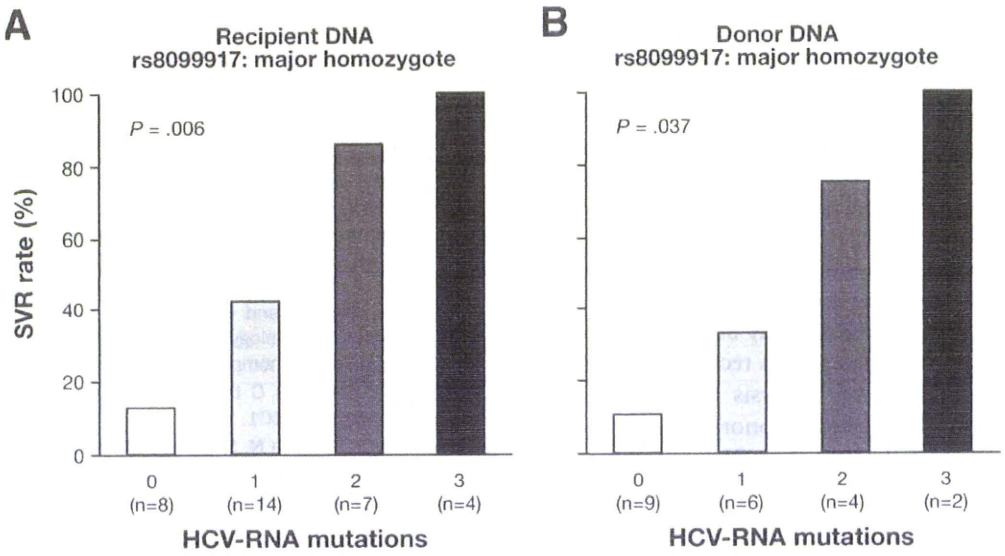

Figure 5. Combined genetic analyses of hepatitis C virus (HCV)-RNA mutations in the Core and nonstructural protein 5A regions for the prediction of sustained viral response (*SVR*) in the recipients carrying the major allele homozygote who received liver transplants from donors carrying the same allele. The interferon sensitivity score was calculated as the total number of cases positive for the following viral factors: double-wild (*DW*) at aa70 and 91, interferon sensitivity-determining region (*ISDR*) ≥2, and interferon/ribavirin resistance-determining region (*IRRDR*) ≥6. The achievement percentage of SVR among the 4 groups is indicated.

in patients predicts that they will be NR to IFN therapy,[15,27,28] and a significant association between the presence of genetic variations in IL28B and the response to PEG-IFN/RBV therapy has been reported in genome-wide association studies.[16-21] In these studies, the expression level of IL28 mRNA in the PBMCs of patients carrying the minor heterozygous or homozygous allele was significantly lower.[18,19] Other studies have shown that IL28B transduces signals through the receptor complexes in a manner different from other type I IFNs, but uses the common Janus activating kinase-signal transducer and activation of transcription pathway to induce ISGs.[29,30] The discrepancies between the impaired transcription of IL28B due to genetic variations and the increased expression of ISGs suggest the participation of other factors in determining the efficacy of IFN therapy.

Although IFN-centered antiviral therapy is significantly associated with post-transplantation graft prognosis in patients infected with HCV,[7] the efficacy of the IFN therapy after OLT is unsatisfactory[8] and the treatment is frequently accompanied by severe side effects.[9] Therefore, in addition to the development of an optimal therapeutic regimen for HCV infection after OLT, establishment of a reliable marker or set of markers to predict the sensitivity to IFN therapy is needed. We have previously reported that viral RNA mutations in the Core and NS5A regions are significantly associated with IFN sensitivity after OLT.[14] In addition, the current study revealed that IL28B genetic variation in both recipients and donors is also associated with IFN sensitivity after OLT. Although the sensitivity and specificity of genetic variations of IL28B for predicting the achievement of SVR have been reported to be high in chronic hepatitis C patients,[16,18,19] the current analysis revealed that the specificity was lower in cases of recurrent hepatitis C after OLT than in chronic hepatitis C patients. By using a combination of genetic analyses, the efficacy of the post-transplantation PEG-IFN/RBV therapy might be predicted before OLT. Large-scale prospective analyses of the association between IFN sensitivity after OLT and genetic variations in both IL28B and HCV will be needed in future studies. In addition, the molecular mechanism underlying the association between IFN sensitivity and genetic variation of IL28B and HCV should be clarified. Furthermore, it might be feasible to predict the IFN sensitivity based on the genetic analyses of viral and host factors, thereby allowing for the individualization of antiviral therapy, including dose-escalated IFN therapy,[31,32] simultaneous splenectomy for pancytopenia,[32,33] and the use of new antivirals such as proteases inhibitors.[34] Further clinical investigation is needed to improve the post-transplantation antiviral therapy for recurrent HCV after OLT.

Previous reports have demonstrated an association between the clinical tolerance of the graft and tissue chimerism, including hepatocytes.[35-37] In the present study, to determine the correlation between the tissue chimerism in the transplanted liver and the impact of IL28B genetic variation in both the recipients and donors, short tandem repeat analysis and evaluation of minor allele frequency in the transplanted liver were performed (Supplementary Figure 2). Although liver tissue chimerism after OLT was demonstrated using short tandem repeat analysis and cloning in this study, we could not deter-

mine the cell types present and whether the cells were made up of hepatocytes, endothelial cells, or infiltrating lymphocytes due to the limited sample volumes. However, the coexistence of cells derived from donors and recipients in the local environment supports the notion that genetic variation of IL28B in both donors and recipients participates in the determination of IFN sensitivity after OLT.

In conclusion, the combination of genetic analysis of IL28B in both the recipient and donor, rather than either alone, together with HCV-RNA may be a reliable predictor of IFN efficacy in patients with recurrent hepatitis C after OLT. In addition, this analysis may also make it possible to select the optimal donors exhibiting high sensitivity to the IFN therapy after OLT.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.07.058.

References

1. Hoofnagle JH. Course and outcome of hepatitis C. Hepatology 2002;36(Suppl):S21–S19.
2. Seeff LB. Natural history of chronic hepatitis C. Hepatology 2002; 36(Suppl):S35–S46.
3. Brown RS. Hepatitis C and liver transplantation. Nature 2005; 436:973–978.
4. Marubashi S, Dono K, Miyamoto A, et al. Liver transplantation for hepatitis C. J Hepatobiliary Pancreat Surg 2006;13:382–392.
5. Forman LM, Lewis JD, Berlin JA, et al. The association between hepatitis C infection and survival after orthotopic liver transplantation. Gastroenterology 2002;122:889–896.
6. Moonka DK, Kim D, Kapke A, et al. The influence of induction therapy on graft and patient survival in patients with and without hepatitis C after liver transplantation. Am J Transplant 2010;10: 590–601.
7. Veldt BJ, Poterucha JJ, Watt KD, et al. Impact of pegylated interferon and ribavirin treatment on graft survival in liver transplant patients with recurrent hepatitis C infection. Am J Transplant 2008;8:2426–2433.
8. Carrion JA, Navasa M, Garcia-Retortillo M, et al. Efficacy of antiviral therapy on hepatitis C recurrence after liver transplantation: a randomized controlled study. Gastroenterology 2007; 132:1746–1756.
9. Berardi S, Lodato F, Gramenzi A, et al. High incidence of allograft dysfunction in liver transplanted patients treated with pegylated-interferon alpha-2b and ribavirin for hepatitis C recurrence: possible de novo autoimmune hepatitis? Gut 2007;56:237–242.
10. Salomon JA, Weinstein MC, Hammitt JK, et al. Cost-effectiveness of treatment for chronic hepatitis C infection in an evolving patient population. JAMA 2003;290:228–237.
11. Akuta N, Suzuki F, Kawamura Y, et al. Predictors of viral kinetics to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b. J Med Virol 2007;79:1686–1695.
12. 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.
13. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 2008;48:38–47.
14. Fukuhara T, Taketomi A, Okano S, et al. Mutations in hepatitis C virus genotype 1b and the sensitivity of interferon-ribavirin therapy after liver transplantation. J Hepatol 2010;52:672–680.
15. Asahina Y, Izumi N, Hirayama I, et al. Potential relevance of cytoplasmic viral sensors and related regulators involving innate immunity in antiviral response. Gastroenterology 2008;134:1396–1405.
16. Montes-Cano MA, Garcia-Lozano JR, Abad-Molina C, et al. IL28B genetic variants and hepatitis virus infection by different viral genotypes. Hepatology 2010;52:33–37.
17. Ge D, Fellay J, Thompson AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399–401.
18. 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.
19. 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.
20. 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, 1345.e1–7.
21. McCarthy JJ, Li JH, Thompson A, et al. Replicated association between an IL28B gene variant and a sustained response to pegylated interferon and ribavirin. Gastroenterology 2010;138: 2307–2314.
22. Soejima Y, Taketomi A, Yoshizumi T, et al. Feasibility of left lobe living donor liver transplantation between adults: an 8-year, single-center experience of 107 cases. Am J Transplant 2006;6: 1004–1011.
23. 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.
24. Maekawa S, Enomoto N. Viral factors influencing the response to the combination therapy of peginterferon plus ribavirin in chronic hepatitis C. J Gastroenterol 2009;44:1009–1015.
25. Pascu M, Martus P, Hohne M, et al. Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences. Gut 2004;53:1345–1351.
26. Sarrazin C, Herrmann E, Bruch K, et al. Hepatitis C virus nonstructural 5A protein and interferon resistance: a new model for testing the reliability of mutational analyses. J Virol 2002;76: 11079–11090.
27. Chen L, Borozan I, Feld J, et al. Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. Gastroenterology 2005;128:1437–1444.
28. Sarasin-Filipowicz M, Oakeley EJ, Duong FH, et al. Interferon signaling and treatment outcome in chronic hepatitis C. Proc Natl Acad Sci U S A 2008;105:7034–7039.
29. Kotenko SV, Gallagher G, Baurin VV, et al. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol 2003;4:69–77.
30. Sheppard P, Kindsvogel W, Xu W, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 2003;4:63–68.
31. Zimmermann T, Bocher WO, Biesterfeld S, et al. Efficacy of an escalating dose regimen of pegylated interferon alpha-2a plus ribavirin in the early phase of HCV reinfection after liver transplantation. Transpl Int 2007;20:583–590.
32. Ikegami T, Taketomi A, Soejima Y, et al. The benefits of interferon treatment in patients without sustained viral response after living

donor liver transplantation for hepatitis C. Transplant Proc 2009;41:4246–4252.
33. Yoshizumi T, Taketomi A, Soejima Y, et al. The beneficial role of simultaneous splenectomy in living donor liver transplantation in patients with small-for-size graft. Transpl Int 2008;21:833–842.
34. Pawlotsky JM, Chevaliez S, McHutchison JG. The hepatitis C virus life cycle as a target for new antiviral therapies. Gastroenterology 2007;132:1979–1998.
35. Kleeberger W, Rothamel T, Glockner S, et al. High frequency of epithelial chimerism in liver transplants demonstrated by microdissection and STR-analysis. Hepatology 2002;35:110–116.
36. Hove WR, van Hoek B, Bajema IM, et al. Extensive chimerism in liver transplants: vascular endothelium, bile duct epithelium, and hepatocytes. Liver Transpl 2003;9:552–556.
37. Ng IO, Chan KL, Shek WH, et al. High frequency of chimerism in transplanted livers. Hepatology 2003;38:989–998.

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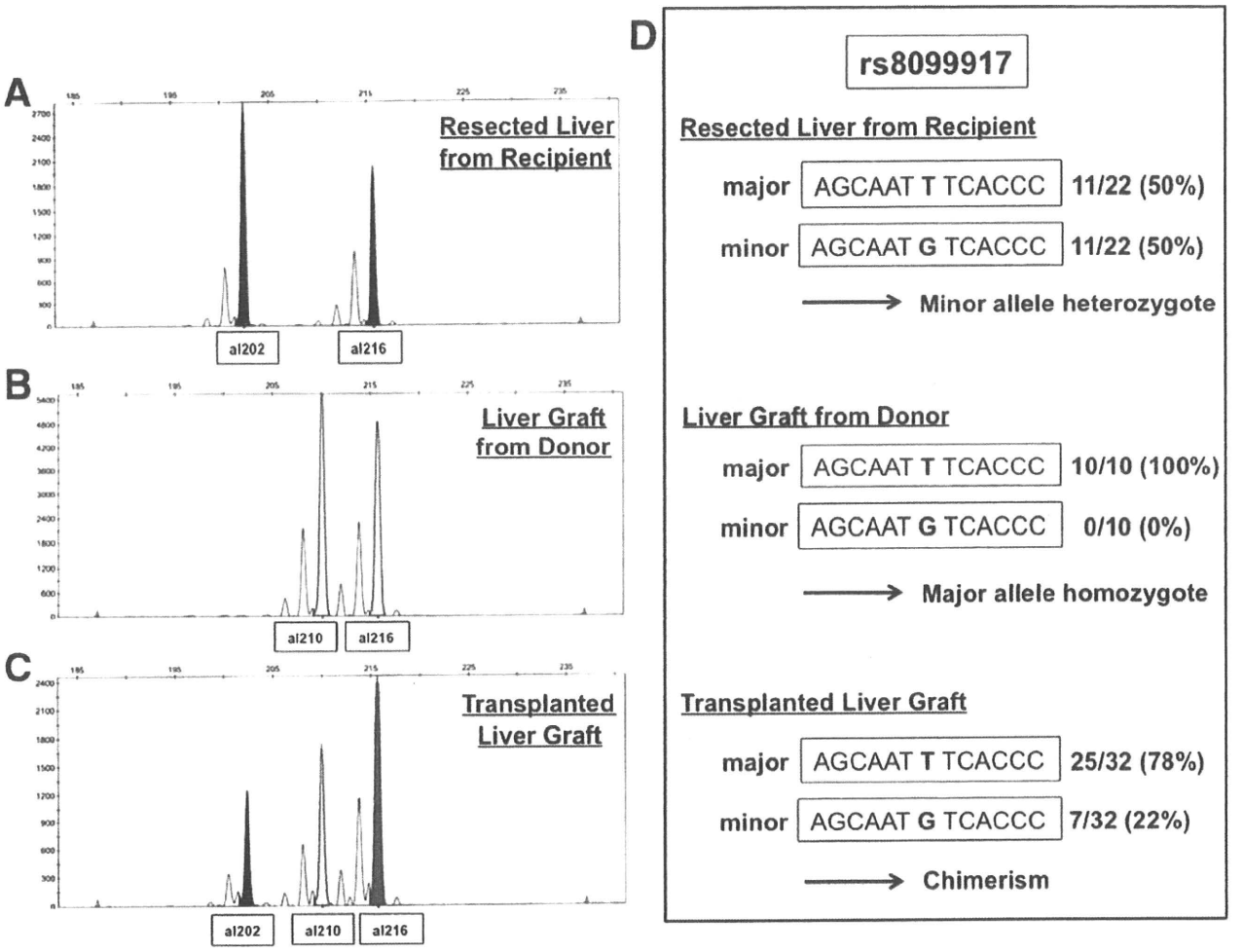
Conflicts of interest

The authors disclose no conflicts.

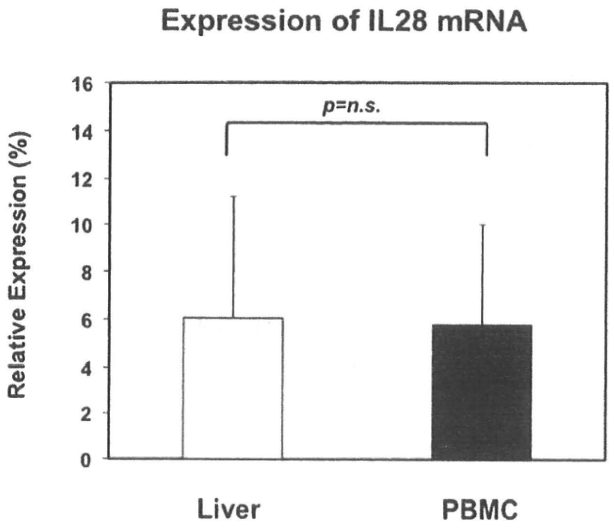
Supplementary Table 1. The Match Rate of Genetic Variation Between rs8099917 and rs12980275

	rs8099917		
	SNP(+), n (%)	SNP(−), n (%)	n (%)
rs12980275			
SNP(+)	26 (24)	3 (2.8)	29 (27)
SNP(−)	4 (3.7)	74 (69)	78 (73)
	30 (28)	77 (72)	108

Concordance rate: 93.5%.



Supplementary Figure 2. To determine the tissue chimerism following orthotopic liver transplantation (OLT), genotyping by short tandem repeat analysis (microsatellite analysis) of DNA samples extracted from the resected liver derived from the recipient, donated liver derived from the donor, and biopsied liver after OLT were performed in 15 cases. Five loci in 5 chromosomes were examined for each sample (D2S123, D5S107, D10S197, D11S904, and D13S175). Polymerase chain reaction products for the loci were analyzed with an Applied Biosystems 3130 genetic analyzer using Genemapper software (Applied Biosystems). To analyze the minor allele-positive rate in the transplanted liver after OLT in the case of the combination of minor allele heterozygotes in the recipient and the major allele homozygote in donor, cloning and sequencing of rs8099917 was performed. Extraction of DNA was performed on the resected liver derived from the recipient, donated liver derived from the donor, and biopsied liver after OLT. The products were cloned by the pT7Blue T-Vector (Takara Bio Inc). Short tandem repeat analyses (microsatellite analyses) at the D2S204 locus of the resected livers from recipients (*A*), the liver grafts from donors (*B*), and the transplanted livers (*C*). The *black* and *gray peaks* correspond to the alleles of recipients and donors, respectively. (*D*) A case of OLT for a recipient who carried the minor allele heterozygote who received the transplant from a donor who had the major allele homozygote.



Supplementary Figure 1. Expression of IL28B messenger RNA (*mRNA*) in livers (*white*) and the peripheral blood mononuclear cells (*PBMC*) (*black*). The statistical analysis was performed using Student's *t* test.

Research Article

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Peripheral B Cells May Serve as a Reservoir for Persistent Hepatitis C Virus Infection

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Key Words

Hepatitis C virus · B cells · Retinoic acid-inducible gene-I · Interferon promoter-stimulator-1 · Interferon regulatory factor-3 · Interferon β

Abstract

A recent study by our group indicated that peripheral B cells in chronic hepatitis C (CHC) patients are infected with hepatitis C virus (HCV). This raised the logical question of how HCV circumvents the antiviral immune responses of B cells. Because type I interferon (IFN) plays a critical role in the innate antiviral immune response, IFNβ expression levels in peripheral B cells from CHC patients were analyzed, and these levels were found to be comparable to those in normal B cells, which suggested that HCV infection failed to trigger antiviral immune responses in B cells. Sensing mechanisms for invading viruses in host immune cells involve Toll-like receptor-mediated and retinoic acid-inducible gene-I (RIG-I)-mediated pathways. Both pathways culminate in IFN regulatory factor-3 (IRF-3) translocation into the nucleus for IFNβ gene transcription. Although the expression levels of RIG-I and its adaptor molecule, IFN promoter-stimulator-1, were substantially enhanced in CHC B cells, dimerization and subsequent nuclear translocation of IRF-3 were not detectable. TANK-binding kinase-1 (TBK1) and IκB kinase ε (IKKε) are essential for IRF-3 phosphorylation. Constitutive expression of both kinases was markedly enhanced in CHC B cells. However, reduced expression of heat shock protein of 90 kDa, a TBK1 stabilizer, and enhanced expression of SIKE, an IKKε suppressor, were observed in CHC B cells, which might suppress the kinase activity of TBK1/IKKε for IRF-3 phosphorylation. In addition, the expression of vesicle-associated membrane protein-associated protein-C, a putative inhibitor of HCV replication, was negligible in B cells. These results strongly suggest that HCV utilizes B cells as a reservoir for persistent infection.

Introduction

Hepatitis C virus (HCV) is an enveloped positive-stranded RNA virus that belongs to the *Flaviviridae* family [1]. It is responsible for public health problems worldwide and affects nearly 200 million people [2]. The liver is regarded as the primary target of HCV infection; however, HCV infection is also associated with B cell lymphoproliferative disorders such as mixed cryoglobulinemia and B cell non-Hodgkin lymphoma [3, 4]. In fact, epidemiological evidence suggests a close link between chronic HCV infection and B cell non-Hodgkin lymphoma [5,

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6]. A pathogenic role for HCV in B cell disorders has been further demonstrated by reports showing clinical resolution of the above-mentioned B cell disorders after successful anti-HCV treatment with interferons (IFNs) [3, 7]. Based on this circumstantial evidence, a possible role for B cells in HCV pathogenesis has been postulated, although this has not been conclusively demonstrated.

A body of evidence suggests that HCV RNA replication occurs in a variety of extrahepatic cells, including peripheral dendritic cells, monocytes and macrophages [8–10]. It has also been suggested that HCV preferentially infects B cells that express CD81, a putative HCV receptor molecule [11–14]. A recent study by our group verified that peripheral CD19+ B cells in chronic hepatitis C (CHC) patients were infected with HCV, which suggested a new viral reservoir during the course of natural HCV infection in humans [15]. Thus, we assume that HCV has an escape strategy for persistent infection of B cells.

Foy et al. [16] found that nonstructural (NS) HCV proteins could inhibit the activation of early signaling pathways, such as Toll-like receptor 3 (TLR3)- and retinoic acid-inducible gene-I (RIG-I)-mediated pathways, which lead to IFNβ production. These results indicated that HCV NS3/4A serine protease blocked IFN regulatory factor-3 (IRF-3) activation upon HCV infection in the human hepatoma cell line HuH-7. Subsequent studies have shown that NS3/4A blocks IFN promoter-stimulator-1 (IPS-1)-mediated signaling pathways by cleaving the IPS-1 molecule and impeding downstream IRF-3 activation [17]. Thus, HCV apparently has a strategy to evade host innate immunity. However, recent studies by Dansako et al. [18, 19] found that the effects of HCV NS3/4A protease on IFN production depended on the cell lines used, because a non-neoplastic human hepatocyte cell line, PH5CH8 [20], retained both TLR3- and RIG-I-mediated pathways, in contrast to HuH-7 cells, which lack the former pathway [21]. However, no studies have examined the effects of HCV infection on IFN responses of nonhepatic cell lines.

In this study, we aimed to understand the mechanisms by which HCV evades innate immune responses in CHC B cells. We found that the antiviral immune response, represented by IFNβ induction, was severely impaired in B cells of CHC patients. Our results strongly suggest that the IRF-3 activation cascade is impeded in B cells upon HCV infection. Thus, IFNβ gene transcription is not augmented, which may result in failed IFNβ-inducible antiviral responses in CHC B cells. Furthermore, the expression of vesicle-associated membrane protein-associated protein-C (VAP-C), a putative inhibitor of HCV replication, was negligible in B cells. These results support the notion that HCV can successfully reside in B cells, resulting in persistent infection. This is the first study describing analysis of the suppressive effects of HCV infection on antiviral innate immunity in peripheral B cells. Thus, this study offers new insights into the role of B cells in the pathogenesis of HCV.

Methods

Patients and Samples

A total of 24 CHC patients were enrolled in this study, with the following characteristics: 14 males and 10 females; mean age 62.4 ± 7.4 years; mean serum ALT levels 67.5 ± 36.0 IU/l; mean serum AST levels 66.7 ± 34.3 IU/l; 21 patients with HCV genotype 1b and 3 with HCV genotype 2a, and mean HCV RNA 1,752 ± 1,188 KIU/ml. All cases were confirmed to be negative for other viral infections, including hepatitis B virus and human immunodeficiency virus. The study protocols were approved by the Review Board of the National Institute of Infectious Diseases. All donors gave written informed consent. Controls were healthy blood donors at the Tokyo Red Cross Blood Center (Tokyo, Japan) who were confirmed to be negative for HCV, hepatitis B virus and human immunodeficiency virus.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia Biotech, Quebec, Que., Canada) density gradient centrifugation. CD19+ B lymphocytes were isolated from PBMCs by negative selection (B Cell Isolation Kit II, human; Miltenyi, Auburn, Calif., USA). The purity of isolated B cells was generally >95%, as assessed by flow cytometry.

Semiquantitative Real-Time PCR

Total RNA was extracted from lymphoid cells using Isogen (Nippon Gene Co. Ltd., Tokyo, Japan). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, Calif., USA) with oligo(dT)12–18 primer (Invitrogen). PCR amplification was performed using SYBR Premix Ex Taq™ II (Takara Shuzo, Kyoto, Japan) with gene-specific primers (Bex Co. Ltd., Tokyo, Japan) available in the public database RTPrimerDB [22] under the codes 3542 for IFNβ and 3539 for GAPDH, and the Universal Probe Library Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp; Roche Applied Science) as follows: IPS-1 (No. 19, 04686926001), TIR domain-containing adaptor inducing IFN (TRIF; No. 37, 04687957001), suppressor of IκB kinase ε (IKKε) (SIKE; No. 56, 04688538001), heat-shock protein of 90 kDa (Hsp90; No. 25, 04686993001) and DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked (DDX3X; No. 69, 04688686001). The primer sequences for RIG-I were 5′-GTG CAA AGC CTT GGC ATG T-3′ (forward) and 5′-TGG CTT GGG ATG TGG TCT ACT C-3′ (reverse) [23], and for TLR3 they were 5′-GTT ACG AAG AGG CTG GAA TGG T-3′ (forward) and 5′-GCC AGG AAT GGA GAG GTC TAG A-3′ (reverse) [24].

Real-time PCR was carried out for 45 cycles at 94°C for 1 min and at 60°C for 25 s (two-step PCR) using a Light Cycler (Roche Diagnostics, Basel, Switzerland). Amplification of predicted fragments was confirmed by melt curve analysis and gel electropho-

resis. Standard curves were established with 10-fold serial dilutions of amplified products. Measured amounts of each mRNA were normalized to GAPDH mRNA. mRNA expression levels in normal B cells were arbitrarily defined as 1.0.

Immunoblot Analysis

To extract whole-cell proteins, cell pellets were suspended in modified RIPA buffer [150 mM NaCl, 50 mM Tris-Cl (pH 7.4), 1 mM EDTA, 1.0% NP-40, 0.5% sodium deoxycholic acid and 0.1% SDS] containing Halt protease inhibitor cocktail (Pierce, Rockford, Ill., USA) and Halt phosphatase inhibitor cocktail (Pierce; 2×10^7 cells/ml). After 20 min of incubation on ice, cell extracts were centrifuged at 12,000 *g* for 10 min at 4°C, transferred to other tubes and stored at –80°C. Nuclear and cytoplasmic proteins were separated using a Nuclear Extraction Kit (Active Motif, Carlsbad, Calif., USA) according to the manufacturer's protocol. Protein concentration was determined using the BCA™ Protein Assay Kit – Reducing Agent Compatible (Pierce). Samples (whole-cell extract, 1 g; fractionated extract, 2×10^5 cells) were loaded onto 7.5 or 12.5% SDS acrylamide gels (Real Gel Plate; Bio Craft, Tokyo, Japan), followed by transfer to polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature using Block Ace™ (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan). They were then sequentially probed with primary and secondary antibodies at 4°C overnight and for 1 h at room temperature, respectively.

For primary antibodies, we used anti-IFN rabbit polyclonal antibody (ab9662, 1/1,000 dilution; Abcam Inc., Cambridge, Mass., USA), anti-ACTB (β-actin) rabbit polyclonal antibody (4967, 1/1,000 dilution; Cell Signaling Technology, Danvers, Mass., USA), anti-TLR3 rabbit polyclonal antibody (ab62566, 1/1,000 dilution; Abcam), anti-TRIF rabbit polyclonal antibody (4596, 1/1,000 dilution; Cell Signaling Technology), anti-RIG-I rabbit polyclonal antibody (29010, 1/100 dilution; Immuno-Biological Laboratories Co. Ltd., Gunma, Japan), anti-IPS-1 rabbit polyclonal antibody (AT107, 1/2,000 dilution; Alexis Biochemicals, Farmingdale, N.Y., USA), anti-IRF-3 rabbit polyclonal antibody (18781, 1/100 dilution; Immuno-Biological Laboratories), anti-GAPDH mouse monoclonal antibody [5G4(6C5), 1/9,000 dilution; HyTest Ltd., Turku, Finland], anti-PARP-1 mouse monoclonal antibody (AM30, 1/500 dilution; Calbiochem, San Diego, Calif., USA), anti-TANK-binding kinase-1 (TBK1) rabbit polyclonal antibody (3504, 1/1,000 dilution; Cell Signaling Technology) and anti-IKKε rabbit polyclonal antibody (ab7891, 1/500 dilution; Abcam). Anti-VAP-C rabbit polyclonal antibody (2.66 g/ml) was produced by a group of the authors (H.K., K. Moriishi and Y.M.).

The secondary antibodies used were horseradish peroxidase-coupled donkey anti-rabbit Ig (NA934, 1/10,000 dilution; GE Healthcare Ltd.; UK, Buckinghamshire, UK) and horseradish peroxidase-coupled sheep anti-mouse Ig (NA931, 1/10,000 dilution; GE Healthcare UK). Protein bands were detected using ECL Plus™ Western Blotting Detection Reagents (GE Healthcare UK) and a LAS-3000 Image Analyzer (Fuji Film, Tokyo, Japan). Densitometric analysis was performed within a linear range using Image Gauge (Fuji Film). The density of each band (the amount of protein) was normalized against that of the corresponding β-actin.

Native PAGE for IRF-3 Dimer Detection

Native PAGE was performed using 7.5% SDS acrylamide gels (Real Gel Plate; Bio Craft). Gels were prerun with 25 mM Tris-Cl (pH 8.4) and 192 mM glycine with and without 0.2% deoxycholate in the cathode and anode chambers, respectively, for 30 min at 40 mA. Samples were extracted in lysis buffer (3×10^7 cells/ml; 50 mM Tris-Cl, pH 8.0, 1% NP40, 150 mM NaCl) containing Halt protease inhibitor cocktail and Halt phosphatase inhibitor cocktail, mixed with equal volumes of Tris-glycine native sample buffer (2×; Invitrogen), applied to the gel and electrophoresed for 60 min at 25 mA.

Immunoblotting was performed as described above. As a positive control for IRF-3 dimerization, HeLa cells were added with 100 g/ml polyriboinosinic-polyribocytidylic acid (poly I:C; kindly provided by Toray Co. Ltd., Tokyo, Japan). Three hours after incubation, cells were harvested and cell lysates were prepared as described above.

Poly I:C Transfection

CD19+ B lymphocytes isolated from PBMCs were cultured in RPMI-1640 medium containing 10% FCS, 2 mM L-glutamine, 1 mM HEPES, 0.05 mM β-mercaptoethanol, penicillin and streptomycin in a flat-bottom 96-well plate for 3 h (2.5×10^6 cells/well). To activate the RIG-I-mediated pathway, cells were transfected with 10 μg/ml poly I:C using Poly(I:C)/LyoVec (Invivogen, San Diego, Calif., USA). After 18 h of culture, transfected or non-transfected cells were dissolved in Isogen (Nippon Gene) for semi-quantitative real-time PCR assay. Three independent triplicate transfection experiments were performed in order to verify the reproducibility of the results.

Statistics

Unpaired (two-tailed) Student's t tests were applied at the 95% confidence level ($p < 0.05$) using Prism (version 4; GraphPad Software Inc., San Diego, Calif., USA) in all cases.

Results

Impaired IFN Responses in Peripheral B Cells of CHC Patients

We recently demonstrated that HCV infected and may have replicated in peripheral B cells of CHC patients [15]. This implied that HCV may have evaded the immune response by B cells, resulting in persistent infection. Because the induction of type I IFNs, including IFNβ, is crucial for host defense against invading viruses, we first examined constitutive IFNβ expression levels in peripheral B cells of CHC patients. As shown in figure 1a, IFNβ mRNA expression levels were not augmented in CHC B cells compared with normal B cells. The results of Western blotting analysis (fig. 1b) indicated that constitutive IFNβ protein expression levels were not enhanced in B cells of CHC patients, which supported the finding of unaltered IFNβ mRNA expression.

We then stimulated normal and CHC B cells using poly I:C transfection, which triggers RIG-I- and melanoma differentiation-associated gene-5-mediated IFNβ path-

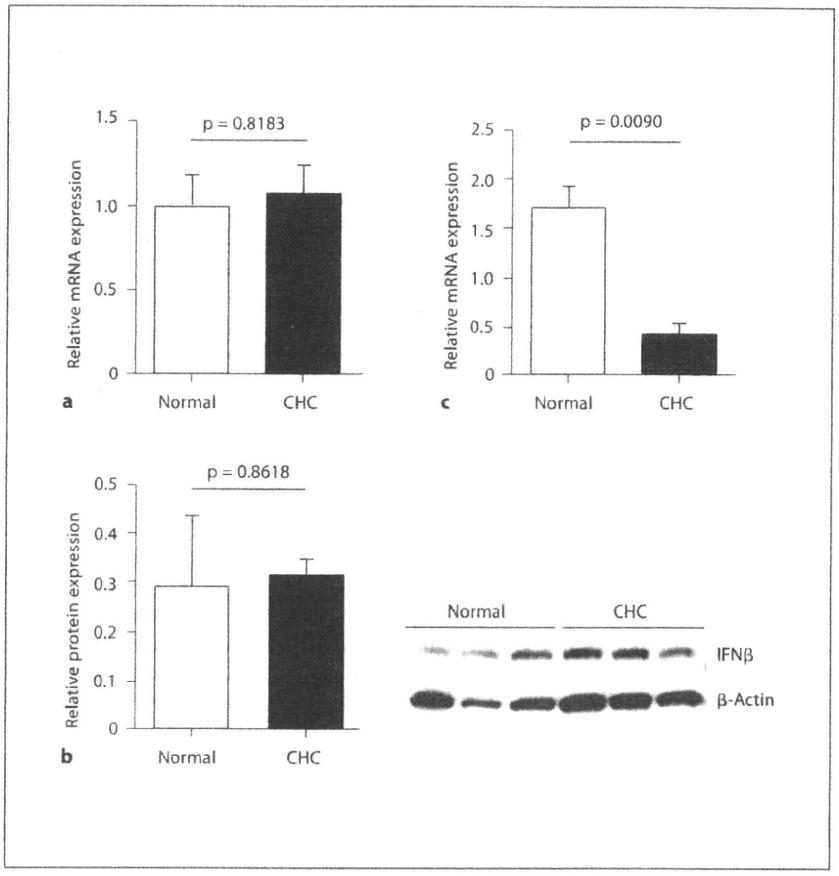

Fig. 1. IFNβ expression in CHC B cells. Fractionation of CD19+ B cells from PBMCs was performed as described in Methods. **a** IFNβ mRNA expression levels in CD19+ B cells isolated from normal individuals (n = 4) and CHC patients (n = 7) were measured in duplicate by quantitative real-time RT-PCR and normalized against those of the housekeeping gene GAPDH. mRNA expression levels in normal B cells were arbitrarily defined as 1.0. **b** Whole-cell extracts prepared from CD19+ B cells isolated from normal individuals (n = 3) and CHC patients (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-IFNβ and anti-ACTB antibodies. Relative IFNβ protein expression levels normalized against β-actin expression are shown. **c** CD19+ B cells isolated from normal individuals (n = 3) or CHC patients (n = 3) were transfected with poly I:C (10 g/ml). Eighteen hours after transfection, cells were harvested and total RNA was isolated. IFNβ mRNA expression levels were measured in duplicate using quantitative real-time RT-PCR and normalized against those of the housekeeping gene GAPDH. mRNA expression levels in untransfected normal or CHC B cells were arbitrarily defined as 1.0. Representative results from at least 2 independent experiments with similar results are shown.

ways. As shown in figure 1c, IFNβ mRNA expression levels in CHC B cells were much lower than those in normal B cells, suggesting that CHC B cells are defective with regard to IFNβ production upon stimulation with the intracellular delivery of poly I:C. In addition, the expression levels of IFN-stimulated genes, such as ISG-15 and ISG-56, in CHC B cells were also much lower than those in normal B cells upon poly I:C stimulation (data not shown).

Taken together, these results indicate that chronic HCV infection fails to induce an IFNβ response in CHC B cells. Subsequent experiments were designed to elucidate the underlying mechanism(s) by which HCV interrupted the IFN responses in CHC B cells.

Expression Levels of HCV Sensor Molecules in Peripheral B Cells of CHC Patients

We next examined the gene expression levels in peripheral B cells of two major viral sensors, TLR3 and RIG-I, as well as their corresponding adaptor molecules, TRIF and IPS-1, which are indispensable for initiating innate immune responses [25]. As shown in figure 2, TLR3, TRIF, RIG-I and IPS-1 expression levels were significantly enhanced in peripheral B cells of CHC patients. Expression of another cytoplasmic sensor molecule, melanoma differentiation-associated gene-5, was also enhanced (data not shown). These results demonstrate that the expression levels of cytoplasmic virus sensors as well as their adaptors are constitutively augmented in CHC B cells.

Expression, Dimerization and Nuclear Translocation of IRF-3 in CHC B Cells

The IRF-3 activation cascade, including phosphorylation, dimerization and nuclear translocation, is essential for IFNβ gene transcription [26]. We found that constitutive IRF-3 expression levels in CHC B cells were significantly lower than those in normal B cells ($p = 0.0018$) as assessed by Western blotting (fig. 3a). Furthermore, IRF-

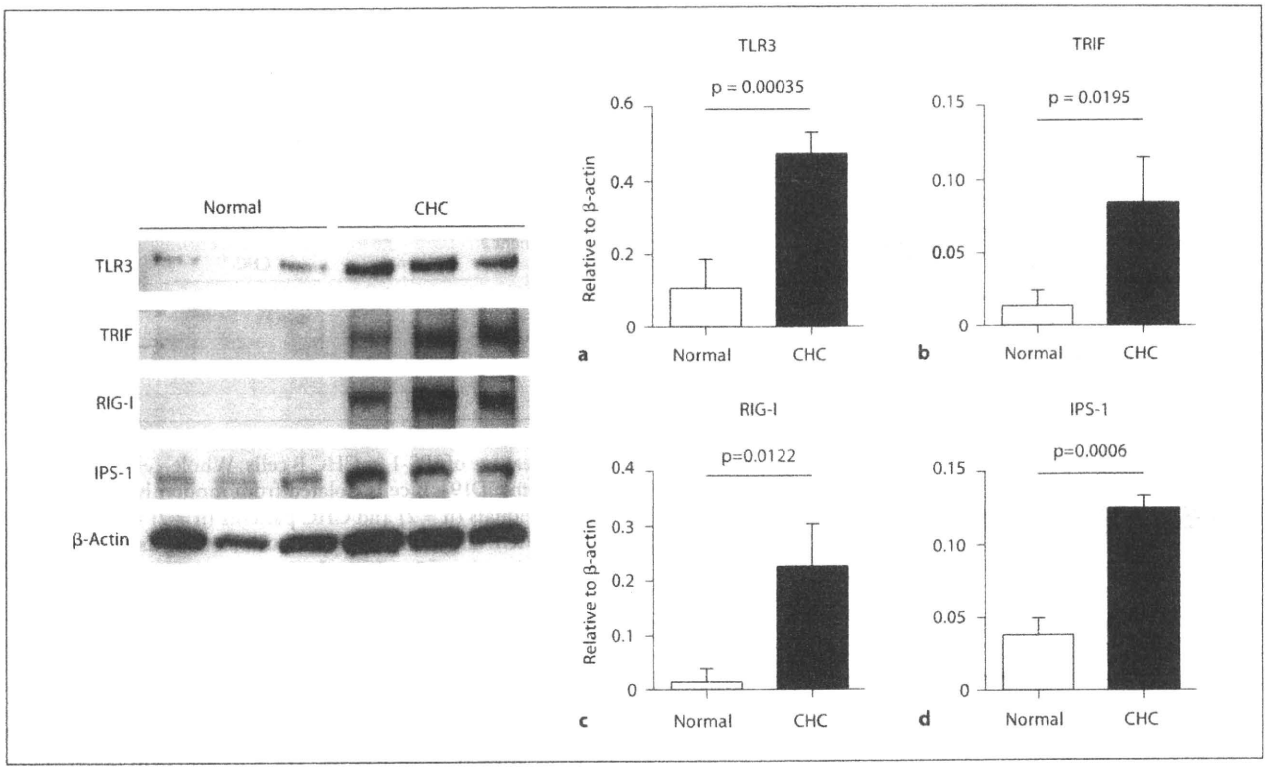

Fig. 2. Expression levels of HCV sensor and adaptor molecules in CHC B cells. Whole-cell extracts prepared from CD19+ B cells isolated from normal individuals (n = 3) and CHC patients (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-TLR3, anti-TRIF, anti-RIG-I, anti-IPS-1 and control anti-ACTB antibodies. Relative protein expression levels normalized against β-actin expression are shown.

3 dimerization assessed by native PAGE was not observed in CHC B cells (fig. 3b). Consequently, IRF-3 nuclear translocation did not occur in CHC B cells (fig. 3c). Thus, these results indicate that the IRF-3 activation cascade does not proceed in CHC B cells, which may explain the lack of IFNβ responses to HCV infection.

Cleavage of IPS-1 in CHC B Cells

Several lines of evidence have indicated an essential role for the adaptor molecule IPS-1, also called Cardif, MAVS or VISA, which acts downstream of RIG-I in the IRF-3 signaling pathway [27]. Recent studies have confirmed that HCV can cleave IPS-1 via its NS3/4A protease activity [28]. As a result of this proteolytic cleavage, IPS-1 is dislodged from the mitochondria and becomes an inactive cytosolic fragment. This causes the failure of downstream signaling for IRF-3 activation. Therefore, we examined IPS-1 cleavage in CHC B cells by native PAGE. As shown in figure 4, IPS-1 cleavage was incomplete, and a substantial amount of uncleaved (intact) IPS-1 was detected. Thus, we concluded that impaired IRF-3 activation cannot be solely explained by IPS-1 cleavage in CHC B cells. These results suggest either that the NS3/4A protease is not expressed in CHC B cells or that IPS-1 in B cells is resistant to this protease. Because NS3 molecules were detected in CHC B cells by Western blotting [15], the latter seems to be more likely.

Stabilization or Inhibition of TBK1/IKKε Kinase Activity in CHC B Cells

Two protein kinases, TBK1 and IKKε, which are both located downstream of IPS-1, are essential for IFNβ production via IRF-3 phosphorylation [29]. Interestingly, the constitutive expression levels of both kinases were markedly enhanced in CHC B cells compared with those in normal B cells (fig. 5), although the downstream IRF-3 activation cascade was severely impaired, as shown in figure 3.

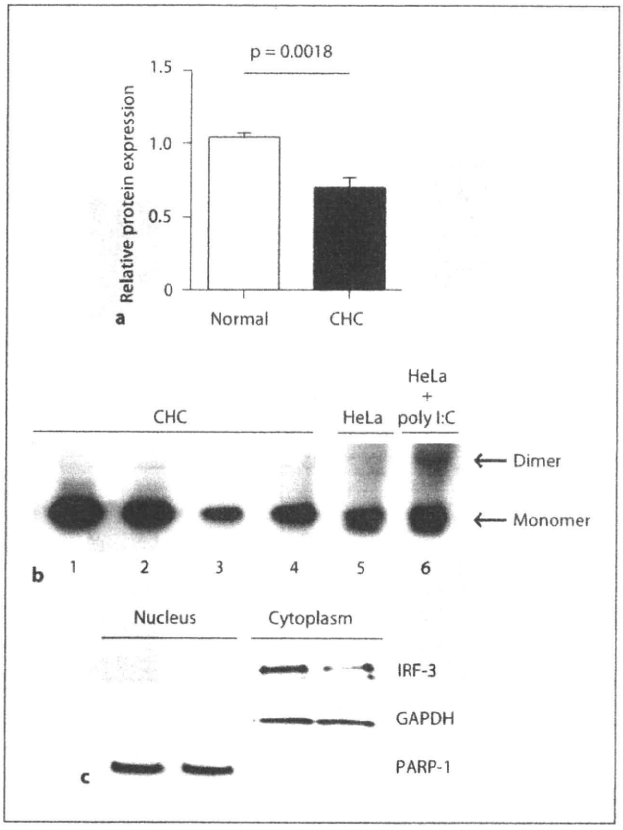

Fig. 3. Expression, dimerization and nuclear translocation of IRF-3 in CHC B cells. **a** Whole-cell extracts prepared from CD19+ B cells isolated from randomly selected normal individuals (n = 3) and CHC patients (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-IRF-3 and anti-ACTB antibodies. Relative protein expression levels of IRF-3 normalized against β-actin expression are shown. **b** Dimerization of IRF-3 in CD19+ B cells isolated from CHC patients (n = 4) were analyzed by native PAGE as described in Methods. As a positive control to detect IRF-3 dimer formation, cell extracts from HeLa cells stimulated with poly I:C were applied (lane 6). **c** Nuclear and cytoplasmic proteins were isolated from CD19+ B cells of CHC patients (n = 2) as described in Methods. Samples were subjected to SDS-PAGE and analyzed by immunoblotting using anti-IRF-3, anti-GAPDH (cytoplasmic marker protein) and anti-PARP-1 (nuclear marker protein) antibodies.

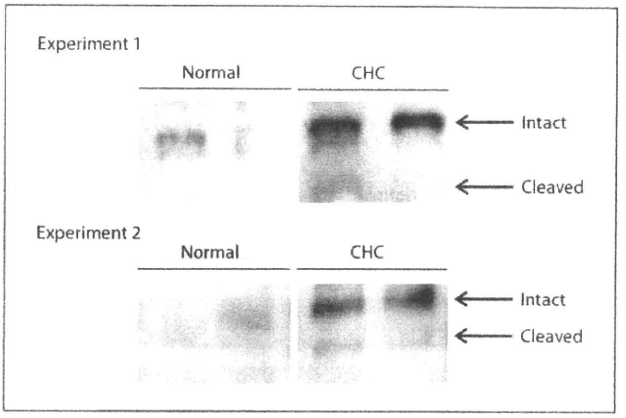

Fig. 4. Cleavage of IPS-1 in CHC B cells. Whole-cell extracts prepared from CD19+ B cells isolated from randomly selected normal individuals (n = 2) and CHC patients (n = 2) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-IPS-1 antibody. Results of 2 independent experiments are shown.

Huang et al. [30] identified a protein called SIKE that interacts with IKKε and TBK1 and has an inhibitory effect on the IRF-3 activation pathway. Yang et al. [31] demonstrated that Hsp90 was important for stabilizing TBK1 and promoting IRF-3 phosphorylation by TBK1 in response to viral infection. The gene expression levels of these two molecules in B cells were analyzed by real-time PCR, as shown in figure 6a. SIKE expression levels were significantly enhanced in CHC B cells ($p = 0.0059$), while those of Hsp90 were significantly reduced ($p = 0.001$). These results strongly suggest that the kinase activities of TBK1 and IKKε are downregulated in CHC B cells, which may be responsible for the failure in IRF-3 activation and subsequent IFNβ transcription.

The DEAD box helicase DDX3X [32] is a critical component of TBK1-dependent type I IFN induction [33, 34]. As shown in figure 6a, DDX3X expression levels were significantly reduced in CHC B cells ($p = 0.0043$). This could be just a concomitant observation; however, this result is of interest assuming that HCV has an additional mechanism by which it interferes with IRF-3 activation.

VAP-C Expression in B Cells

Human VAP subtype A (VAP-A) and subtype B (VAP-B) are essential host factors for HCV replication because they bind to both NS5A and NS5B [35]. VAP-C is a splicing variant of VAP-B that lacks two thirds of the C terminus [36, 37]; therefore, it cannot interact with VAP-A, VAP-B or NS5A. A physiological role of VAP-C was recently demonstrated by Kukihara et al. [38], who found that VAP-C inhibited the association between VAP-A/B and NS5B, thereby reducing HCV replication efficiency. Interestingly, VAP-C expression in hepatocytes was found to be negligible, which may be advantageous for

HCV replication in the liver [38]. These results prompted us to examine VAP-C expression in B cells. As shown in figure 6b, VAP-C was expressed in CD19– cells (i.e. non-B cells), but not in CD19+ B cells. Together with the defect in antiviral immune responses of CHC B cells described above, this observation further supports the notion that HCV utilizes B cells as a reservoir for persistent infection.

Discussion

HCV infection of hepatocytes has long been an implicit assumption. However, this does not necessarily imply that hepatocytes are the exclusive target for HCV infection. HCV may seek other cellular compartments as reservoirs in the event that the liver becomes unsuitable for replication, possibly due to cellular destruction caused by the host immune response and/or the development of conditions such as cirrhosis and hepatocellular carcinoma. Our previous study suggested the possibility that HCV persistently infects peripheral B cells [15]. Consequently, we were extremely interested in how HCV is able to escape host innate immunity and persistently remain in peripheral B cells. The results of this study may provide plausible answers to these questions.

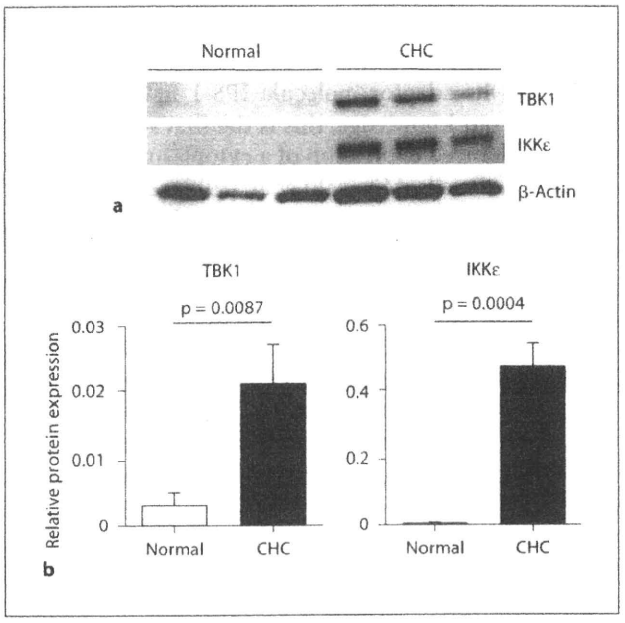

Fig. 5. Expression of TBK1 and IKKε in CD19+ B cells. **a** Whole-cell extracts prepared from CD19+ B cells isolated from normal individuals (n = 3) and CHC patients (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-TBK1, anti-IKKε and anti-ACTB antibodies. **b** Relative protein expression levels of each protein normalized against β-actin expression are shown.

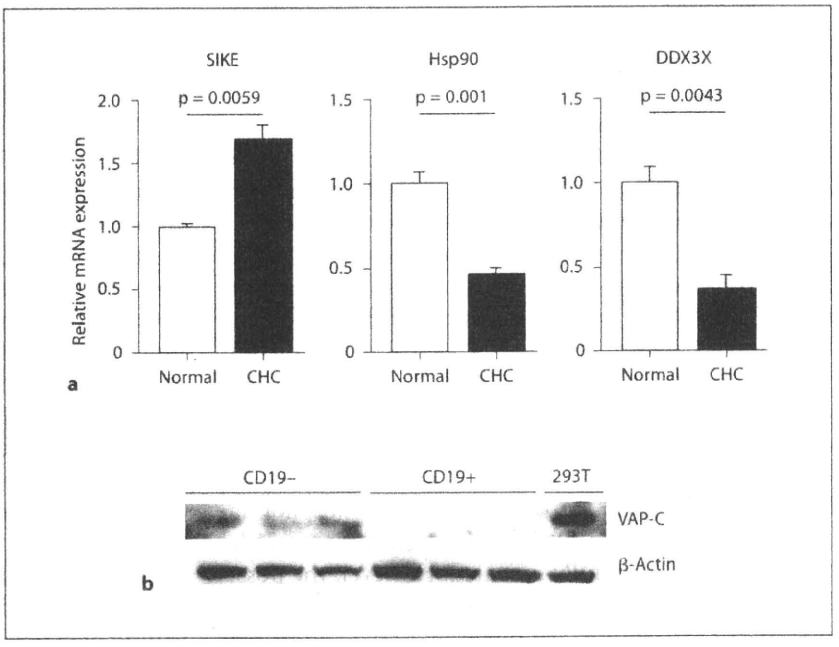

Fig. 6. Expression of SIKE, Hsp90, DDX3X and VAP-C in CD19+ B cells. **a** mRNA expression levels of SIKE, Hsp90 and DDX3X in CD19+ B cells isolated from normal individuals (n = 3–7) and CHC patients (n = 3–5) were measured in duplicate by quantitative real-time RT-PCR and normalized against those of the housekeeping gene GAPDH. mRNA expression levels in normal B cells were arbitrarily defined as 1.0. **b** Whole-cell extracts prepared from CD19– and CD19+ B cells isolated from normal individuals (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-VAP-C antibody. Whole-cell extracts from 293T cells were used as a positive control.

In this study, we observed augmented expression of RIG-I, a cytoplasmic double-stranded RNA sensor molecule, and its adaptor molecule IPS-1 in CHC B cells (fig. 2). To our knowledge, this is the first study describing the enhanced expression of a cytoplasmic HCV sensor in CHC B cells. Miyazaki et al. [39] recently reported similar findings on examining myeloid dendritic cells in CHC patients, thus suggesting that HCV infection augments the expression of a cytoplasmic double-stranded RNA sensor regardless of the cell type. The same may be true for expression of the RIG-I adaptor molecule IPS-1, because Miyazaki et al. [39] also reported enhanced IPS-1 expression in myeloid dendritic cells in CHC patients. These results support the notion that HCV triggers innate immunity by immune cells, including peripheral B cells.

The primary cause for the failure to induce innate immunity in CHC B cells appears to be a defect in promoting the IRF-3 activation cascade (fig. 3). HCV is thought to evade an innate antiviral response by the actions of the viral NS3/4A protease-helicase complex, which inhibits RIG-I signaling via proteolytic cleavage and IPS-1 inactivation [28], thereby preventing its downstream activation (i.e. IRF-3 activation). This hypothesis was essentially derived from in vitro experiments using human hepatoma cell lines such as HuH-7. In the present study, IPS-1 cleavage was analyzed for the first time in peripheral B cells of CHC patients. The results shown in figure 4 indicate only marginal cleavage of IPS-1 in CHC B cells, such that this could not be the main cause of the defect in downstream IRF-3 activation. We therefore sought additional explanations for the IRF-3 activation defect in CHC B cells.

The results shown in figure 5 indicate markedly enhanced expression of TBK1 and IKKε in CHC B cells compared with normal B cells, thus suggesting that IPS-1 signaling is mainly intact and not completely abolished by HCV infection as expected. In the present study, it was not possible to identify the mechanism underlying the expression of TBK1 and IKKε induced by HCV infection. However, as shown in figure 3, IRF-3 activation was markedly suppressed in CHC B cells. The defect in IRF-3 activation in CHC B cells may be linked to the enhanced expression of SIKE and the reduced expression of Hsp90 (fig. 6a), which together may affect the kinase activities of both TBK1 and IKKε. Consequently, IRF-3 phosphorylation (i.e. the first step of the IRF-3 activation cascade) would not be successfully executed. However, the possibilities that IPS-1 signaling is disrupted along the cascade despite the existence of the signal molecule and/or that IRF-3 activation is directly blocked by HCV proteins should also be taken into consideration.

It was recently demonstrated that the expression of DDX3X, a DEAD box RNA helicase [32], enhanced IFNβ promoter induction by TBK1/IKKε, whereas its knockdown inhibited IRF3 activation [33]. The results shown in figure 6a confirm that DDX3X expression levels were significantly downregulated in CHC B cells, which is in agreement with previous studies. However, Ariumi et al. [40] reported that DDX3X was required for HCV RNA replication, which seems contradictory to our results. We currently do not know the reasons for this discrepancy; however, the fact that in their study they used the HuH-7 hepatoma cell line, while we examined naturally occurring HCV-infected B cells, may account for these conflicting results.

The role of VAP proteins in HCV replication is another interesting aspect. HCV NS5A is associated with a range of cellular proteins, including VAP-A [41], which are involved in cell signaling pathways. VAP-B has been identified as another NS5A-binding protein by screening human libraries using the yeast two-hybrid system with NS5A as bait [35]. Both VAP-A and VAP-B are involved in HCV replication via interactions with NS5A and NS5B [35], while VAP-C inhibits the association between VAP-A/B and NS5B, which results in reduced HCV replication efficiency [38]. Therefore, the absence of VAP-C expression in B cells, similar to hepatocytes, may be favorable for HCV replication. This could be another strategy for maintaining persistent HCV infection in B cells. In fact, our recent study demonstrated that a high copy number of HCV RNA was detected in CHC B cells, but not in CHC non-B cells [15], suggesting that the absence of VAP-C expression in B cells is, at least in part, responsible for the HCV replication. Further analyses are required to examine if the forced expression of VAP-C could inhibit HCV replication in B cells.

Several lines of evidence indicate that chronic infection with HCV can induce molecular alterations in lymphocytes that may subsequently play a role in the multistep process of malignant lymphocyte transformation with the induction of clonal B cell expansion. Lymphoid cells from patients with chronic HCV overexpress the antiapoptotic protein Bcl-2 with a high incidence of *t* (14;18) translocations involving the *bcl-2* gene [42, 43]. Our recent study revealed that the expression level of activation-induced cytidine deaminase, which promotes B cell lymphomagenesis by its overexpession [44, 45], in CHC B cells was significantly increased [15]. Furthermore, enhanced expression of putative lymphomagenesis-related

genes such as cyclin D1, cyclin D2, B aggressive lymphoma gene, serine/threonine kinase 15 and galectin-3 was observed in CHC B cells [15]. In addition, expression of HCV core protein and NS3 was detected in CD19+ B cells of CHC patients [15]. HCV core protein has been demonstrated to promote immortalization in different cell lines as well as being capable of blocking c-myc-induced apoptosis [46]. NS3 has also been shown to promote oncogenic transformation and to interact with p53 and interfere with apoptosis [47]. Thus, persistent infection with HCV via the suppression of innate immunity responses in CHC B cells would cause functional disorders and lead to B cell lymphoma.

Interestingly, it has been shown that interaction between HCV E2 and CD81 on B cells triggers enhanced expression of activation-induced cytidine deaminase, which induces double-strand DNA breaks and hypermutation, specifically in the VH gene of B cells [48]. Stamataki et al. [49] demonstrated that peripheral blood B cells could bind infectious HCV in the cell strain JFH-1. Accordingly, it seems likely that mere interaction between envelope proteins of HCV and signaling receptors on the surface of B cells could generate lymphoproliferative disorders.

The difficulty in collecting an adequate number of purified B cells from CHC patients prevented us from analyzing a large sample size in each experiment, which may weaken the impact of these results. However, in the light of the fact that potentially heterogeneous patient samples were examined in this study, we believe that our considerably homogeneous results do have a certain biological impact. In addition, it may be worth noting that most CHC patients enrolled in this study (21 of 24) were infected with HCV of genotype 1b, which is prevalent in Asia. It would have been ideal to perform these experiments with different genotypes to observe if this phenomenon is noted across all genotypes or if it is specific only to genotype 1b. A study enrolling CHC patients infected with other HCV genotypes will be required in order to draw more robust conclusions, although it is very difficult to enroll such CHC subjects in Japan.

Taken together, the present results strongly suggest that HCV utilizes B cells as an extrahepatic reservoir for persistent infection. Whether the apparent suppression of innate immune responses in B cells is restricted to HCV infection or if this is a phenomenon seen in other B cell tropic viruses such as Epstein-Barr virus is currently unknown. This intriguing question could be answered by further elucidating the suppression mechanisms in CHC B cells as well as by investigating innate immune responses in Epstein-Barr virus-infected B cells in future studies.

We assume that memory B cells are the main reservoir of HCV infection because of their long life span. In support of this proposal, one of our current studies indicated that CD19+CD27+ cells (i.e. memory B cells) are recruited to the livers of CHC patients via interactions between CXCR3 expressed on CD19+CD27+ cells and IP-10 produced in the liver [Mizuochi et al., in press]. This would be a robust strategy for HCV in order to secure sites for long-lasting infection. Interestingly, a recent study by Stamataki et al. [49] indicated that HCV associated with B cells had the potential to transfect HuH-7.5 in vitro. Our results strongly suggest that such HCV transfection may occur in vivo under physiological conditions. This would offer new therapeutic insights for HCV clearance by eliminating peripheral B cells with anti-B cell antibodies and drugs such as rituximab in conjunction with a combination therapy using peginterferon and ribavirin.

In conclusion, we propose that peripheral B cells serve as a reservoir for persistent HCV infection. Based on this proposal and from a therapeutic perspective, it may be beneficial to eliminate peripheral B cells in CHC patients. Together with antiviral treatment to eliminate circulating HCV in the blood, this could lead to a synergistic effect for HCV clearance in CHC patients.

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References

1 Suzuki T, Ishii K, Aizaki H, Wakita T: Hepatitis C viral life cycle. Adv Drug Deliv Rev 2007;59:1200–1212.

2 Lauer GM, Walker BD: Hepatitis C virus infection. N Engl J Med 2001;345:41–52.

3 Agnello V, Mecucci C, Casato M: Regression of splenic lymphoma after treatment of hepatitis C virus infection. N Engl J Med 2002; 347:2168–2170, author reply 2168–2170.

4 Zuckerman E, Zuckerman T, Levine AM, Douer D, Gutekunst K, Mizokami M, Qian DG, Velankar M, Nathwani BN, Fong TL: Hepatitis C virus infection in patients with B-cell non-Hodgkin lymphoma. Ann Intern Med 1997;127:423–428.

5 de Sanjose S, Benavente Y, Vajdic CM, Engels EA, Morton LM, Bracci PM, Spinelli JJ, Zheng T, Zhang Y, Franceschi S, Talamini R, Holly EA, Grulich AE, Cerhan JR, Hartge P, Cozen W, Boffetta P, Brennan P, Maynadie M, Cocco P, Bosch R, Foretova L, Staines A, Becker N, Nieters A: Hepatitis C and non-Hodgkin lymphoma among 4784 cases and 6269 controls from the international lymphoma epidemiology consortium. Clin Gastroenterol Hepatol 2008;6:451–458.
6 Turner NC, Dusheiko G, Jones A: Hepatitis C and B-cell lymphoma. Ann Oncol 2003;14:1341–1345.
7 Mazzaro C, Franzin F, Tulissi P, Pussini E, Crovatto M, Carniello GS, Efremov DG, Burrone O, Santini G, Pozzato G: Regression of monoclonal B-cell expansion in patients affected by mixed cryoglobulinemia responsive to alpha-interferon therapy. Cancer 1996;77:2604–2613.
8 Caussin-Schwemling C, Schmitt C, Stoll-Keller F: Study of the infection of human blood derived monocyte/macrophages with hepatitis C virus in vitro. J Med Virol 2001;65:14–22.
9 Goutagny N, Fatmi A, De Ledinghen V, Penin F, Couzigou P, Inchauspe G, Bain C: Evidence of viral replication in circulating dendritic cells during hepatitis C virus infection. J Infect Dis 2003;187:1951–1958.
10 Navas MC, Fuchs A, Schvoerer E, Bohbot A, Aubertin AM, Stoll-Keller F: Dendritic cell susceptibility to hepatitis C virus genotype 1 infection. J Med Virol 2002;67:152–161.
11 Blackard JT, Kemmer N, Sherman KE: Extrahepatic replication of HCV: insights into clinical manifestations and biological consequences. Hepatology 2006;44:15–22.
12 Ducoulombier D, Roque-Afonso AM, Di Liberto G, Penin F, Kara R, Richard Y, Dussaix E, Feray C: Frequent compartmentalization of hepatitis C virus variants in circulating B cells and monocytes. Hepatology 2004;39:817–825.
13 Morsica G, Tambussi G, Sitia G, Novati R, Lazzarin A, Lopalco L, Mukenge S: Replication of hepatitis C virus in B lymphocytes (CD19+). Blood 1999;94:1138–1139.
14 Muller HM, Kallinowski B, Solbach C, Theilmann L, Goeser T, Pfaff E: B-lymphocytes are predominantly involved in viral propagation of hepatitis C virus (HCV). Arch Virol Suppl 1994;9:307–316.
15 Ito M, Murakami K, Suzuki T, Mochida K, Suzuki M, Ikebuchi K, Yamaguchi K, Mizuochi T: Enhanced expression of lymphomagenesis-related genes in peripheral blood B cells of chronic hepatitis C patients. Clin Immunol 2010;135:459–465.
16 Foy E, Li K, Wang C, Sumpter R Jr, Ikeda M, Lemon SM, Gale M Jr: Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science 2003;300:1145–1148.
17 Foy E, Li K, Sumpter R Jr, Loo YM, Johnson CL, Wang C, Fish PM, Yoneyama M, Fujita T, Lemon SM, Gale M Jr: Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. Proc Natl Acad Sci USA 2005;102:2986–2991.
18 Dansako H, Ikeda M, Kato N: Limited suppression of the interferon-beta production by hepatitis C virus serine protease in cultured human hepatocytes. FEBS J 2007;274:4161–4176.
19 Dansako H, Naka K, Ikeda M, Kato N: Hepatitis C virus proteins exhibit conflicting effects on the interferon system in human hepatocyte cells. Biochem Biophys Res Commun 2005;336:458–468.
20 Ikeda M, Sugiyama K, Mizutani T, Tanaka T, Tanaka K, Sekihara H, Shimotohno K, Kato N: Human hepatocyte clonal cell lines that support persistent replication of hepatitis C virus. Virus Res 1998;56:157–167.
21 Li K, Chen Z, Kato N, Gale M Jr, Lemon SM: Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. J Biol Chem 2005;280:16739–16747.
22 Pattyn F, Robbrecht P, De Paepe A, Speleman F, Vandesompele J: RTPrimerDB: the real-time PCR primer and probe database, major update 2006. Nucleic Acids Res 2006;34(Database issue):D684–D688.
23 Imaizumi T, Yagihashi N, Kubota K, Yoshida H, Sakaki H, Yagihashi S, Kimura H, Satoh K: Expression of retinoic acid-inducible gene-I (RIG-I) in macrophages: possible involvement of RIG-I in atherosclerosis. J Atheroscler Thromb 2007;14:51–55.
24 Xu J, Yang Y, Sun J, Ding Y, Su L, Shao C, Jiang B: Expression of Toll-like receptors and their association with cytokine responses in peripheral blood mononuclear cells of children with acute rotavirus diarrhoea. Clin Exp Immunol 2006;144:376–381.
25 Seth RB, Sun L, Chen ZJ: Antiviral innate immunity pathways. Cell Res 2006;16:141–147.
26 Hiscott J, Pitha P, Genin P, Nguyen H, Heylbroeck C, Mamane Y, Algarte M, Lin R: Triggering the interferon response: the role of IRF-3 transcription factor. J Interferon Cytokine Res 1999;19:1–13.
27 Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, Akira S: IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol 2005;6:981–988.
28 Li XD, Sun L, Seth RB, Pineda G, Chen ZJ: Hepatitis C virus protease NS3/4a cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci USA 2005;102:17717–17722.
29 Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, Maniatis T: IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003;4:491–496.
30 Huang J, Liu T, Xu LG, Chen D, Zhai Z, Shu HB: SIKE is an IKK epsilon/TBK1-associated suppressor of TLR3- and virus-triggered IRF-3 activation pathways. EMBO J 2005;24:4018–4028.
31 Yang K, Shi H, Qi R, Sun S, Tang Y, Zhang B, Wang C: Hsp90 regulates activation of interferon regulatory factor 3 and TBK-1 stabilization in Sendai virus-infected cells. Mol Biol Cell 2006;17:1461–1471.
32 Cordin O, Banroques J, Tanner NK, Linder P: The DEAD-box protein family of RNA helicases. Gene 2006;367:17–37.
33 Schroder M, Baran M, Bowie AG: Viral targeting of dead box protein 3 reveals its role in TBK1/IKKepsilon-mediated IRF activation. EMBO J 2008;27:2147–2157.
34 Soulat D, Burckstummer T, Westermayer S, Goncalves A, Bauch A, Stefanovic A, Hantschel O, Bennett KL, Decker T, Superti-Furga G: The DEAD-box helicase DDX3X is a critical component of the TANK-binding kinase 1-dependent innate immune response. EMBO J 2008;27:2135–2146.
35 Hamamoto I, Nishimura Y, Okamoto T, Aizaki H, Liu M, Mori Y, Abe T, Suzuki T, Lai MM, Miyamura T, Moriishi K, Matsuura Y: Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. J Virol 2005;79:13473–13482.
36 Loewen CJ, Levine TP: A highly conserved binding site in vesicle-associated membrane protein-associated protein (VAP) for the FFAT motif of lipid-binding proteins. J Biol Chem 2005;280:14097–14104.
37 Nishimura Y, Hayashi M, Inada H, Tanaka T: Molecular cloning and characterization of mammalian homologues of vesicle-associated membrane protein-associated (VAMP-associated) proteins. Biochem Biophys Res Commun 1999;254:21–26.
38 Kukihara H, Moriishi K, Taguwa S, Tani H, Abe T, Mori Y, Suzuki T, Fukuhara T, Taketomi A, Maehara Y, Matsuura Y: Human VAP-C negatively regulates hepatitis C virus propagation. J Virol 2009;83:7959–7969.
39 Miyazaki M, Kanto T, Inoue M, Itose I, Miyatake H, Sakakibara M, Yakushijin T, Kakita N, Hiramatsu N, Takehara T, Kasahara A, Hayashi N: Impaired cytokine response in myeloid dendritic cells in chronic hepatitis C virus infection regardless of enhanced expression of Toll-like receptors and retinoic acid inducible gene-I. J Med Virol 2008;80:980–988.
40 Ariumi Y, Kuroki M, Abe K, Dansako H, Ikeda M, Wakita T, Kato N: DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication. J Virol 2007;81:13922–13926.

41 Tu H, Gao L, Shi ST, Taylor DR, Yang T, Mircheff AK, Wen Y, Gorbalenya AE, Hwang SB, Lai MM: Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. Virology 1999;263:30–41.
42 Zuckerman E, Zuckerman T, Sahar D, Streichman S, Attias D, Sabo E, Yeshurun D, Rowe J: bcl-2 and immunoglobulin gene rearrangement in patients with hepatitis C virus infection. Br J Haematol 2001;112:364–369.
43 Zignego AL, Giannelli F, Marrocchi ME, Mazzocca A, Ferri C, Giannini C, Monti M, Caini P, Villa GL, Laffi G, Gentilini P: t(14;18) translocation in chronic hepatitis C virus infection. Hepatology 2000;31:474–479.
44 Komeno Y, Kitaura J, Watanabe-Okochi N, Kato N, Oki T, Nakahara F, Harada Y, Harada H, Shinkura R, Nagaoka H, Hayashi Y, Honjo T, Kitamura T: AID-induced T-lymphoma or B-leukemia/lymphoma in a mouse BMT model. Leukemia 2010;24:1018–1024.
45 Robbiani DF, Bunting S, Feldhahn N, Bothmer A, Camps J, Deroubaix S, McBride KM, Klein IA, Stone G, Eisenreich TR, Ried T, Nussenzweig A, Nussenzweig MC: AID produces DNA double-strand breaks in non-Ig genes and mature B cell lymphomas with reciprocal chromosome translocations. Mol Cell 2009;36:631–641.
46 Ray RB, Meyer K, Ray R: Suppression of apoptotic cell death by hepatitis C virus core protein. Virology 1996;226:176–182.
47 Sakamuro D, Furukawa T, Takegami T: Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. J Virol 1995;69:3893–3896.
48 Machida K, Cheng KT, Pavio N, Sung VM, Lai MM: Hepatitis C virus E2-CD81 interaction induces hypermutation of the immunoglobulin gene in B-cells. J Virol 2005;79:8079–8089.
49 Stamataki Z, Shannon-Lowe C, Shaw J, Mutimer D, Rickinson AB, Gordon J, Adams DH, Balfe P, McKeating JA: Hepatitis C virus association with peripheral blood B lymphocytes potentiates viral infection of liver-derived hepatoma cells. Blood 2009;113:585–593.

Network based analysis of hepatitis C virus Core and NS4B protein interactions†

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Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. Here we attempt to further our understanding of the biological context of protein interactions in HCV pathogenesis, by investigating interactions between HCV proteins Core and NS4B and human host proteins. Using the yeast two-hybrid (Y2H) membrane protein system, eleven human host proteins interacting with Core and 45 interacting with NS4B were identified, most of which are novel. These interactions were used to infer overall protein interaction maps linking the viral proteins with components of the host cellular networks. Core and NS4B proteins contribute to highly compact interaction networks that may enable the virus to respond rapidly to host physiological responses to HCV infection. Analysis of the interaction networks highlighted enriched biological pathways likely influenced in HCV infection. Inspection of individual interactions offered further insights into the possible mechanisms that permit HCV to evade the host immune response and appropriate host metabolic machinery. Follow-up cellular assays with cell lines infected with HCV genotype 1b and 2a strains validated Core interacting proteins ENO1 and SLC25A5 and host protein PXN as novel regulators of HCV replication and viral production. ENO1 siRNA knockdown was found to inhibit HCV replication in both the HCV genotypes and viral RNA release in genotype 2a. PXN siRNA inhibition was observed to inhibit replication specifically in genotype 1b but not in genotype 2a, while SLC25A5 siRNA facilitated a minor increase in the viral RNA release in genotype 2a. Thus, our analysis can provide potential targets for more effective anti-HCV therapeutic intervention.

1. Introduction

Hepatitis C virus (HCV) is the causative agent of chronic liver disease including liver steatosis, cirrhosis and hepatocellular carcinoma (HCC) and infects nearly 3% of the population worldwide. HCV is a positive single-stranded RNA virus with a single 9600 nucleotide ORF flanked by 5′ and 3′-UTRs. The HCV ORF encodes a 3000 amino acid polyprotein, which undergoes proteolytic processing by host and viral proteases to yield four structural (Core, E1, E2 and p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins.[1–3] HCV variants span six genotypes that display phylogenetic heterogeneity, differences in infectivity and interferon sensitivity.[4] However, despite a wealth of concerted research, a precise understanding of the molecular mechanisms underlying HCV pathology remains elusive.

Most genes and proteins function in a complex web of interactions. Thus, the study of protein–protein interactions (PPIs) is critical to understanding the cellular networks that regulate the physiology of a living organism. The increasing availability of PPI data for human and host–pathogen interactions has led to increasing efforts in understanding the network basis of human diseases and pathogenesis.[5,6] In particular, the increasing availability of large scale interaction data between viral and human host proteins is likely to lead to a better understanding of viral pathogenesis and help identify novel targets for experimental and therapeutic intervention.[7–9] Comprehensive analyses of yeast two-hybrid (Y2H) screens have been employed to investigate the interactions of HCV,[7] Epstein–Barr virus[10] herpesviral[11] proteins with host factors. Analysis of such interactions suggests that viral (and bacterial) pathogens preferably interact with host proteins either engaged in a large number of interactions or critical to the integrity of the host cellular networks.[7,10]

Here, we report the host biological processes likely to be perturbed by HCV Core and NS4B proteins by virtue of inferred PPI networks. Core, also known as capsid protein, is spliced from the polyprotein by signal peptidase and further processed into a highly conserved 21 kDa mature form by the signal peptide peptidase; this processing facilitates its transfer to the detergent-resistant membrane fraction where virus replication and assembly take place.[12] Core also localises to the nucleus, which is essential for efficient viral propagation and development of HCV pathogenicity.[3,13] Core is a multi-functional protein implicated in RNA binding and as a pathogenic factor, which induces steatosis and HCC and thus, a promising target for anti-HCV therapy.[14,15] NS4B, the least

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characterised HCV protein, is a 27 kDa non-structural integral membrane protein located in the ER membrane, which induces membrane changes and facilitates HCV replication in the host cells, though recent reports suggest that it may function in HCV pathogenesis and viral assembly.[14,16] Since Core and NS4B proteins are primarily associated with the ER membrane, they were employed as baits to screen against a library of human cDNAs using the Y2H membrane protein approach, which identifies PPIs involving integral membrane proteins and membrane-associated proteins in an *in vivo* setting.[17] We identified 11 interactions for Core and 45 interactions for NS4B, nearly all of which are previously uncharacterised. By extending these interactions to include human protein interaction data, our analysis provided insights into the functional pathways likely to be associated with HCV–host interactions in HCV pathogenesis, a better understanding of which may help identify new targets for anti-HCV therapeutic intervention.

2. Results and discussion

2.1 Identifying host proteins that interact with HCV Core and NS4B proteins

Since Core and NS4B are primarily localised to the ER membrane, to investigate their biological associations, we performed a series of Y2H screens customised for characterising the PPIs involving integral membrane and membrane-associated proteins (see Materials and Methods). Analysis of positive colonies revealed 11 interactors for Core protein and 45 interactors for NS4B protein (Table 1).

Nine of the 11 host proteins interacting with Core (Table 1) are novel findings but the other two interactions are known; signal peptide peptidase (HM13), an ER localised protein, is crucial for the intramembrane processing of the Core protein, facilitating its localisation and viral propagation;[12] proteasome subunit alpha type 7 (PSMA7) is involved in regulating HCV internal ribosome entry site (IRES), which is essential for HCV replication.[18] These results suggest that the PPIs detected by our screening approach may closely reflect Core interactions *in vivo*. Among the other interacting proteins, four localise to mitochondria and are likely involved in oxidative electron transfer (ETFB; NDUFS2) and solute transport (SLC25A5; TOMM20), which may be a consequence of known Core localisation to the mitochondrial outer membrane.[19] Additionally, Core interacting proteins Alpha Enolase (ENO1), Ferritin light chain (FTL) and SLC25A5 are perturbed in cancerous tissues from HCC patients with HCV infection.[20–22] These observations suggest potential roles for the above-identified Core protein interactions in HCV infection.

NS4B protein was found to interact with 45 host proteins (Table 1), nearly all of which are novel interactions. A significant proportion of these mapped to either the membrane component (GO:0016020; 17 of 45, 38%; $p = 0.04243$), or the extracellular region (GO:0005576; 12 of 45, 27%; $p = 3.64 \times 10^{-4}$), while five (APOA1, APOB, F2, FGG, LRG1) localise to both compartments. The NS4B interactions with a large number of host proteins (especially membrane proteins) may be crucial to its ability to induce membrane alterations termed membranous webs (MW), which host the HCV replication complex. It appears consistent with the suggested role of NS4B protein as an important hub in the virus–host interaction network.[16,23]

The absence of overlap between the PPIs identified in our approach and a previous large scale study[7] may be attributed to differences in screening approaches and experimental settings. Since our approach seeks to investigate the interactions associated with Core and NS4B in their membrane setting, it is more likely to fish out associations that may not be easily detected using the standard Y2H screening and co-immunoprecipitation assays such as those employed by de Chassey *et al.*[7] This situation would be especially true for NS4B, which unlike Core is not detected outside the membrane fraction, thus explaining the 45 interactions for NS4B reported by our approach compared to the one reported previously.[7] Our observations also highlight the significance of employing specific approaches to investigating different aspects of host–pathogen interactions in general.

2.2 Topological analysis of Core and NS4B protein interaction networks

To further understand the biological processes likely targeted by HCV, we expanded the Y2H-derived interactions by incorporating the secondary interactors of the human proteins that interact with the Core and NS4B proteins to derive extended PPI networks (Fig. 1a and b). The Core extended PPI network was made up of 208 entities (genes) with 1063 interactions between them (Table S1–S3, ESI†). For comparison, we also derived an extended PPI network for Core interactions reported by de Chassey *et al.*[7] (Table S4, ESI†). The NS4B extended PPI network was made up of 253 entities (genes) with 481 interactions between them (Table S1–S3, ESI†). First, we computed *node degree distribution* and *characteristic/average path length* measures to capture the topology of the Core and NS4B extended PPI networks (Fig. 3A and B). The degree of a protein, which corresponds to the number of its interacting partners, provides some insights into its biological relevance, since a higher degree may likely correspond to a higher ability to influence biological networks. It is also a useful measure to distinguish real world and random networks. In most interactome networks, a few nodes called "hubs" have a high degree and most nodes have a low degree, while in random networks the degree is uniformly distributed. Average path lengths provide an approximate measure of the relative ease and speed of dissemination of signalling information among network components.

Our analysis revealed that the average degree of the Core membrane protein yeast two-hybrid (MY2H) network (9.75) is on par with the human interactome (9.3), though shorter than the average degree estimated for the Core de Chassey extended network (14.7) (Table S4, ESI†). The Core MY2H network has a shorter characteristic path length vis-à-vis the human interactome (2.9 *versus* 4.04) and on par with that of Core de Chassey network (2.97), which is consistent with previous observations on the HCV protein infection network.[7] While the average degree of the NS4B MY2H network (3.4) is substantially lower than that of the

Table 1 List of host proteins interacting with HCV Core and NS4B proteins, identified by Y2H screens

List of host proteins interacting with the Core protein		
Gene ID	Official symbol	Description
1937	EEF1G	Eukaryotic translation elongation factor 1 gamma
1964	EIF1AX	Eukaryotic translation initiation factor 1A, X-linked
2023	ENO1	Enolase 1 (alpha)
2109	EFTB	Electron-transfer-flavoprotein, beta polypeptide
2512	FTL	Ferritin, light polypeptide
292	SLC25A5	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5
4720	NDUFS2	NADH dehydrogenase (ubiquinone) Fe–S protein 2, 49 kDa (NADH-coenzyme Q reductase)
5265	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
5688	PSMA7	Proteasome (prosome, macropain) subunit, alpha type, 7
81502	HM13	Histocompatibility (minor) 13
9804	TOMM20	Translocase of outer mitochondrial membrane 20 homolog (yeast)
List of host proteins interacting with the HCV NS4B protein		
Gene ID	Official symbol	Description
10130	PDIA6	Protein disulfide isomerase family A, member 6
10682	EBP	Emopamil binding protein (sterol isomerase)
116844	LRG1	Leucine-rich alpha-2-glycoprotein 1
1209	CLPTM1	Cleft lip and palate associated transmembrane protein 1
132299	OCIAD2	OCIA domain containing 2
1528	CYB5A	Cytochrome b5 type A (microsomal)
154467	C6orf129	Chromosome 6 open reading frame 129
1571	CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1
196410	METTL7B	Methyltransferase like 7B
200185	KRTCAP2	Keratinocyte associated protein 2
2013	EMP2	Epithelial membrane protein 2
2147	F2	Coagulation factor II (thrombin)
2220	FCN2	Ficolin (collagen/fibrinogen domain containing lectin) 2 (hucolin)
2266	FGG	Fibrinogen gamma chain
2267	FGL1	Fibrinogen-like 1
27173	SLC39A1	Solute carrier family 39 (zinc transporter), member 1
2731	GLDC	Glycine dehydrogenase (decarboxylating)
286451	YIPF6	Yip1 domain family, member 6
334	APLP2	Amyloid beta (A4) precursor-like protein 2
335	APOA1	Apolipoprotein A-I
338	APOB	Apolipoprotein B (including Ag(x) antigen)
3732	CD82	CD82 molecule
4267	CD99	CD99 molecule
4513	COX2	Cytochrome c oxidase subunit II
4538	ND4	NADH dehydrogenase, subunit 4 (complex I)
4924	NUCB1	Nucleobindin 1
51075	TMX2	Thioredoxin-related transmembrane protein 2
51643	TMBIM4	Transmembrane BAX inhibitor motif containing 4
517	ATP5G2	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit C2 (subunit 9)
5265	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
5355	PLP2	Proteolipid protein 2 (colonic epithelium-enriched)
5446	PON3	Paraoxonase 3
54657	UGT1A4	UDP glucuronosyltransferase 1 family, polypeptide A4
54658	UGT1A1	UDP glucuronosyltransferase 1 family, polypeptide A1
5479	PPIB	Peptidylprolyl isomerase B (cyclophilin B)
563	AZGP1	Alpha-2-glycoprotein 1, zinc-binding
56851	C15orf24	Chromosome 15 open reading frame 24
57817	HAMP	Hepcidin antimicrobial peptide
5950	RBP4	Retinol binding protein 4, plasma
6048	RNF5	Ring finger protein 5
6522	SLC4A2	Solute carrier family 4, anion exchanger, member 2 (erythrocyte membrane protein band 3-like 1)
7905	REEP5	Receptor accessory protein 5
84975	MFSD5	Major facilitator superfamily domain containing 5
9204	ZMYM6	Zinc finger, MYM-type 6
967	CD63	CD63 molecule