 (6.6–48.5)	< .001
	Non-SVR	17.7 (1.1–59.9)	31.3 (10.3–90.6)	17.4 (5.4–52.0)	< .001

NOTE. Data are median (5th–95th percentile) values. IL, interleukin; SVR, sustained virological response.

10 production and is associated with a high risk of inefficient HCV clearance [23, 24] and resistance to IFN treatment [25–28].

In agreement with our findings, recent studies have indicated that Gln70 substitutions in the HCV core region are associated with treatment failure [11, 29–32]. Additionally, patients with Gln70 had higher IL-10 levels compared with those with Arg70. Among the 28 HCV patients who had Gln70, all 14 non-responders had higher IL-10 (≥ 5.0 pg/mL), whereas 11 of 14 responders had lower IL-10 levels ($P < .001$). This association between Gln70 and elevated IL-10 levels is intriguing. Dolganiuc et al reported that HCV core and NS3 proteins in monocytes and dendritic cells induce IL-10 [33], so further studies are needed to clarify the relationship between IL-10 and core region amino acid substitutions.

This report demonstrates the beneficial role of IL-12 in achieving an SVR during PEG-IFN and ribavirin therapy. IL-12 is a proinflammatory cytokine that promotes the differentiation of Th1 cells, suppresses Th2 function, and amplifies the cytotoxicity of cytotoxic T lymphocytes and natural killer cells [34]. Thus, production of IL-12 is directed toward the elimination of intracellular pathogens and viruses. Elevated serum IL-12 has been noted in patients with chronic HBV or HCV infection, and is even more prominent among responders to IFN- α treatment [35, 36]. In our study, we noted significantly higher serum IL-12p70 in participants carrying mutant-type ISDR than in those with intermediate- or wild-type ISDR. This correlation between IL-12 and ISDR substitutions is striking and requires further study to verify its favorable effect during PEG-IFN and ribavirin therapy.

It is believed that the dynamics of the Th1/Th2 response determine the outcome of antiviral therapy to chronic hepatitis C [10] and that IL-18 is an important mediator of the Th1/Th2 balance. IL-18 plays a critical role in host defense against infection by intracellular microbes but also induces autoimmune diseases and propagates inflammation [37]. IL-18 is significantly upregulated in patients with chronic HCV infection and is correlated with hepatic injury [38, 39], indicating a key role in disease pathogenesis. However, the effect of IL-18 on antiviral therapy for chronic hepatitis C is still unclear. We found that IL-18 levels were significantly higher in patients with chronic HCV infection compared with healthy controls, but they were also higher at baseline in patients who achieved an SVR than in those who did not. In addition, there was a significant correlation between IL-18 and IL-12; in the presence of IL-12, IL-18 stimulates *IFNG* expression, thus promoting the Th1-mediated immune response. Without IL-12, IL-18 stimulates Th2 responses [37]. In this study, because serum IFN- γ levels were below detection thresholds, we could not assess the association of such cytokines.

Lastly, we observed that pretreatment serum VEGF levels were associated with an SVR. A previous study showed no association between baseline VEGF and treatment outcome, but only 36

patients, including 19 with genotype 1, were studied [40]. Hence, it is still unclear if this angiogenesis marker plays a critical role in response to antiviral therapy in chronic HCV infection. Furthermore, we correlated VEGF with IL-12 and IL-18 in our study. In particular, IL-18 enhances the production of VEGF in rheumatoid arthritis synovial fibroblasts, suggesting that IL-18 could be an angiogenic mediator with triggering effects on VEGF production [41]. Although the preoperative serum VEGF level was found to be a significant predictor of tumor recurrence and overall survival in patients with HCC [42], there have been no reports regarding treatment response in patients with chronic hepatitis C during antiviral therapy.

In multivariate analysis of our cohort, low IL-10, high IL-12p40, and high IL-18 were independent factors related to an SVR in patients treated with PEG-IFN and ribavirin. Our results indicate that such 3-cytokine profiling may offer clinicians another tool in predicting treatment outcome of HCV infection. Further investigation must be done in vitro and using many samples to validate the significance of our findings.

In conclusion, several cytokines were seen to be elevated in patients with chronic hepatitis C using the multiplex bead assay. Serum IL-10 levels and amino acid substitutions at the 70 aa core region of HCV are useful for predicting a nonresponse to PEG-IFN and ribavirin therapy in patients with chronic hepatitis C genotype 1. A higher level of serum IL-12 is considered to be favorable for response to antiviral therapy, and is correlated with substitutions in the ISDR. Lastly, IL-18 is notably high in patients with chronic HCV infection, and is correlated with IL-12.

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References

1. Alter HJ, Purcell RH, Shih JW, et al. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N Engl J Med* 1989; 321:1494–500.
2. Kiyosawa K, Sodeyama T, Tanaka E, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: Analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; 12:671–5.
3. Umemura T, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *Hepatol Res* 2007; 37:S95–S100.
4. Umemura T, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *J Gastroenterol* 2009; 44:102–7.

5. Manns MP, McHutchison JG, Gordon SC, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomised trial. *Lancet* 2001; 358:958–65.
6. Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347:975–82.
7. Wan L, Kung YJ, Lin YJ, et al. Th1 and Th2 cytokines are elevated in HCV-infected SVR(-) patients treated with interferon- α . *Biochem Biophys Res Commun* 2009; 379:855–60.
8. Umemura T, Wang RY, Schechterly C, Shih JW, Kiyosawa K, Alter HJ. Quantitative analysis of anti-hepatitis C virus antibody-secreting B cells in patients with chronic hepatitis C. *Hepatology* 2006; 43:91–9.
9. Umemura T, Zen Y, Hamano H, Kawa S, Nakanuma Y, Kiyosawa K. Immunoglobulin G4-hepatopathy: Association of immunoglobulin G4-bearing plasma cells in liver with autoimmune pancreatitis. *Hepatology* 2007; 46:463–71.
10. Shirakawa H, Matsumoto A, Joshita S, et al. Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. *Hepatology* 2008; 48:1753–60.
11. Akuta N, Suzuki F, Sezaki H, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005; 48:372–80.
12. Kato N, Hijikata M, Ootsuyama Y, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci USA* 1990; 87:9524–8.
13. Enomoto N, Sakuma I, Asahina Y, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334:77–81.
14. Kuzushita N, Hayashi N, Katayama K, et al. High levels of serum interleukin-10 are associated with a poor response to interferon treatment in patients with chronic hepatitis C. *Scand J Gastroenterol* 1997; 32:169–74.
15. Marín-Serrano E, Rodríguez-Ramos C, Díaz F, Martín-Herrera L, Girón-González JA. Modulation of the anti-inflammatory interleukin 10 and of proapoptotic IL-18 in patients with chronic hepatitis C treated with interferon alpha and ribavirin. *J Viral Hepat* 2006; 13:230–4.
16. Takaki A, Wiese M, Maertens G, et al. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med* 2000; 6:578–82.
17. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 1989; 170:2081–95.
18. Fiorentino DF, Zlotnik A, Vieira P, et al. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 1991; 146:3444–51.
19. McGeachy MJ, Bak-Jensen KS, Chen Y, et al. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol* 2007; 8:1390–7.
20. Stumhofer JS, Silver JS, Laurence A, et al. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol* 2007; 8:1363–71.
21. Crawley E, Kon S, Woo P. Hereditary predisposition to low interleukin-10 production in children with extended oligoarthritic juvenile idiopathic arthritis. *Rheumatology (Oxford)* 2001; 40:574–8.
22. Reuss E, Fimmers R, Kruger A, Becker C, Rittner C, Höhler T. Differential regulation of interleukin-10 production by genetic and environmental factors—a twin study. *Genes Immun* 2002; 3:407–13.
23. Oleksyk TK, Thio CL, Truelove AL, et al. Single nucleotide polymorphisms and haplotypes in the IL10 region associated with HCV clearance. *Genes Immun* 2005; 6:347–57.
24. Paladino N, Fainboim H, Theiler G, et al. Gender susceptibility to chronic hepatitis C virus infection associated with interleukin 10 promoter polymorphism. *J Virol* 2006; 80:9144–50.
25. Edwards-Smith CJ, Jonsson JR, Purdie DM, Bansal A, Shorthouse C, Powell EE. Interleukin-10 promoter polymorphism predicts initial response of chronic hepatitis C to interferon alfa. *Hepatology* 1999; 30:526–30.
26. Yee LJ, Tang J, Gibson AW, Kimberly R, Van Leeuwen DJ, Kaslow RA. Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. *Hepatology* 2001; 33:708–12.
27. Knapp S, Hennig BJ, Frodsham AJ, et al. Interleukin-10 promoter polymorphisms and the outcome of hepatitis C virus infection. *Immunogenetics* 2003; 55:362–9.
28. Morgan TR, Lambrecht RW, Bonkovsky HL, et al. DNA polymorphisms and response to treatment in patients with chronic hepatitis C: Results from the HALT-C trial. *J Hepatol* 2008; 49:548–56.
29. Mori N, Imamura M, Kawakami Y, et al. Randomized trial of high-dose interferon-alpha-2b combined with ribavirin in patients with chronic hepatitis C: Correlation between amino acid substitutions in the core/NS5A region and virological response to interferon therapy. *J Med Virol* 2009; 81:640–9.
30. Kurbanov F, Tanaka Y, Matsuura K, et al. Positive selection of core 70Q variant genotype 1b hepatitis C virus strains induced by pegylated interferon and ribavirin. *J Infect Dis* 2010; 201:1663–71.
31. Nakagawa M, Sakamoto N, Ueyama M, et al. Mutations in the interferon sensitivity determining region and virological response to combination therapy with pegylated-interferon alpha 2b plus ribavirin in patients with chronic hepatitis C-1b infection. *J Gastroenterol* 2010; 45:656–65.
32. Hayashi K, Katano Y, Ishigami M, et al. Mutations in the core and NS5A region of hepatitis C virus genotype 1b and correlation with response to pegylated-interferon-alpha 2b and ribavirin combination therapy [published online ahead of print 28 March 2010]. *J Viral Hepat*. doi: 10.1111/j.1365-2893.2010.01305.x.
33. Dolganiuc A, Kodys K, Kopasz A, et al. Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation. *J Immunol* 2003; 170:5615–24.
34. Trinchieri G. Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 1995; 13:251–76.
35. Rossol S, Marinos G, Carucci P, Singer MV, Williams R, Naoumov NV. Interleukin-12 induction of Th1 cytokines is important for viral clearance in chronic hepatitis B. *J Clin Invest* 1997; 99:3025–33.
36. Quiroga JA, Martín J, Navas S, Carreño V. Induction of interleukin-12 production in chronic hepatitis C virus infection correlates with the hepatocellular damage. *J Infect Dis* 1998; 178:247–51.
37. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol* 2001; 19:423–74.
38. Loffreda S, Muratori P, Muratori L, Mele L, Bianchi FB, Lenzi M. Enhanced monocyte Th1 cytokine production in HCV-infected cryoglobulinemic patients. *J Hepatol* 2003; 38:230–6.
39. Schvoerer E, Navas MC, Thumann C, et al. Production of interleukin-18 and interleukin-12 in patients suffering from chronic hepatitis C virus infection before antiviral therapy. *J Med Virol* 2003; 70:588–93.
40. Salcedo X, Medina J, Sanz-Cameno P, et al. The potential of angiogenesis soluble markers in chronic hepatitis C. *Hepatology* 2005; 42:696–701.
41. Cho ML, Jung YO, Moon YM, et al. Interleukin-18 induces the production of vascular endothelial growth factor (VEGF) in rheumatoid arthritis synovial fibroblasts via AP-1-dependent pathways. *Immunol Lett* 2006; 103:159–66.
42. Chao Y, Li CP, Chau GY, et al. Prognostic significance of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin in patients with resectable hepatocellular carcinoma after surgery. *Ann Surg Oncol* 2003; 10:355–62.

Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor

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Hepatitis C virus (HCV) is a leading cause of liver disease worldwide. With ~170 million individuals infected and current interferon-based treatment having toxic side effects and marginal efficacy, more effective antivirals are crucially needed¹. Although HCV protease inhibitors were just approved by the US Food and Drug Administration (FDA), optimal HCV therapy, analogous to HIV therapy, will probably require a combination of antivirals targeting multiple aspects of the viral lifecycle. Viral entry represents a potential multifaceted target for antiviral intervention; however, to date, FDA-approved inhibitors of HCV cell entry are unavailable. Here we show that the cellular Niemann-Pick C1-like 1 (NPC1L1) cholesterol uptake receptor is an HCV entry factor amenable to therapeutic intervention. Specifically, NPC1L1 expression is necessary for HCV infection, as silencing or antibody-mediated blocking of NPC1L1 impairs cell culture-derived HCV (HCVcc) infection initiation. In addition, the clinically available FDA-approved NPC1L1 antagonist ezetimibe^{2,3} potentially blocks HCV uptake *in vitro* via a virion cholesterol-dependent step before virion-cell membrane fusion. Moreover, ezetimibe inhibits infection by all major HCV genotypes *in vitro* and *in vivo* delays the establishment of HCV genotype 1b infection in mice with human liver grafts. Thus, we have not only identified NPC1L1 as an HCV cell entry factor but also discovered a new antiviral target and potential therapeutic agent.

HCV is thought to enter cells via receptor-mediated endocytosis beginning with interaction of the viral particle with a series of cell surface receptors, including the tetraspanin CD81 protein⁴, scavenger receptor class B member I (SR-BI, also known as SCARB1)⁵ and the tight-junction proteins claudin-1 (CLDN1)⁶ and occludin (OCLN)^{7,8}, followed by clathrin-mediated endocytosis and fusion between the virion envelope and the endosomal membrane^{9,10}. Although the specifics of each interaction are not fully understood, it is now recognized that multiple cellular factors, as well as many components of the viral particle, not just the viral glycoproteins, participate in the entry process. For example, the HCVcc particle is associated with cellular lipoproteins (for example, low-density lipoprotein and very-low-density lipoprotein)^{11,12} and is enriched in cholesterol¹³, the latter

of which has been shown to be necessary for HCV cell entry^{13,14}. Apart from the likely function of cholesterol in viral membrane stabilization and organization, the dependence of HCV infectivity on cholesterol led us to reason that other cholesterol-uptake receptors (apart from SR-BI and LDLR), such as NPC1L1, might also have a role in HCV cell entry.

NPC1L1, a 13-transmembrane-domain cell surface cholesterol-sensing receptor (Fig. 1a) expressed on the apical surface of intestinal enterocytes and human hepatocytes, including Huh7 cells (Supplementary Fig. 1), is responsible for cellular cholesterol absorption and whole-body cholesterol homeostasis^{15,16}. Similar to what has been observed for other HCV entry factors⁸, we observed downregulation of NPC1L1 in HCVcc-infected Huh7 cultures. Specifically, as early as day 4 after infection NPC1L1 protein levels were markedly reduced and remained downregulated until the end of the experiment at day 12 after infection (Fig. 1b). Having observed a correlation between NPC1L1 expression and HCV infection, we next determined whether NPC1L1 expression levels affect HCV infection by transfecting Huh7 cells with siRNAs targeting NPC1L1 or the known HCV entry factors CD81 or SR-BI. Compared to cells transfected with an irrelevant control siRNA, CD81-, SR-BI- and NPC1L1-silenced cells were significantly less susceptible to HCVcc infection (Fig. 1c). The inhibition was HCV specific, as silencing of these proteins had no effect on vesicular stomatitis virus G protein-pseudotyped particle (VSVGpp) infection (Supplementary Fig. 2a). Inhibition of HCV also correlated with a reduction in NPC1L1 mRNA and protein and was NPC1L1 specific and not the result of off-target effects (Fig. 1d,e, Supplementary Figs. 3 and 4a,b). Although protein amounts were only marginally lowered by siRNA knockdown, the effect on HCV was considerable, highlighting the sensitivity of HCV to small changes in NPC1L1 levels. Notably, SR-BI mRNA expression has been shown to be reduced by NPC1L1 knockdown in nonhepatic cells¹⁷ and SR-BI is an HCV entry factor⁵, but we found that SR-BI expression was not adversely affected by NPC1L1 silencing in Huh7 cells (Supplementary Fig. 4c,d). Finally, NPC1L1 silencing had no effect on HCV subgenomic RNA replication, full-length infectious HCVcc RNA replication or secretion of *de novo* HCVcc (Supplementary Fig. 5).

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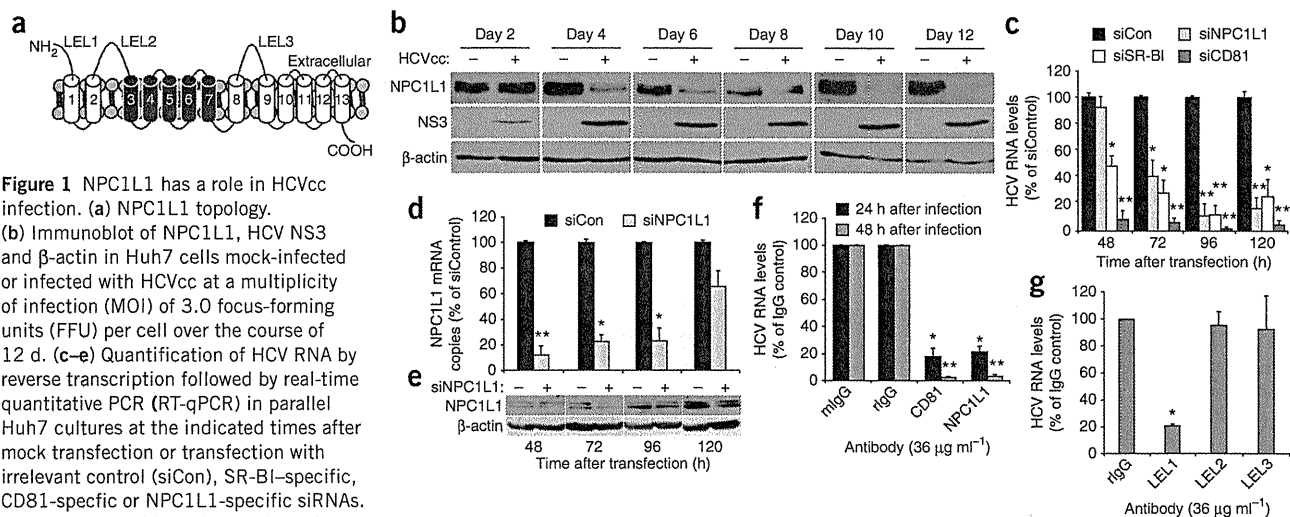


Figure 1 NPC1L1 has a role in HCVcc infection. (a) NPC1L1 topology. (b) Immunoblot of NPC1L1, HCV NS3 and β -actin in Huh7 cells mock-infected or infected with HCVcc at a multiplicity of infection (MOI) of 3.0 focus-forming units (FFU) per cell over the course of 12 d. (c–e) Quantification of HCV RNA by reverse transcription followed by real-time quantitative PCR (RT-qPCR) in parallel Huh7 cultures at the indicated times after mock transfection or transfection with irrelevant control (siCon), SR-BI-specific, CD81-specific or NPC1L1-specific siRNAs. (c) Percentage of infection achieved in siCon-transfected cultures. Data are normalized to GAPDH. (d) NPC1L1 transcript levels quantified by RT-qPCR, normalized to GAPDH and graphed as a percentage of the maximum number of copies determined in siCon-transfected cultures at each time point examined. (e) Immunoblot of NPC1L1 and β -actin protein in siCon-transfected (–) and siNPC1L1-transfected cultures (+). (f,g) Intracellular HCV RNA levels detected in parallel Huh7 cell cultures treated with $36 \mu\text{g ml}^{-1}$ of a mouse (mIgG) or rabbit (rIgG) isotype control antibody, a CD81-specific antibody or antibodies specific for each of the three LELs of NPC1L1 for 1 h before and during HCVcc infection. Shown are HCV RNA levels, determined by RT-qPCR and normalized to GAPDH levels, 24 h (f) or 48 h (f,g) after infection. Results are graphed as a percentage of infection achieved in the respective IgG control-treated cultures. In all cases, significant differences relative to controls (one-way analysis of variance (ANOVA) and Tukey's post-hoc *t* test) are denoted as **P* < 0.05 or ***P* < 0.01. All results are graphed as means \pm s.d. for triplicate samples. The data presented are representative of three independent experiments.

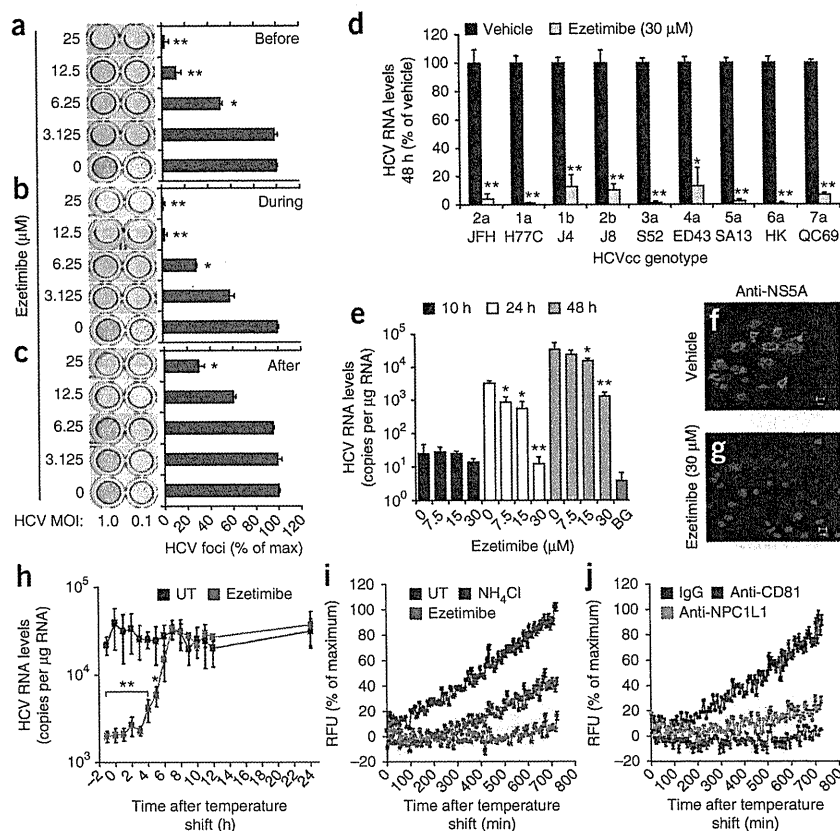
Because siRNA-mediated knockdown of NPC1L1 suggested that HCV infection is inhibited at a step before RNA replication or secretion, we next assessed whether HCV infection was susceptible to inhibition by antibody-mediated blocking of cell surface NPC1L1. Compared to cells treated with irrelevant IgG control antibodies, HCVcc infection, as measured by intracellular HCV RNA levels, was significantly reduced in cells treated with an antibody specific for the known HCV cell entry factor CD81 (Fig. 1f). When we incubated cells with an NPC1L1-specific antibody, HCVcc infection was similarly reduced (Fig. 1f), and the inhibition was HCV specific, as antibody-mediated blocking had no effect on VSVGpp entry (Supplementary Fig. 2b,c). To determine the NPC1L1 domains necessary for HCV entry, we treated cells with antibodies targeting each of the three large extracellular loops (LELs) of NPC1L1 and observed that HCV infection was reduced only when NPC1L1 LEL1, but not LEL2 or LEL3, was blocked (Fig. 1g). Thus, NPC1L1 silencing and antibody-mediated blocking of NPC1L1 LEL1 reduced HCV infection as effectively as targeting other known HCV cell entry factors.

Ezetimibe is a 2-azetidinone-class drug that has been approved by the FDA as a cholesterol-lowering medication¹⁸. As ezetimibe has been shown to be a direct inhibitor of NPC1L1 internalization^{19,20}, we next used this high-affinity, specific pharmacological agent as an alternate means of targeting NPC1L1 before, during or after viral inoculation while additionally evaluating its anti-HCV potential. Specifically, we performed HCVcc foci-reduction assays and quantified foci (that is, clusters of ≥ 5 HCV E2-positive cells) after ezetimibe treatment. Ezetimibe reduced HCVcc foci formation in a dose-dependent manner when present before infection and then removed (Fig. 2a) or only during virus inoculation (Fig. 2b). However, when we added ezetimibe to cells after inoculation (Fig. 2c), the initiation of HCV-positive foci was unaffected, as would be expected for a viral entry inhibitor. Notably, the highest dose of ezetimibe ($25 \mu\text{M}$) reduced

the size of the HCV-positive foci observed from the typical ≥ 5 HCV E2-positive cells to only 1–3 HCV E2-positive cells per focus, which accounts for the lower number of foci with ≥ 5 HCV E2-positive cells being counted in those cultures, suggesting that NPC1L1 may also affect HCV cell-to-cell spread (data not shown). Dose-responsive, time-of-addition-dependent inhibition of HCV infection was also evident when HCV RNA levels were measured (Supplementary Fig. 6). We additionally observed ezetimibe sensitivity across a panel of HCVcc intergenotypic clones containing the structural regions of diverse HCV genotypes (1–7)²¹ (Fig. 2d). Finally, because NPC1L1 and SR-BI are both involved in cellular cholesterol uptake and SR-BI has been reported to be a rate-limiting HCV cell entry factor²², we overexpressed SR-BI before ezetimibe treatment and found that this overexpression did not overcome the dependence of HCV entry on NPC1L1 (Supplementary Fig. 7). Likewise, we confirmed that the potent antiviral effect of ezetimibe was not due to drug-mediated cytotoxicity (Supplementary Figs. 2d,e and 8a), changes in cell proliferation (Supplementary Fig. 8b), reduced expression of the other known HCV cell surface receptors (Supplementary Fig. 8c–g), inhibition of HCV RNA replication (Supplementary Fig. 9a–c) or inhibition of virus secretion (Supplementary Fig. 9d). Hence, the data support the conclusion that direct pharmacological inhibition of NPC1L1 reduces HCV infection by directly inhibiting viral cell entry.

We next assessed whether ezetimibe inhibits HCVcc binding or a post-binding step by examining cell-associated HCV RNA and protein expression from internalized RNA in vehicle- and ezetimibe-treated HCVcc-infected cultures. At 10 h after infection, a time before detectable HCV RNA replication occurs (Supplementary Fig. 10), ezetimibe did not affect cell-bound HCV RNA levels (Fig. 2e). In contrast, at later time points, HCV RNA levels (Fig. 2e) and *de novo* NS5A protein expression (Fig. 2f,g) were reduced in ezetimibe-treated cultures, suggesting HCV can efficiently bind ezetimibe-treated cells, but a post-binding step is prevented. To further test this hypothesis

Figure 2 Ezetimibe-mediated inhibition of NPC1L1 reduces HCV entry at a post-binding, pre-fusion step. (a–c) Quantification of HCV foci observed in Huh7 cultures treated with vehicle or increasing concentrations of ezetimibe for 6 h before infection and then removed (Before) (a), for 12 h coincident with viral inoculation and then removed (During) (b) or after viral inoculation (After) (c). HCV foci are expressed as a percentage of the foci obtained in vehicle-treated (0 μM) cultures \pm s.d. ($n = 3$). MOI, multiplicity of infection. (d,e) Intracellular HCV RNA levels detected in parallel Huh7 cultures treated with vehicle or the indicated concentrations of ezetimibe beginning 1 h before and during infection with HCVcc containing the structural region of the indicated genotypes (d) or HCVcc JFH-1 (e). Shown are HCV RNA levels, determined by RT-qPCR and normalized to GAPDH, at the indicated times after infection. Results are graphed as a percentage of infection in vehicle-treated cultures or as mean HCV RNA copies per μg total cellular RNA \pm s.d. ($n = 3$). Assay background (BG) is the HCV RNA level detected in uninfected samples. (f,g) Indirect immunofluorescence analysis of HCV NS5A in vehicle-treated (f) and ezetimibe-treated (30 μM) (g) cultures 24 h after infection with HCVcc JFH-1. Scale bars, 20 μm . (h) Intracellular HCV RNA levels detected in synchronized infections performed in parallel Huh7 cultures treated with vehicle or ezetimibe (30 μM) at the indicated times. Shown are HCV RNA levels, determined by RT-qPCR and normalized to GAPDH, 30 h after infection. Results are graphed as mean HCV RNA copies per μg total cellular RNA \pm s.d. ($n = 3$). * $P < 0.05$ or ** $P < 0.01$ for HCV relative to vehicle-treated cultures (one-way ANOVA and Tukey's post-hoc t test). (i,j) HCV fusion, as measured by DiD fluorescence dequenching in Huh7 cells treated with vehicle (UT), NH_4Cl (10 mM), ezetimibe (30 μM), IgG control antibody (36 $\mu\text{g ml}^{-1}$), CD81-specific antibody (anti-CD81, 36 $\mu\text{g ml}^{-1}$), or an antibody to NPC1L1 LEL1 (anti-NPC1L1, 36 $\mu\text{g ml}^{-1}$). Results are graphed as a percentage of maximum background-corrected relative fluorescence units (RFU) achieved in vehicle-treated or IgG control-treated cultures. All data presented are representative of three independent experiments.



and determine when during the entry process NPC1L1 functions, we assessed the ability of ezetimibe to block HCVcc infection when added at various times after virus binding at 4 $^{\circ}\text{C}$. Ezetimibe retained inhibitory activity after temperature shift to 37 $^{\circ}\text{C}$ for up to 5 h (half-maximal inhibition at 4 h), confirming that NPC1L1 functions after binding, probably late in viral entry (Fig. 2h).

To determine whether ezetimibe acts before fusion, we developed a fluorescence-based HCVcc fusion assay. Specifically, we labeled HCVcc with the hydrophobic fluorophore DiD²³, which incorporates into biological membranes and, at high concentrations, is self-quenching. Upon fusion of viral and target membranes, the DiD fluorophores diffuse away from each other, causing dequenching and allowing for the progression or inhibition of fusion to be measured in real time (Supplementary Fig. 11). Compared to NH_4Cl , an inhibitor of endosomal acidification⁹, ezetimibe more potently inhibited HCVcc^{DiD} fusion, such that by 12 h after binding we measured only ~10% HCVcc^{DiD} dequenching in ezetimibe-treated cultures as compared to vehicle-treated controls (Fig. 2i). Analogously, antibody-mediated inhibition of both CD81 and NPC1L1 also reduced HCVcc^{DiD} fusion (Fig. 2j), indicating that the inhibition observed in ezetimibe-treated wells (Fig. 2i) was not drug specific. We also observed similar results using HCVcc^{DiD} alternatively purified by iodixanol density gradient centrifugation (Supplementary Fig. 12a,b).

As not all viral membrane-incorporated DiD is self-quenched, DiD can also serve as a fluorescent tag to monitor virions during cell entry²⁴. Taking advantage of this, we performed fluorescence microscopy analysis of HCVcc^{DiD}-infected cultures and noted that although we observed little DiD on the surface of vehicle-treated cells, indicative of successful viral entry and fusion, we observed markedly more DiD on the surface of ezetimibe-treated cells (Supplementary Fig. 12c,d). Together with the DiD-fusion data, this indicates that inhibition of NPC1L1 prevents HCVcc cell entry at or before virion-host cell fusion.

Given that antibody-mediated blocking of only NPC1L1 LEL1 (Fig. 1g) reduced HCVcc infection, LEL1 has been shown to bind cholesterol^{20,25} and infectious HCV particles are enriched in cholesterol^{13,26}, we next investigated whether the dependence of HCV cell entry on NPC1L1 might be related to the cholesterol contained within the HCV virions¹³. To address this hypothesis, we used viruses containing the E1/E2 glycoproteins derived from the HCV JFH-1 consensus clone but that differ in their virion-associated cholesterol content and assessed their relative dependence on NPC1L1. Specifically, we found that lentivirus particles pseudotyped with the JFH-1 HCV glycoproteins (HCVpp) contained 94% less cholesterol than the authentic JFH-1 HCVcc particles (HCVcc^{WT}). In contrast, cell culture-adapted HCVcc^{G451R} produced from a JFH-1 viral clone with a G451R point mutation in the viral E2 glycoprotein (HCVcc^{G451R}) contained ~50% more cholesterol than HCVcc^{WT} (Fig. 3a). These cholesterol profiles are consistent with



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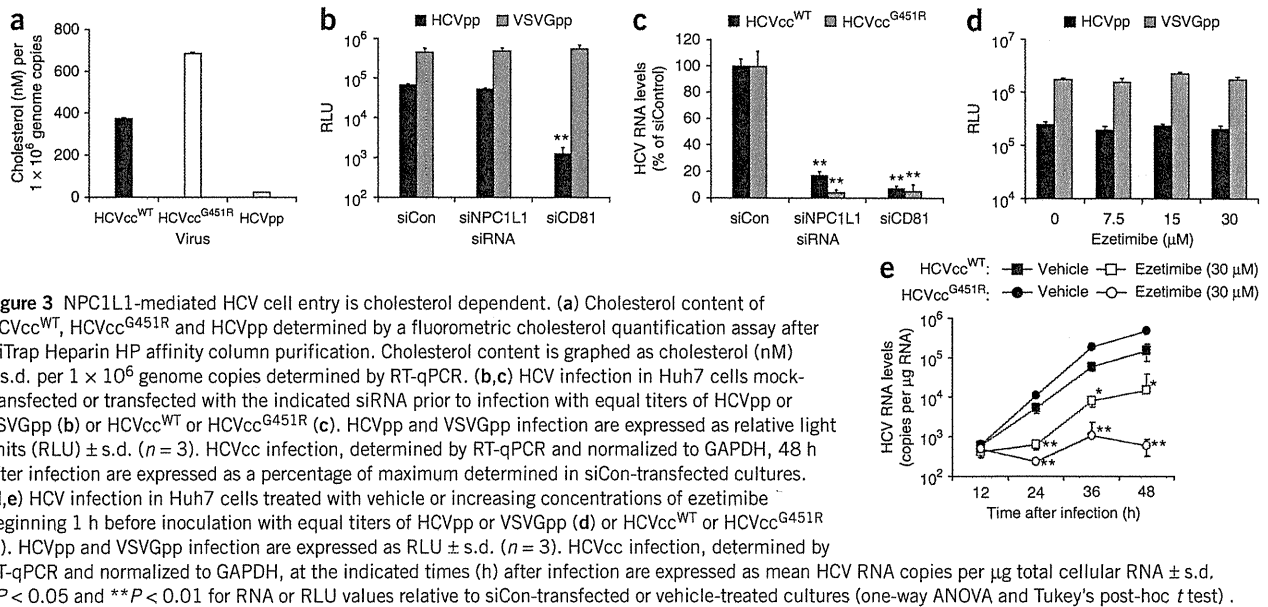


Figure 3 NPC1L1-mediated HCV cell entry is cholesterol dependent. (a) Cholesterol content of HCVcc^{WT}, HCVcc^{G451R} and HCVpp determined by a fluorometric cholesterol quantification assay after HiTrap Heparin HP affinity column purification. Cholesterol content is graphed as cholesterol (nM) \pm s.d. per 1×10^6 genome copies determined by RT-qPCR. (b,c) HCV infection in Huh7 cells mock-transfected or transfected with the indicated siRNA prior to infection with equal titers of HCVpp or VSVGpp (b) or HCVcc^{WT} or HCVcc^{G451R} (c). HCVpp and VSVGpp infection are expressed as relative light units (RLU) \pm s.d. ($n = 3$). HCVcc infection, determined by RT-qPCR and normalized to GAPDH, 48 h after infection are expressed as a percentage of maximum determined in siCon-transfected cultures. (d,e) HCV infection in Huh7 cells treated with vehicle or increasing concentrations of ezetimibe beginning 1 h before inoculation with equal titers of HCVpp or VSVGpp (d) or HCVcc^{WT} or HCVcc^{G451R} (e). HCVpp and VSVGpp infection are expressed as RLU \pm s.d. ($n = 3$). HCVcc infection, determined by RT-qPCR and normalized to GAPDH, at the indicated times (h) after infection are expressed as mean HCV RNA copies per μ g total cellular RNA \pm s.d. * $P < 0.05$ and ** $P < 0.01$ for RNA or RLU values relative to siCon-transfected or vehicle-treated cultures (one-way ANOVA and Tukey's post-hoc t test).

the fact that HCVpp is produced from 293T embryonal kidney cells, which do not produce cholesterol-associated lipoproteins²⁷, and are therefore compositionally distinct from HCVcc, whereas HCVcc^{G451R} has a narrower density range with a higher average mean density than the original JFH-1-derived HCVcc^{WT} (ref. 28). As expected, when CD81 was silenced, both HCVpp (Fig. 3b) and HCVcc^{G451R} (Fig. 3c) cell entry was reduced; however, when NPC1L1 was silenced or inhibited by ezetimibe, the cholesterol-scarce HCVpp showed NPC1L1-independent cell entry and insensitivity to ezetimibe inhibition (Fig. 3b,d). In contrast, the cholesterol-abundant HCVcc^{G451R} showed enhanced NPC1L1-dependent cell entry and hypersensitivity to ezetimibe-mediated inhibition (Fig. 3c,e). Together, these data reveal a correlation between the amount of virion-associated cholesterol and dependence on NPC1L1 for HCV cell entry.

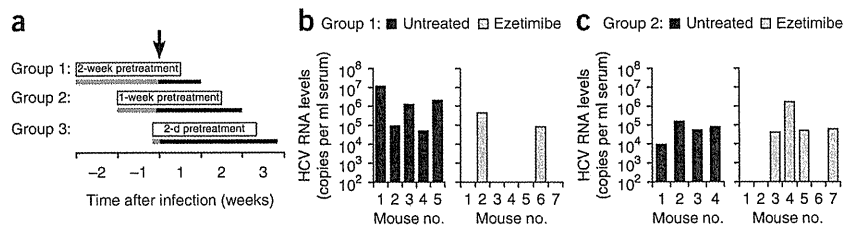
Finally, to assess the involvement of NPC1L1 in HCV cell entry *in vivo*, we evaluated the ability of ezetimibe to inhibit infection of a genotype 1 clinical isolate in a hepatic xenorepopulation model of acute HCV infection²⁹. Specifically, we repopulated urokinase-type plasminogen activator-severe combined immunodeficiency (uPA-SCID) mice with human hepatocytes and treated them via oral gavage with ezetimibe (10 mg per kg body weight per day) or diluent alone for a total of 3 weeks, with treatment beginning 2 weeks, 1 week or 2 d before challenge with HCV genotype 1b positive serum (Fig. 4a).

Ezetimibe treatment delayed the establishment of HCV infection in mice pretreated for 2 weeks before infection (Fig. 4b, $P = 0.0192$), confirming the ability of this drug to inhibit HCV infection *in vivo*. However, when mice were pretreated for only 1 week before infection, ezetimibe was less effective at delaying infection ($P = 0.062$), and it was completely ineffective when treatment was initiated only 2 d before challenge or after infection had been established (data not shown). Specifically, 100% of the nine control diluent-treated mice were serum positive for HCV 1 week after challenge, whereas 71% (five out of seven) and 43% (three out of seven) of mice treated with ezetimibe for 2 weeks and 1 week before infection were HCV negative, respectively (Fig. 4b,c). Although the majority of ezetimibe-treated mice eventually became HCV positive, of the five mice in the 2-week ezetimibe pretreatment group that were HCV negative at week 1, two were completely protected, remaining HCV negative at weeks 2 and 3 after infection (and one mouse died during gavage) (Supplementary Fig. 13). Thus, similar to what was recently reported for another potential HCV entry inhibitor, erlotinib³⁰, ezetimibe was able to delay initial infection *in vivo*. Notably, since NPC1L1 is highly expressed on the apical surface of intestinal enterocytes^{15,16}, a considerable amount of orally administered ezetimibe initially binds to these cells following oral administration³¹. Thus it is plausible that development of alternate non-oral delivery or drug-targeting methods

Figure 4 Ezetimibe delays the establishment of HCV infection in hepatic xenorepopulated mice.

(a) Schematic diagram of an experiment in which uPA-SCID mice transplanted with human hepatocytes³⁵ were pretreated with diluent alone ($n = 4$ or 5) or ezetimibe (10 mg per kg body weight per day) ($n = 7$), via oral gavage, starting 2 weeks, 1 week or 2 d before infection (indicated by gray bars). The arrow indicates when the mice were intravenously inoculated

on day 0 with HCV human serum containing 1.0×10^5 genome copies of HCV genotype 1b and the black bars indicate the time period post-HCV inoculation that treatments were continued. (b,c) HCV RNA levels (genome copies per milliliter of serum) 1 week after infection from mice pretreated for 2 weeks (b) or 1 week (c). The lower limit of HCV RNA detection is equal to 100 genomic copies per milliliter of serum. A two-tailed Fisher's exact test was performed to compare categorical variables. In all cases, $P < 0.05$ was used to reject the null hypothesis that the distribution of HCV-positive and HCV-negative mice between ezetimibe-treated and nine diluent-treated mice at specific weeks after infection were the same.



might improve transport of ezetimibe to hepatocytes and increase its anti-HCV efficacy. Nevertheless, our finding that ezetimibe can delay the establishment of HCV genotype 1 infection in mice confirms the involvement of NPC1L1 in HCV infection *in vivo* and highlights the therapeutic potential of further pursuing the refinement or development of anti-NPC1L1 therapies³² for the treatment of HCV.

Here we have shown that NPC1L1 is an HCV cell entry factor that functions after binding, at or before fusion. These findings, together with the facts that NPC1L1 is a cellular cholesterol receptor, the HCV particle is enriched in cholesterol, and relative dependence on NPC1L1 is correlated with HCV particle cholesterol levels, support and expand upon previous reports suggesting that virion cholesterol is involved in HCV cell entry^{13,14,26}. Whether NPC1L1 directly interacts with HCV or indirectly participates in HCV entry by removing virion-associated cholesterol to perhaps reveal protected viral glycoprotein binding sites or confer a required conformational change remains to be determined. Lastly, as NPC1L1 is expressed only on human and primate hepatocytes^{33,34}, this discovery additionally highlights NPC1L1 as a potential HCV tropism determinant, which may facilitate the future development of a small-animal model of HCV infection.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

B.S. made the initial discovery. B.S. and S.L.U. designed the project, analyzed the results and wrote the manuscript. B.S., N.B., D.N.M., S.H., K.A.M. and X.Y. performed experimental work. B.S., S.L.U., M.I. and K.C. designed the hepatic xenorepopulation mouse experiments, and N.H. performed the *in vivo* studies. W.A.A. was involved in the initial conception of the project and provided valuable expertise.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturemedicine/>.

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- Uprichard, S.L. Hepatitis C virus experimental model systems and antiviral drug research. *Viral. Sin.* **25**, 227–245 (2010).
- Gupta, E.K. & Ito, M.K. Ezetimibe: the first in a novel class of selective cholesterol-uptake inhibitors. *Heart Dis.* **4**, 399–409 (2002).
- Garcia-Calvo, M. *et al.* The target of ezetimibe is Niemann-Pick C1-like 1 (NPC1L1). *Proc. Natl. Acad. Sci. USA* **102**, 8132–8137 (2005).
- Pileri, P. *et al.* Binding of hepatitis C virus to CD81. *Science* **282**, 938–941 (1998).
- Scarselli, E. *et al.* The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J.* **21**, 5017–5025 (2002).
- Evans, M.J. *et al.* Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**, 801–805 (2007).
- Ploss, A. *et al.* Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* **457**, 882–886 (2009).
- Liu, S. *et al.* Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. *J. Virol.* **83**, 2011–2014 (2009).
- Tscherne, D.M. *et al.* Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. *J. Virol.* **80**, 1734–1741 (2006).
- Meertens, L., Bertaux, C. & Dragic, T. Hepatitis C virus entry requires a critical postinternalization step and delivery to early endosomes via clathrin-coated vesicles. *J. Virol.* **80**, 11571–11578 (2006).
- Huang, H. *et al.* Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. *Proc. Natl. Acad. Sci. USA* **104**, 5848–5853 (2007).
- Gastaminza, P. *et al.* Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. *J. Virol.* **82**, 2120–2129 (2008).
- Atzaki, H. *et al.* Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. *J. Virol.* **82**, 5715–5724 (2008).
- Kapadia, S.B., Barth, H., Baumert, T., McKeating, J.A. & Chisari, F.V. Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. *J. Virol.* **81**, 374–383 (2007).
- Yu, L. The structure and function of Niemann-Pick C1-like 1 protein. *Curr. Opin. Lipidol.* **19**, 263–269 (2008).
- Altmann, S.W. *et al.* Niemann-Pick C1 like 1 protein is critical for intestinal cholesterol absorption. *Science* **303**, 1201–1204 (2004).
- Sané, A.T. *et al.* Localization and role of NPC1L1 in cholesterol absorption in human intestine. *J. Lipid Res.* **47**, 2112–2120 (2006).
- Bays, H.E., Neff, D., Tomassini, J.E. & Terhakovec, A.M. Ezetimibe: cholesterol lowering and beyond. *Expert Rev. Cardiovasc. Ther.* **6**, 447–470 (2008).
- Chang, T.Y. & Chang, C. Ezetimibe blocks internalization of the NPC1L1/cholesterol complex. *Cell Metab.* **7**, 469–471 (2008).
- Weinglass, A.B. *et al.* Extracellular loop C of NPC1L1 is important for binding to ezetimibe. *Proc. Natl. Acad. Sci. USA* **105**, 11140–11145 (2008).
- Gottwein, J.M. *et al.* Development and characterization of hepatitis C virus genotype 1–7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. *Hepatology* **49**, 364–377 (2009).
- Grove, J. *et al.* Scavenger receptor BI and BII expression levels modulate hepatitis C virus infectivity. *J. Virol.* **81**, 3162–3169 (2007).
- Zaitseva, E., Yang, S.T., Melikov, K., Pourmal, S. & Chernomordik, L.V. Dengue virus ensures its fusion in late endosomes using compartment-specific lipids. *PLoS Pathog.* **6**, e1001131 (2010).
- Coller, K.E. *et al.* RNA interference and single particle tracking analysis of hepatitis C virus endocytosis. *PLoS Pathog.* **5**, e1000702 (2009).
- Zhang, J.H. *et al.* The N-terminal domain of NPC1L1 protein binds cholesterol and plays essential roles in cholesterol uptake. *J. Biol. Chem.* **286**, 25088–25097 (2011).
- Yamamoto, M. *et al.* Structural requirements of virion-associated cholesterol for infectivity, buoyant density and apolipoprotein association of hepatitis C virus. *J. Gen. Virol.* **92**, 2082–2087 (2011).
- Farquhar, M.J. & McKeating, J.A. Primary hepatocytes as targets for hepatitis C virus replication. *J. Viral Hepat.* **15**, 849–854 (2008).
- Zhong, J. *et al.* Persistent hepatitis C virus infection *in vitro*: coevolution of virus and host. *J. Virol.* **80**, 11082–11093 (2006).
- Kneteman, N.M. & Toso, C. *In vivo* study of HCV in mice with chimeric human livers. *Methods Mol. Biol.* **510**, 383–399 (2009).
- Lupberger, J. *et al.* EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nat. Med.* **17**, 589–595 (2011).
- Sweeney, M.E. & Johnson, R.R. Ezetimibe: an update on the mechanism of action, pharmacokinetics and recent clinical trials. *Expert Opin. Drug Metab. Toxicol.* **3**, 441–450 (2007).
- Bettlers, J.L. & Yu, L. Transporters as drug targets: discovery and development of NPC1L1 inhibitors. *Clin. Pharmacol. Ther.* **87**, 117–121 (2010).
- Davies, J.P., Scott, C., Oishi, K., Liapis, A. & Ioannou, Y.A. Inactivation of NPC1L1 causes multiple lipid transport defects and protects against diet-induced hypercholesterolemia. *J. Biol. Chem.* **280**, 12710–12720 (2005).
- Davis, H.R. Jr. *et al.* Niemann-Pick C1 like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. *J. Biol. Chem.* **279**, 33586–33592 (2004).
- Matsumura, T. *et al.* Amphipathic DNA polymers inhibit hepatitis C virus infection by blocking viral entry. *Gastroenterology* **137**, 673–681 (2009).





ONLINE METHODS

HCVcc. Plasmids containing the full-length HCV JFH-1 genome (pJFH1)³⁶, full-length HCV JFH-1 genome with a G451R mutation in the E2 glycoprotein (pJFH-1^{G451R})³⁷ and the eight intergenotypic clones (described in ref. 21) were XbaI linearized and transcribed using MEGAscript T7 (Ambion), and 10 µg *in vitro*-transcribed RNA was electroporated (BioRad) into Huh7 cells³⁸. We generated HCVcc viral stocks by infecting naive Huh7 cells at an MOI of 0.01 FFU per cell with medium from Huh7 cells electroporated with *in vitro*-transcribed RNA from pJFH-1-based vectors, as previously described³⁸.

Treatments and analysis. Huh7 cultures were established as previously described³⁸. We performed RNA silencing experiments by reverse transfection (Lipofectamine RNAiMAX, Invitrogen) of siRNAs into Huh7 cells. Transfected cells were infected with equal titers of HCVpp or VSVGpp or HCVcc at an MOI of 0.05 FFU per cell at the indicated times after transfection (Fig. 1c–e). For antibody experiments, we treated cells with 36 µg ml⁻¹ of antibody before and during infection with HCVcc at an MOI of 0.05 FFU per cell. For ezetimibe inhibition experiments, cells were vehicle-treated or treated with increasing concentrations of ezetimibe (Sequoia Research Products) before infection, during the time of virus inoculation and/or after virus inoculation with HCVcc at an MOI of 0.1–1.0 FFU per cell. The ezetimibe concentrations of 3.125–30 µM (that is, 1.5–12.28 µg per ml culture medium) used in this study are consistent with previously published reports^{19,20,39} and are additionally in line with patient daily intake concentrations of 10 mg d⁻¹ (that is, 2.0–3.3 µg per ml of serum). For RT-qPCR analysis, we isolated total cellular RNA from triplicate culture wells after infection or transfection. For HCV E2-positive foci analysis, we fixed infected cells with 4% (wt/vol) paraformaldehyde 72 h after infection, and immunocytochemical staining for HCV E2 was performed. See **Supplementary Methods** for further details.

HCV infection in chimeric mice. All mouse studies were conducted with protocols approved by the Ethics Review Committee for Animal Experimentation

of the Graduate School of Biomedical Sciences, Hiroshima University. Male uPA-SCID mice transplanted with human hepatocytes (BD Biosciences³⁵) were purchased from PhenixBio⁴⁰. Mice were treated daily with 10 mg per kg body weight ezetimibe via oral gavage of a 0.02 mg ml⁻¹ solution of ezetimibe resuspended in corn oil (100 µl 20g⁻¹) for a total of 3 weeks, with treatment initiation beginning 2 weeks, 1 week or 2 d before infection¹⁶. Control mice were treated via oral gavage with corn oil alone (100 µl per 20 g body weight). A total of four to seven mice were included in each group. On day 0, we intravenously inoculated mice with serum from HCV-infected humans containing 1.0 × 10⁵ copies of HCV genotype 1b. Mouse serum samples were obtained for HCV RNA or human albumin determination by RT-qPCR and Alb-II Kit (Eiken Chemical), respectively.

Statistical analyses. Data are presented as the means ± s.d. We determined significant differences by one-way ANOVA followed by Tukey's post-hoc *t* test (GraphPad Prism software). To compare categorical variables we used a two-tailed Fisher's exact test (SPSS). In all cases, a *P* value <0.05 was considered statistically significant.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

36. Wakita, T. *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* **11**, 791–796 (2005).
37. Sabahi, A. *et al.* The rate of hepatitis C virus infection initiation *in vitro* is directly related to particle density. *Virology* **407**, 110–119 (2010).
38. Yu, X. & Uprichard, S.L. Cell-based hepatitis C virus infection fluorescence resonance energy transfer (FRET) assay for antiviral compound screening. *Curr. Protoc. Microbiol.* **18**, 17.5.1–17.5.27 (2010).
39. Ge, L. *et al.* The cholesterol absorption inhibitor ezetimibe acts by blocking the sterol-induced internalization of NPC1L1. *Cell Metab.* **7**, 508–519 (2008).
40. Tateno, C. *et al.* Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am. J. Pathol.* **165**, 901–912 (2004).

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