HCV infection. For instance, hepatic RELA mRNA levels are suppressed in chronic hepatitis C patients and associated with increased liver fibrosis,[35] while RELA and TRAF2 knockdowns with siRNA resulted in a substantial reduction in HCV replication.[36] Additionally, the interaction of HCV protein NS5A with TRAF2 inhibits NFKB activation and thereby disrupts the host immune response,[37] which also suggests probable links between HCV replication and NFKB activation.[36] The expression of NFKBIA, a critical regulator of NFKB activation, is known to be increased by HCV Core protein, resulting in suppression of pro-inflammatory genes downstream of NFKB.[38] IKBKG (IKK Gamma), an anti-apoptotic protein, is essential for NFKB activation and modulates tumour necrosis factor (TNF)-mediated apoptosis.[39] IKBKG mutations are associated with immune deficiency phenotype (Table S5, ESI†) and disruption of IKBKG activity may contribute to impaired immune response in HCV infection.

Some genes (PRKAA1 and PRKAB1) associated with the pro-inflammatory "Adipocytokine signaling pathway" also function in "Insulin signaling pathway", the disruption of which may contribute to insulin resistance (IR). IR is commonly observed in HCV infection and is associated with steatosis and fibrosis progression and impaired response to interferon-α anti-HCV therapy[40,41] and overexpression of HCV Core protein can induce IR in transgenic mice.[42] PRKAA1 expression is implicated in HCV infection,[43] suggesting that it may function in Core-induced IR.

The bulk of these genes (RELA, NFKB1, IKBKG, NFKBIA, TRAF2, PRKAA1) interact with SLC25A5 (Fig. 4a), suggesting that SLC25A5 may play an important role in Core perturbation of host innate immune response and liver fibrosis and possibly HCV replication.

2.3.1.2 Oxidative stress. HCV and other pathogens have evolved mechanisms to modulate host metabolism to facilitate their survival and propagation. ER stress, oxidative stress and mitochondrial dysfunction are some characteristic features associated with chronic hepatitis C infection.[44–46] Overexpression of HCV Core, NS3 and NS5A proteins is associated with increased production of reactive oxygen species (ROS), disrupted mitochondrial electron transport and altered Ca^{2+} homeostasis leading to perturbed Cytochrome c release from the mitochondria. This reaction together with induced insulin resistance eventually leads to accelerated fibrosis, HCC and DNA damage, while ensuring cell survival.[1,46–48]

Our Y2H screening identified NDUFS2, a mitochondrial protein essential for NADH to ubiquinone electron transfer, and ETFB, a mitochondrial electron-transfer flavoprotein as interacting partners of Core protein (Table 1). These interactions may permit Core protein to perturb oxidative electron transfer and consequently induce mitochondrial aberrations, which would be consistent with oxidative modification of mitochondrial respiratory complexes in pathogen infection.[49] Mutations in NDUFS2 (KEGG pathway "Oxidative phosphorylation" (10 of 208, $p = 0.0072$)) are associated with the OMIM phenotype "Mitochondrial complex I deficiency" (Table S5, ESI†), which causes several clinical disorders including liver disease. ETFB and its interacting partner ETFA are involved in beta-oxidation of fatty acids; ETFA displays a decreased activity during HCV replication, possibly contributing to steatosis,[50] suggesting that its interaction with ETFB may permit Core protein to interfere with mitochondrial fatty acid metabolism. This interference may contribute to lipid accumulation in hepatocytes, which facilitates viral entry, replication and assembly, insulin resistance and steatosis.[40,51–53]

In addition, Core may also induce mitochondrial perturbations *via* SLC25A5 protein which interacts with the subunits of ATP synthase (ATP5A1, ATP5B) and ATPase (ATP6V1A, ATP6V1B2, ATP6V0D1) enzymes. Interestingly, SLC25A5 (ANT2) in association with ATP synthase is involved in glycolytic ATP import into mitochondria, thus facilitating a shift to almost exclusively glycolytic metabolism in cancer cells.[54] Thus, Core interactions with SLC25A5 may provide an important link between oxidative stress and a shift in energy metabolism in HCV infection (see below).

2.3.1.3 Host energy metabolism and cell adhesion. HCV induction of oxidative stress is accompanied by a shift towards non-oxidative glucose metabolism to facilitate viral growth and is often characterised by elevated levels of glycolytic enzymes in the infected cells.[22,55] Our Y2H screening identified a novel interaction between Core protein and Alpha Enolase (ENO1), a key enzyme in the glycolytic pathway implicated in several disorders including metastatic cancer.[56] ENO1 was upregulated in response to HCV infection[22] and may be a key regulator in the shift towards glycolytic metabolism and viral replication. However, there is little understanding of the role of ENO1 in HCV infection and to the best of our knowledge no physical interaction between HCV proteins and ENO1 had been reported earlier.

Our network analysis revealed that some proteins interacting with ENO1 (ACTB, PXN) mapped to KEGG "Focal adhesion" pathway (Fig. 4b; Table 1, Table S3, ESI†). Focal adhesion regulates cell migration and its deregulation is linked to tumour progression and probably HCV propagation in the host.[7] Paxillin (PXN) is involved in cytoskeleton remodelling and a disruption in its activity by human papilloma virus (HPV) E6 protein is an important aspect of HPV pathogenesis.[57] Thus, the Core interaction with ENO1 may be important in HCV mediated cytoskeleton remodelling to facilitate viral propagation. SLC25A5 (ANT2), also implicated in cancer cell glycolysis,[54] interacts with proteins (ACTB, EGFR, PXN, VCL) involved in "Focal adhesion" (Fig. 4b; Table 1, Table S3, ESI†). Most of these interactions are associated with reasonable confidence levels.[58,59] Enhancement of EGFR signalling by HCV NS3/4A and NS5A proteins is important for viral replication and persistence.[60,61] Interestingly, EGFR is upregulated in lymph node metastasis in HCC, while SLC25A5 levels are downregulated,[20] suggesting that the Core interaction with SLC25A5 may be involved in regulating EGFR activity and host energy metabolism and consequently HCV replication and propagation.

HCV infection is also characterised by elevated hepatic iron levels (induced by ROC), which contributes to abnormalities in glucose metabolism and induction of insulin resistance, eventually leading to fibrosis.[62,63] Our Y2H screening identified interaction between Core and FTL, a subunit of the

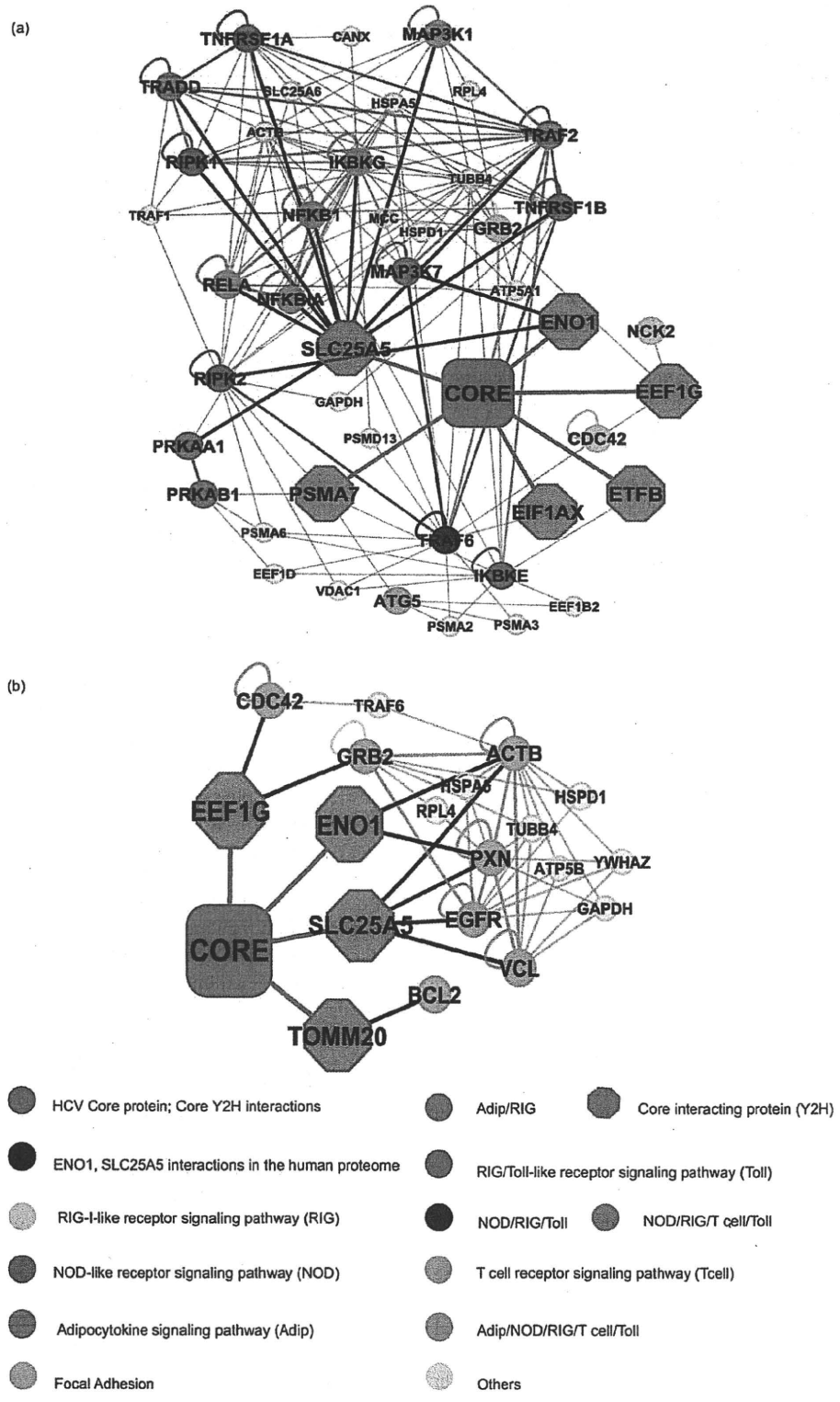

Fig. 4 Functional associations of the Core network. (a) Network illustration of interactions between HCV Core interacting proteins and host proteins mapped to five KEGG pathways— "RIG-I-like receptor signaling", "T cell receptor signaling", "NOD-like receptor signaling", "Toll-like receptor signaling" and "Adipocytokine signaling". SLC25A5 and ENO1 interactions are highlighted in black. (b) Network illustration of interactions between HCV Core interacting proteins and host proteins mapped to KEGG "Focal adhesion" pathway. The node sizes differ for better clarity and do not reflect any topological attributes.

intracellular iron storage protein Ferritin, which may facilitate Core induced disruptions in host iron metabolism.

2.3.2 Functional analysis of NS4B interaction network. To further understand the biological context of NS4B interactions, we examined the overrepresentation of specific biological associations within the NS4B PPI network. The analysis revealed an enrichment of three KEGG pathways ($p \leq 0.05$) "Complement and coagulation cascades", "Hematopoietic cell lineage" and "Oxidative phosphorylation" likely to be significantly affected by NS4B interactions in HCV infection (Table 2). To assess the robustness of enriched KEGG pathway associations, we explored the overlap of PPIs in the NS4B network with those documented in the I2D database.[27] We observed that 396 of the 436 secondary interactions (see Materials and Methods) in the NS4B interaction network were represented in I2D;[27] the genes associated with these interactions were mapped to two enriched KEGG pathways ("Complement and coagulation cascades" and "Hematopoietic cell lineage"; data not shown), both of which were among the enriched KEGG pathway associations for the NS4B interaction network.

2.3.2.1 Complement and coagulation. A significant number of proteins in the NS4B network mapped to KEGG pathway "Complement and coagulation cascades" (21 of 254, 5%; $p = 2.49 \times 10^{-14}$; Fig. 5, Table 2, Table S3, ESI†), which functions in the host innate immune response against pathogen invasion and clearance of viral antigens from the blood of the infected hosts. Overexpression of HCV Core protein activates the coagulation pathway *via* hepatic inflammation, which contributes to fibrosis, disruption of host T-cell mediated

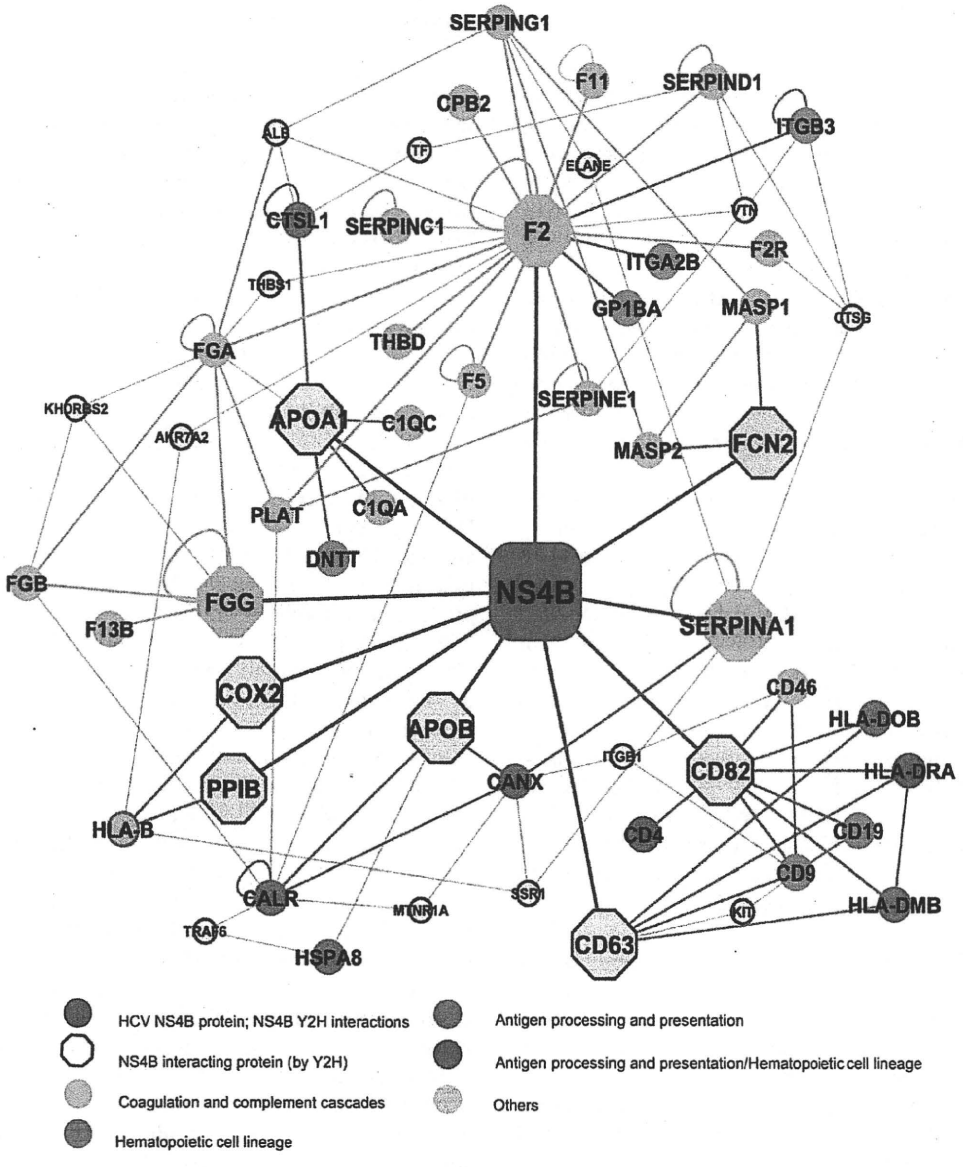

Fig. 5 Network illustration of HCV NS4B interactions associated with KEGG pathways Complement and coagulation cascades, Hematopoietic cell lineage and Antigen processing and presentation. The node sizes differ for better clarity and do not reflect any topological attributes.

immune response and viral persistence. Blocking complement activation can ameliorate the effects of HCV induced hepatic inflammation, suggesting the complement pathway as an attractive target for anti-HCV therapy.[64–67]

Our Y2H screening identified thrombin (F2), serine protease inhibitor SERPINA1 and fibrinogen gamma chain (FGG) as primary interacting partners of NS4B (Table 1, Fig. 2 and 5). Thrombin is implicated in liver cell fibrosis by inducing hepatic stellate cell proliferation,[68] while SERPINA1 defects are implicated in chronic liver disease, hepatitis C and HCC[69–71] and elevated FGG expression and plasma fibrinogen levels are associated with HCC progression.[72] To the best of our knowledge, however, no interactions between these proteins and NS4B had been reported earlier. Interactions with thrombin and SERPINA1 may allow NS4B to directly perturb the host immune response *via* complement activation and thus contribute to HCV pathogenesis and HCC.

2.3.2.2 Hematopoietic development and antigen presentation. Chronic HCV infection induces a reduced natural killer (NK) cell frequency and activity, resulting in impaired cytokine secretion, a stunted immune response and viral persistence.[73] Analysis of the NS4B network revealed an enrichment of proteins (9 of 254, 4%; $p = 0.0213$) mapped to KEGG pathway "Hematopoietic cell lineage", which functions in the generation of NK cells and T and B lymphocytes. The T and B lymphocytes play an important role in innate and adaptive immune response.[74] Our analysis suggested that interactions with host proteins CD63, CD82 and APOA1 (Apolipoprotein A-I) that physically interact with the components of "Hematopoietic cell lineage" may permit NS4B to influence and possibly impair NK cell development and host immune response (Fig. 5).

We also observed an enrichment of proteins (9 of 254, 4%; $p = 0.0155$) mapped to the KEGG pathway "Antigen processing and presentation". Our network analysis showed that these proteins interact with four NS4B interacting proteins: CD63 and CD82 (interacting with HLA-DMB; HLA-DOB and HLA-DRB); APOB (Apolipoprotein B; interacting with CALR, CANX and HSPA8) and APOA1 (interacting with CTSL1 and DNTT) (Fig. 5). The dysfunction of CD63 and CD82 is implicated in cell-to-cell transmission of HIV-I,[75] suggesting that their interaction with NS4B may be crucial to the spread of HCV in the host. APOB associated cholesterol is positively associated with HCV assembly and entry[76,77] thus, NS4B interactions with APOB (and possibly APOA1) may modulate host lipid metabolism and immune response to facilitate HCV pathogenesis and steatosis.[16]

2.3.2.3 Oxidative stress. NS4B overexpression induces ER stress, unfolded protein response (UPR) and production of ROS, which eventually triggers oxidative stress.[78] However, whether NS4B may induce mitochondrial dysfunction by direct associations remains unclear.

Our Y2H screening identified three mitochondrial proteins COX2 (MT-CO2; Cytochrome c oxidase II), ND4 (mitochondrially encoded NADH dehydrogenase 4) and ATP5G2 (ATP synthase subunit C2) to interact with NS4B (Table 1). These proteins map to the KEGG pathway "Oxidative phosphorylation" and are components of the mitochondrial oxidative machinery. Disruptions in ND4 activity[79] and Cytochrome c oxidase deficiency[80] are associated with oxidative stress. Thus, NS4B interactions with the mitochondrial oxidative machinery may allow the virus to influence host oxidative metabolism and potentially induce insulin resistance, steatosis and fibrosis.

The analysis of NS4B interactions may help unravel further associations in HCV infection. Our Y2H screening identified RBP4 (retinol binding protein 4; Table 1) to interact with NS4B, which is suggested to be inversely correlated with chronic HCV infection.[81] However, a precise understanding of the significance of the interactions identified here would be apparent only with further experimental investigations.

2.4 Validation of novel interactions for their role in HCV replication and release

Traditionally, viral and host proteins associated with various steps in HCV lifecycle (internalisation, replication, assembly and release) have been the primary targets in studies focused on anti-HCV strategies. Due to the lack of a suitable model system for HCV infection, cell culture-based systems for HCV RNA replication and infectious viral particle production have been extensively exploited to understand HCV–host interactions and identify potential anti-HCV drug targets.[4,82–84] Our observations by virtue of extended PPI networks suggested novel and potentially crucial roles of host proteins ENO1, PXN, SLC25A5 and VCL (vinculin), an important component of cell–cell junctions,[85] in HCV replication and persistence in the host.

To further explore the roles of these proteins in HCV life cycle, we performed cellular assays to assess the impact of ENO1, PXN, SLC25A5 and VCL siRNA knockdowns on HCV replication and release. Since HCV-production systems established with HCV JFH1 infectious strain (genotype 2a) isolates alone are capable of both efficient replication and production of infectious viral particles,[86,87] JFH1 was used to infect the Huh7OK1 cell line 24 h after transfection with each siRNA (see Materials and Methods). The infected cells were harvested after 72 h post-infection and the expression of each host protein was assessed by qRT-PCR (Fig. 6A). Supernatant viral RNA was significantly decreased by the knockdown of ENO1, but was not affected by the knockdown of PXN in the infected cells, while SLC25A5 knockdown resulted in a slight but statistically significant increase in the amount of the supernatant viral RNA (Fig. 6B). Intracellular viral RNA was significantly reduced by the knockdown of ENO1, but was unaffected by the knockdown of PXN and SLC25A5 (Fig. 6C), suggesting that ENO1 and SLC25A5 regulate HCV replication and assembly/secretion, respectively. VCL knockdown had no significant effect on the intracellular and supernatant virus RNA in the infected cells (data not shown).

To assess the impact of the knockdown of these genes on other HCV genotypes, we repeated the HCV replication assays using Huh-7 cells including HCV replicons derived from JFH1 and Con1 (genotype 1b) infectious strains. Unlike Huh7OK1 cells infected with JFH1, these replicon systems facilitate studies on HCV replication but not infectious virus

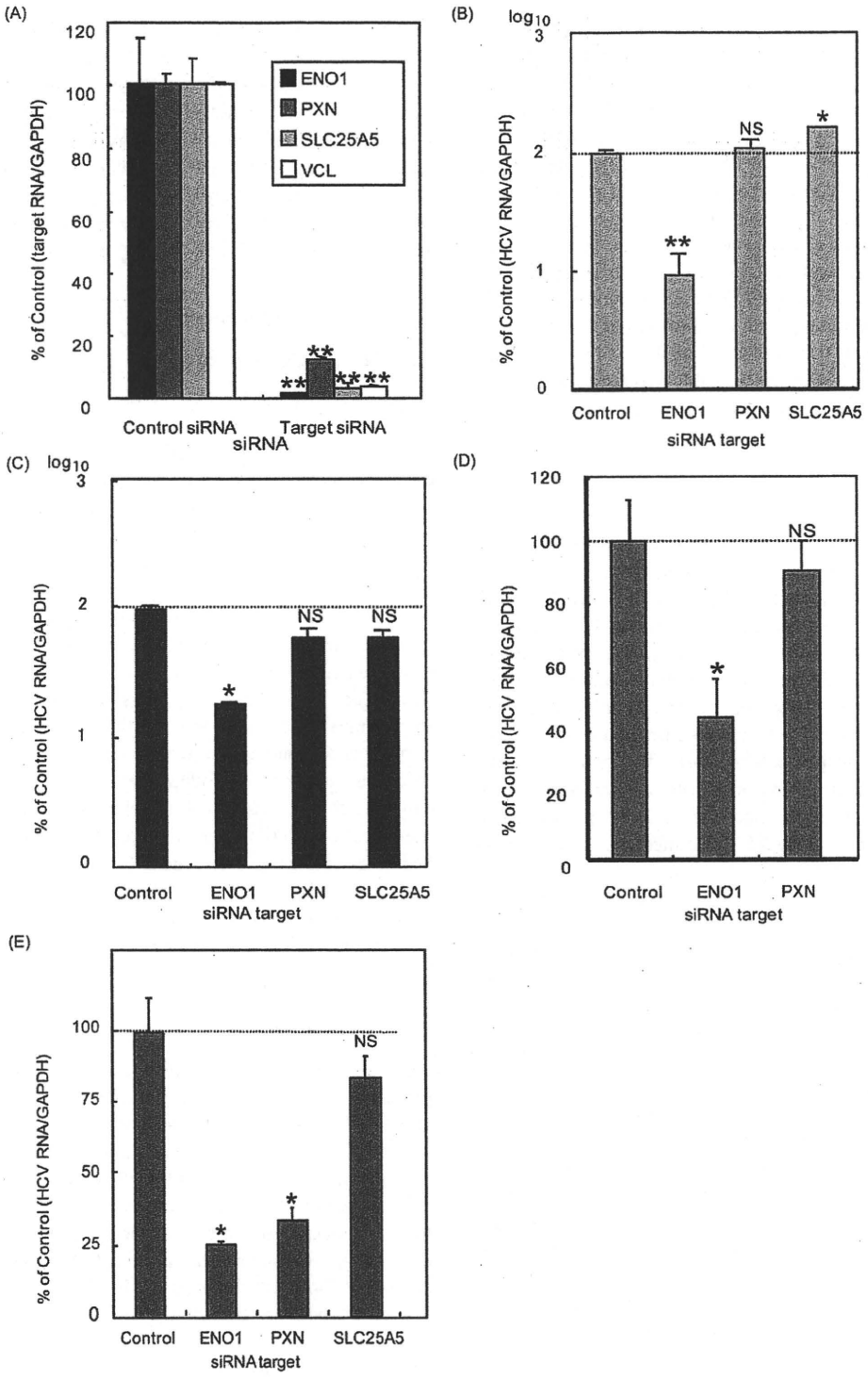

Fig. 6 Effects of knockdown of host protein candidates on HCV propagation and replication. Host proteins ENO1, PXN, SLC25A5 and VCL were suppressed by RNAi in Huh7OK1 cells infected with HCV JFH1 strain (genotype 2a; A, B and C) and in Huh-7 cells including JFH1 replicon RNA (D) or Con1 replicon (genotype 1b; E). Amounts of mRNA of the intracellular host proteins (A), the supernatant viral RNA (B) and the intracellular viral RNA (C, D and E) were estimated. Each value was represented as the percentage of the corresponding quantity measured for the cells transfected with the control siRNA. $*P < 0.05$; $**P < 0.01$; NS: not statistically significant.

secretion.[88] ENO1 knockdown suppressed HCV replication in Huh-7 cells, including either subgenomic replicon RNA of JFH1, which does not encode structural proteins (Fig. 6D), or of Con1, which encodes all viral proteins (Fig. 6E). On the other hand, PXN knockdown in Huh-7 cells including Con1 impaired HCV replication significantly (Fig. 6E), in contrast

to no effect in HuhOK1 cells including JFH1, as described above. This result was not due to the differences in cell lines and the expression of Core protein, because PXN knockdown in Huh-7 cells including JFH1 had no significant effect on intracellular HCV RNA production (Fig. 6D). This observation suggests that PXN possibly regulates the replication of HCV genotype 1b but not of genotype 2a. The standard therapy of pegylated interferon-α plus rebavirin treatment typically achieves less than 50% sustained virological response in HCV genotype 1b infected patients compared to 80% in genotype 2 and 3 infected patients.[89] Therefore, the identification of novel specific (PXN) and non-specific (ENO1) regulators of replication in different HCV genotypes provides potentially attractive targets for developing more effective combinatorial therapies with interferon/RBV treatment.

3. Conclusions

We describe here our observations of PPIs between HCV encoded and host proteins. We first derived a set of experimentally determined interactions between HCV proteins Core and NS4B and host proteins *via* Y2H screens customised for detecting membrane protein interactions. We proceeded to map these interactions onto an overall interaction network that comprised a repertoire of connections potentially required for the two viral proteins to link up with and modulate the components of the host cellular networks. We then employed a network-based approach to further understanding the biological context of these connections in HCV pathogenesis. Core interacting protein SLC25A5 manifested as a potentially important link between the Core protein and the host immune machinery. ENO1, by virtue of gathered interactions, may also function in regulating HCV replication and propagation. We identified 45 previously uncharacterised interactions for NS4B protein that may be crucial for NS4B to function as an important hub in HCV–host interactions. Further investigation of these interactions may help expand substantially our understanding of NS4B function in viral pathogenesis and its potential as an anti-HCV target.

Our observations were then used to prioritise four of the 459 potential candidates in the two extended PPI networks for follow-up experimental investigations through cellular assays based on siRNA knockdowns for HCV genotypes 1b and 2a. These assays validated Core interacting protein ENO1 as a novel regulator of HCV replication and a potentially minor role of SLC25A5 in HCV secretion. In addition, our assays also suggested a genotype 1b specific role of the host protein PXN (which interacts with ENO1 and SLC25A5) in HCV replication. These observations highlighted the attractiveness of the selected host proteins as suitable targets in potentially more effective targeted anti-HCV strategies. The genetic variability of HCV has facilitated the emergence of drug resistance against antiviral drugs that target HCV components. Therefore, antivirals that target less mutable host proteins critical to viral pathogenesis, preferably with minimal adverse side effects, may provide attractive alternatives to existing therapies. That we were able to experimentally validate three of the four genes selected for experimental characterisation reinforces the strengths of elaborate network-based approaches employing diverse functional associations and knowledge-based inputs for identification and prioritisation of suitable targets for experimental and therapeutic investigation. Our study also provides a generic framework for investigating host–pathogen interactions. Such investigation may help identify common themes associated with pathogen (especially viral) infection and help develop effective broad spectrum strategies aimed at ameliorating pathogen (viral) infections.

4. Materials and methods

4.1 Y2H membrane protein assay

Screening for the genes encoding the host proteins that interact with HCV Core and NS4B proteins derived from genotype 1b Con1 strain was performed with a Y2H membrane protein kit system (MoBiTec, Göttingen, Germany) as per the manufacturers specifications. Human adult liver libraries that were constructed based on pPR3 were purchased from MoBiTec and expressed as a fusion protein fused to the N-terminal or the C-terminal end of Nub-G. The cDNA of the Core (or NS4B) encoding region of the HCV polyprotein from the Con1 strain (genotype 1b) was amplified by polymerase chain reaction (PCR) and cloned into the pBT3-N vector (MoBiTec). The screening process was repeated three times to maximise the confidence in the interactions. The total number of screened transformants was 4×10^6, which is about twice the amount of independent clones in the libraries employed for the screening. The clones including genes encoding the Core (or NS4B) interacting proteins were grown on the histidine- and adenosine-deficient culture plate containing a high concentration (10 mM) of 3-amino-1,2,4-triazole (3AT), to remove weak interactions and minimise false positive data. The positive colonies were identified from the blue colour by beta-galactosidase assay (data not shown).

4.2 PPI resources

Secondary interactors of the Core and NS4B interacting proteins were retrieved from BioGRID[90] (version 2.0.63) and PPIView[91] databases. These secondary interactions were merged, filtered for redundancy and appended to the Y2H interactions to infer an extended PPI network. To estimate the robustness of the interactions employed to construct extended PPI networks and infer enriched functional associations (see below), we examined the Human PPI dataset from the I2D database[27] for its overlap with the BioGRID[90]- and PPIView[91]-derived secondary interactions.

4.3 Network topology analysis

Network components were visualised using Cytoscape 2.6,[92] while the network properties such as *node degree distribution* and *average shortest path* measures were computed using Cytoscape NetworkAnalyzer plugin.[93] The degree of node v is defined as the number of nodes directly connected to it, *i.e.*, its first neighbours. Node degree distribution $P(k)$ is the number of nodes with a degree k for $k = 0,1,2\ldots$. By fitting a line on datasets, such as node degree distribution data, the

pattern of their dependencies can be visualised. NetworkAnalyzer considers only data points with positive coordinate values for fitting the line where the power law curve of the form $y = \beta x^a$ is transformed into a linear model $\ln y = \ln \beta + a \ln x$ and the *R*-squared value (coefficient of determination) is computed on logarithmised data, which provides a measure of how well the data points fit to the curve. The *average shortest path length*, also known as the *characteristic path length*, gives the expected distance between two connected nodes.

4.4 Functional analysis by characterisation of enriched biological associations

GO associations retrieved from the GO consortium,[26] biological pathway data from KEGG[24] and disease phenotype associations from OMIM[25] were used to assign functional annotations to the genes in the extended networks. The enrichment of specific biological associations within each network was estimated by Fisher's exact test ($p \leq 0.05$) using the module fisher.test from the R statistical package (http://www.R-project.org). The inferred *p*-values were further adjusted for multiple test correction to control the false discovery rate using the Benjamini and Hochberg procedure.[94,95]

4.5 RNAi and transfection

The siRNA pair targets to ENO1, PXN and SLC25A5 and VCL were purchased from Ambion (Ambion, Austin, TX). Stealth™ RNAi Negative Control Low GC Duplex (Invitrogen) was used as a control siRNA. Each siRNA duplex was introduced into the cell lines using lipofectamine RNAiMax (Invitrogen, Carlsbad, CA). Ambion ID numbers of siRNA duplex of ENO1, PXN, SLC25A5 and VCL were S4682, S44629, S1375 and S14764, respectively. The replicon cell line, described below, was transfected with each siRNA at a final concentration of 20 nM as per the manufacturer's protocol and then seeded at 2.5×10^4 cells per well of a 24-well plate. The transfected cells were harvested at 72 h posttransfection. The Huh7OK1 cell line, described below, was transfected with each siRNA at a final concentration of 20 nM as per the manufacturer's protocol and then seeded at 2.5×10^4 cells per well of a 24-well plate. The transfected cells were infected with HCV JFH1 infectious strain (genotype 2a) at a MOI of 0.05 at 24 h posttransfection. The resulting cells were harvested at the indicated times.

4.6 Quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was prepared from cell and culture supernatant using the RNeasy mini kit (QIAGEN) and QIAamp Viral RNA Mini Kit (QIAGEN), respectively. First-strand cDNA was synthesised using High capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA) with random primers. Each cDNA was estimated by Platinum SYBR Green qPCR Super Mix UDG (Invitrogen) as per the manufacturer's protocol. Fluorescent signals of SYBR Green were analysed with ABI PRISM 7000 (Applied Biosystems). The HCV internal ribosomal entry site (IRES) region and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were amplified with the primer pairs 5′-GAGTGTCGTGCAGCCTCCA-3′ and 5′-CACTCGCAAGCACCCTATCA-3′, and 5′-GAAGGTCGGAGTCAACGGATT-3′ and 5′-TGATGACAAGCTTCCCGTTCTC-3′, respectively.[96] The quantities of the HCV genome and other host mRNAs were normalised with that of GAPDH mRNA. ENO1, PXN, and SLC25A5 genes were amplified using the primer pairs 5′-TGCCTCCTGCTCAAAGTCAACCAGA-3′ and 5′-GGTTTCTGAAGTTCCTGCCGGCAAA-3′; 5′-TACTGTCGCAAGGACTACTTCGAC-3′ and 5′-AAGAAGCTGCCGTTCACGAATG-3′; 5′-AGTCTGCCTCCTCTTTCAACATGAC-3′ and 5′-GGACCACGCAGTCTATAATGCCTTT-3′, respectively. VCL gene was amplified using the primer pair 5′-GGTATTTGATGAGAGGGCAGCTAAC-3′ and 5′-GGCTGAATGTTGGCCATAGCTAC-3′.

4.7 Cell lines and virus infection

Cells from the Huh7OK1 cell line are highly permissive to HCV JFH1 strain (genotype 2a) infection compared to Huh 7.5.1 and exhibit highest propagation efficiency for JFH1.[96] These cells were maintained at 37 °C in humidified atmosphere and 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with nonessential amino acids (NEAA), sodium pyruvate, and 10% fetal calf serum (FCS). The human hepatoma cell line Huh-7, harbouring the full genome of HCV Con1 strain (genotype 1b), was prepared as described by Pietschmann *et al.*[88] We also established Huh-7 cell line harbouring the subgenome of the JFH1 strain by the transfection of the plasmid pSGR-JFH1.[97] The Huh7-derived cell lines harbouring a full length HCV replicon were maintained in DMEM containing 10% FCS, nonessential amino acids, sodium pyruvate and 1 mg ml^{-1} G418 (Nakarai Tesque, Tokyo, Japan). The viral RNA of JFH1 was introduced into Huh7OK1 as described by Wakita *et al.*[86] The viral RNA of JFH1 derived from the plasmid pJFH1 was prepared as described by Wakita *et al.*[86]

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Factors affecting efficacy in patients with genotype 2 chronic hepatitis C treated by pegylated interferon alpha-2b and ribavirin: reducing drug doses has no impact on rapid and sustained virological responses

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SUMMARY. Reducing the dose of drug affects treatment efficacy in pegylated interferon (Peg-IFN) and ribavirin combination therapy for patients with hepatitis C virus (HCV) genotype 1. The aim of this study was to investigate the impact of drug exposure, as well as the baseline factors and the virological response on the treatment efficacy for genotype 2 patients. Two-hundred and fifty patients with genotype 2 HCV who were to undergo combination therapy for 24 weeks were included in the study, and 213 completed the treatment. Significantly more patients who achieved a rapid virological response (RVR), defined as HCV RNA negativity at week 4, achieved a sustained virological response (SVR) (92%, 122/133) compared with patients who failed to achieve RVR (48%, 38/80) ($P < 0.0001$). Multivariate logistic-regression analysis showed that only platelet counts [odds ratio (OR), 1.68; confidence interval (CI), 1.002–1.139] and RVR (OR, 11.251; CI, 5.184–24.419) were independently associated with SVR, with no correlation being found for the mean dose of Peg-IFN and ribavirin for RVR and SVR. Furthermore, in the stratification analysis of the timing of viral clearance, neither mean dose of Peg-IFN ($P = 0.795$) nor ribavirin ($P = 0.649$) affected SVR in each group. Among the patients with RVR, the lowest dose group of Peg-IFN (0.77 ± 0.10 μg/kg/week) and ribavirin (6.9 ± 0.90 mg/kg/day) showed 100% and 94% of SVR. Hence, RVR served as an important treatment predictor, and drug exposure had no impact on both SVR and RVR in combination therapy for genotype 2 patients.

Keywords: chronic hepatitis C, drug exposure, genotype 2, peginterferon and ribavirin combination therapy.

INTRODUCTION

The current standard of care for chronic hepatitis C (CHC) patients consists of combination therapy using pegylated interferon (Peg-IFN) and ribavirin [1–3]. Large, randomized clinical trials have demonstrated that 42–52% of hepatitis C virus (HCV) genotype 1 'difficult-to-treat' patients achieved sustained virological response (SVR), whereas 76–84% of HCV genotype 2 or 3 infected patients treated with Peg-IFN and ribavirin achieved SVR [4–6]. It also has been shown that in HCV genotype 2 and 3 infected patients, 24-week treatment regimens are just as effective as 48-week regimens [6,7]. Therefore, current guidelines recommend a 24-week treatment for these patients in contrast to 48 weeks for genotype 1 patients [1–3]. However, as side effects are common and treatment is expensive for this therapy, it would be ideal to be able to further reduce the total amount of drug medication

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CHC, chronic hepatitis C; c-EVR, complete early virological response; ETR, end of treatment response; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; IFN, interferon; NPV, negative predictive value; Peg-IFN, pegylated interferon; RVR, rapid virological response; SVR, sustained virological response.

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without loss of treatment efficacy for genotype 2 and 3 patients.

In HCV genotype 1 patients, reducing drug doses affects treatment efficacy. In our investigation of HCV genotype 1 patients, the rate of complete early virological response (c-EVR), defined as HCV RNA negativity at week 12, was affected by the mean dose of Peg-IFN during the first 12 weeks dose-dependently ($P < 0.0001$) [8]. Furthermore, we showed that only 4% relapse was found in patients given ≥12 mg/kg/day of ribavirin among those with c-EVR, and the relapse rate showed a decline in relation to the increase in the dose of ribavirin ($P = 0.0002$) [9]. On the contrary, it remains to be determined whether treatment efficacy can be preserved by further reducing both drug doses in genotype 2 and 3 patients. Because lower doses are expected to cause fewer adverse effects, it is important to find whether reduced drug doses can be used while retaining efficacy.

In the present study, we retrospectively evaluated the efficacy of Peg-IFN alpha-2b and ribavirin combination therapy for 24 weeks in patients infected with HCV genotype 2 and analysed the factors that affected the treatment efficacy, with particular interests in the drug impact of Peg-IFN and ribavirin.

PATIENTS AND METHODS

Patient selection and study design

Patients considered to be eligible for this study were those infected with HCV genotype 2 who underwent Peg-IFN alpha-2b (Schering-Plough K.K., Tokyo, Japan) and ribavirin (Schering-Plough K.K.) combination therapy from December 2005 to July 2007 at 29 medical institutions taking part in the Osaka Liver Forum and had completed the 24-week observation after a clinical course of 24 weeks. Patients with the following criteria were excluded: hepatitis B virus or human immunodeficiency virus coinfection, decompensated liver disease, severe cardiac, renal, haematological or chronic pulmonary disease, poorly controlled psychiatric disease, poorly controlled diabetes and immunologically mediated disease. Liver biopsy had been performed within 24 months prior to the treatment, and histological results were classified according to the METAVIR scoring system [10].

Written informed consent was obtained from each patient, and the study protocol was reviewed and approved according to the ethical guidelines of the 1975 Declaration of Helsinki by institutional review boards at the respective sites.

Patients were treated with Peg-IFN alpha-2b plus ribavirin for the duration of the study of 24 weeks. Peg-IFN alpha-2b and ribavirin dosages were based on body weight according to the manufacturer's instructions: Peg-IFN alpha-2b was given subcutaneously weekly (45 kg or less, 60 μg/dose; 46–60 kg, 80 μg/dose; 61–75 kg, 100 μg/dose; 76–90 kg, 120 μg/dose; 91 kg or more, 150 μg/dose), and ribavirin was given orally daily (60 kg or less, 600 mg/day; 61–80 kg, 800 mg/day; 81 kg or more, 1000 mg/day). The drug doses were also modified based on the manufacturer's instructions according to the intensity of the haematologic adverse effects.

Virological tests

Serum HCV RNA level was quantified by PCR assay (COBAS Amplicor HCV Test v2.0, Chugai-Roche Diagnostics, Tokyo, Japan), with a sensitivity limit of 5000 IU/mL and a dynamic range from 5000 to 5 000 000 IU/mL [11].

Serum HCV RNA was assessed by qualitative PCR assay (COBAS Amplicor HCV Monitor Test v2.0, Chugai-Roche Diagnostics), with a detection limit of 50 IU/mL [12].

Assessment of efficacy

Serum HCV RNA (qualitatively or quantitatively) was measured at weeks 4, 8, 12 and 24 during treatment and after 24 weeks of follow-up without treatment. Patients were classified as having a rapid virological response (RVR) if serum HCV RNA was undetectable (<50 IU/mL) at week 4 and at the end of treatment response (ETR) at week 24 of treatment. SVR was defined as undetectable HCV RNA at week 24 after treatment. Patients with an ETR who seroreverted to HCV RNA during follow-up were classified as relapsers.

Drug exposure

The amounts of Peg-IFN alpha-2b and ribavirin actually taken by each patient during the treatment period were evaluated by reviewing the medical records. The mean doses of both drugs were calculated individually as averages on the basis of body weight at baseline; Peg-IFN alpha-2b expressed as μg/kg/week and ribavirin as mg/kg/day.

Data collection

The medical records were retrospectively reviewed and the factors necessary for this examination were extracted: age, sex, body weight, body mass index (BMI), basic laboratory assessments, liver histology, quantitative and qualitative HCV RNA, dose of Peg-IFN alpha-2b and ribavirin received at each administration, and the response to treatment.

Statistical analysis

This study was a retrospective study and, for treatment results and the analysis of related factors, analysis was carried out only for cases in which the treatment had been completed (per-protocol analysis). Continuous variables are reported as the mean with standard deviation (SD) or

median level, while categorical variables are shown as the count and proportion. In univariate analysis, the Mann–Whitney *U*-test was used to analyse continuous variables, while chi-squared and Fisher's exact tests were used for analysis of categorical data. Variables with $P < 0.05$ at univariate analysis were retained for the multivariate logistic-regression analysis. Stepwise and multivariate logistic-regression models were used to explore the independent factors that could be used to predict a virological response. The significance of trends in values was determined with the Mantel–Haenszel chi-square test. For all tests, two-sided *P*-values were calculated and the results were considered statistically significant if $P < 0.05$. Statistical analysis was performed using the SPSS program for Windows, version 15.0J (SPSS, Chicago, IL, USA).

RESULTS

The baseline characteristics for the total cohort are shown in Table 1. Most of the patients were female (56%) with a mean age of 54 years. Seventy per cent of the patients were treatment naïve. Of the 250 patients, liver biopsies were performed for 174 patients, and 18 of them had advanced fibrosis (F 3–4).

Of the total of 250 patients, 37 (15%) were withdrawn from treatment because of adverse events: decreased haemoglobin ($n = 10$), psychiatric problems including depression ($n = 9$), fatigue ($n = 3$), thrombocytopenia, neutropenia, pyrexia, rash, cerebral haemorrhage, bleeding of ocular fundus, dyspnea, dizziness, jaundice, transaminase rise, gastrointestinal symptoms ($n = 1$) and other adverse events ($n = 4$). Eight of these patients who discontinued treatment prematurely had SVR (8/37; 22%).

Drug adherence

Seventy-nine of the 213 patients (37%) required dose reduction of Peg-IFN alpha-2b, 99 (46%) of ribavirin because of adverse events (not including patients who later discontinued treatment because of adverse event). Neutropenia (24/79; 30%) and thrombocytopenia (24/79; 30%) were the most common adverse events for dose reduction of Peg-IFN alpha-2b, and decreased haemoglobin (82/99; 83%) for that of ribavirin.

Virological response

Of the 213 patients who completed 24 weeks of treatment and 24 weeks of follow-up, 160 (75%) patients were clear of HCV RNA at week 4, 191 (90%) at week 8, 196 (92%) at week 12. ETR was observed for 195 (92%), and SVR for 160 (75%). The relapse rate was 18% (35/195).

Virological response according to the timing of viral clearance

Positive and negative prediction of sustained virological response according to the timing of viral clearance

We examined SVR rates according to the timing of viral clearance for the case in which HCV RNA was cleared during the treatment (Fig. 1a). The SVR rate was 92% (122/133) for patients clear of HCV RNA until week 4, 64% (37/58) from week 5 until week 8, 20% (1/5) from week 9 until

Table 1 Baseline demographic and viral characteristics of patients

Number of cases	250	
Age (years)*	54.0 ± 12.4	(22–76)
Sex (male/female)	110/140	
Body weight (kg)*	60.3 ± 11.7	(39–99)
Body mass index (kg/m^2)*	23.1 ± 3.2	(16–35)
Past IFN therapy (naïve/experienced)†	175/70	
HCV RNA (KIU/mL)‡	1700	(4–5000 <)
Fibrosis (0/1/2/3/4)§	18/98/40/14/4	
Activity (0/1/2/3)§	15/81/70/8	
White blood cells (/mm^3)*	5210 ± 1,750	(2100–13 870)
Neutrophils (/mm^3)*	2700 ± 1,250	(590–9020)
Red blood cells (×10^4/mm^3)*	436 ± 48	(307–554)
Haemoglobin (g/dL)*	13.9 ± 1.4	(10–18)
Platelets (×10^4/mm^3)*	18.3 ± 6.4	(4–41)
ALT (IU/L)*	79 ± 77	(13–581)
γ-GTP (U/L)*	56 ± 65	(7–479)
Creatinine(mg/dL)*	0.7 ± 0.1	(0.4–1.1)

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase. *Values expressed as mean ± SD (range), †interferon treatment history was not known for five patients, ‡values expressed as median (range), §data for 76 patients are missing.

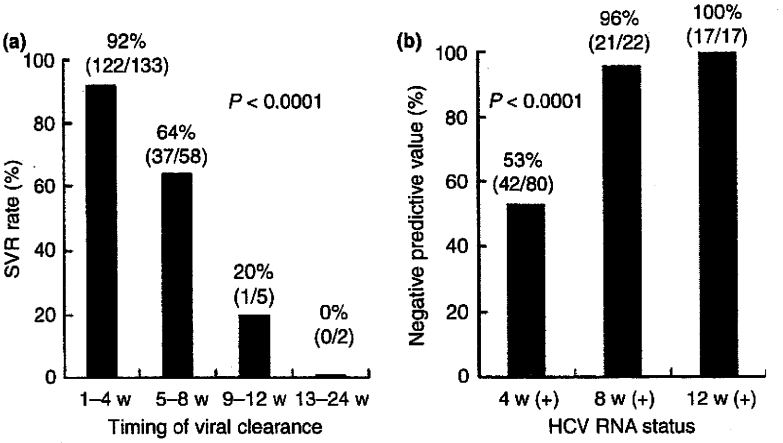

Fig. 1 (a) SVR rates according to timing of viral clearance. The number above each bar shows the percentage, and the numbers inside parentheses show the number of patients showing responses over the total number in the subgroup. The timing of viral clearance was time-dependently correlated with SVR ($P < 0.0001$). (b) Negative predictive values according to time of HCV RNA positivity. The number above each bar shows the percentage, and the numbers inside parentheses show the number of patients showing responses over the total number in the subgroup. The time of HCV RNA positivity was time-dependently correlated with NPV ($P < 0.0001$).

week 12 and 0% (0/2) from week 13 until week 24. The Mantel–Haenszel chi-square test showed that SVR rates were diminished with a delay in the timing of viral clearance becoming late ($P < 0.0001$). Significantly, more patients who attained RVR achieved final SVR (92%, 122/133) than patients who failed to attain RVR (48%, 38/80; $P < 0.0001$).

Next, we examined the negative predictive value (NPV) for the proportion of patients with treatment failure among those with HCV RNA persistence at week 4, 8 and 12 (Fig. 1b). NPV was 53% at week 4, 96% at week 8 and 100% at week 12. Only one of the 22 patients with positive HCV RNA at week 8 reached SVR.

Predictors of sustained virological response

Both pretreatment and treatment factors that could be associated with the response to Peg-IFN and ribavirin combination therapy were compared between patients with and without SVR in Table 2. This univariate analysis showed that age ($P = 0.029$), baseline HCV RNA level ($P = 0.033$), past IFN treatment history ($P = 0.028$), platelets counts ($P = 0.020$) and having RVR ($P < 0.0001$) contributed to achievement of SVR. Factors that were significantly associated with SVR by univariate analysis were then analysed by multivariate logistic regression analysis. SVR was attained independent of high platelet counts [odds ratio (OR) 1.070, 95% confidence interval (CI) 1.003–1.140, $P = 0.040$] and having RVR (OR 11.526, 95% CI 5.317–24.984, $P < 0.0001$; Table 3). As for drug doses, the mean dose of Peg-IFN alpha-2b was 1.32 ± 0.27 μg/kg/week in patients with SVR and 1.27 ± 0.29 μg/kg/week in those without SVR ($P = 0.130$), while that of ribavirin was 10.2 ± 1.9 and 10.2 ± 2.0 mg/kg/day ($P = 0.949$), respectively. Thus, neither Peg-IFN nor ribavirin drug exposure during the full treatment period affected attainment of SVR.

Predictors of rapid virological response

To delineate features that might help identify patients most likely to reach RVR, we also analysed these factors because having RVR turned out to be one of the most powerful predictors of SVR attainment. By univariate and multivariate logistic-regression analyses, RVR was attained independent of younger age (OR 0.648, 95% CI 0.494–0.850, $P = 0.002$) and lower baseline HCV RNA level (OR 0.964, 95% CI 0.944–0.984, $P < 0.0001$; Tables 4 & 5). The mean dose of Peg-IFN alpha-2b during the first 4 weeks was 1.31 ± 0.27 μg/kg/week in patients with RVR and 1.31 ± 0.29 μg/kg/week in those without RVR ($P = 0.259$), that of ribavirin was 10.1 ± 1.8 mg/kg/day and 10.3 ± 2.1 mg/kg/day ($P = 0.637$), respectively. Thus, neither Peg-IFN nor ribavirin drug exposure during the first 4 weeks had an impact on attainment of RVR.

Virological response according to drug exposure and the timing of viral clearance

Impact of drug exposure on sustained virological response

To more closely evaluate the impact of drug exposure on virological response, we classified the average doses of both drugs into four categories (Peg-IFN alpha-2b: up to 0.9 μg/kg/week, from 0.9 to >1.2 μg/kg/week, from 1.2 to >1.5 μg/kg/week, from 1.5 μg/kg/week; ribavirin: up to

Table 2 Factors associated with SVR among patients who completed the treatment – univariate analysis

Factor	SVR (n = 160)	Non-SVR (n = 53)	P-value
Age (years)*	52.4 ± 12.6	56.9 ± 10.2	0.029
Sex (male/female)	66 / 94	26 / 27	0.202
Body weight (kg)*	59.5 ± 11.5	59.9 ± 12.5	0.896
Body mass index (kg/m^2)*	22.8 ± 3.1	22.8 ± 3.5	0.817
HCV RNA (KIU/mL)†	1170	1600	0.033
Past IFN therapy (naive/experienced)‡	116/41	31/22	0.028
Fibrosis (F 0–2/3–4)§	106/10	30/5	0.247
Activity (A 0–1/2–3)§	62/54	20/15	0.847
White blood cells (/mm^3)*	5260 ± 1680	4720 ± 1500	0.078
Neutrophils (/mm^3)*	2740 ± 1270	2420 ± 1020	0.186
Red blood cells (×10^4/mm^3)*	435 ± 44	437 ± 55	0.820
Haemoglobin (g/dL)*	13.9 ± 1.3	14.0 ± 1.5	0.441
Platelets (×10^4/mm^3)*	19.0 ± 6.0	16.5 ± 6.2	0.020
ALT (IU/L)*	86 ± 89	64 ± 45	0.514
γ-GTP (U/L)*	54 ± 67	58 ± 59	0.512
Creatinine (mg/dL)*	0.7 ± 0.1	0.7 ± 0.1	0.457
Mean Peg-IFN dose (μg/kg/week)*	1.32 ± 0.27	1.27 ± 0.29	0.130
Mean ribavirin dose (mg/kg/day)*	10.2 ± 1.9	10.2 ± 2.0	0.949
RVR (yes/no)	122/11	38/42	<0.0001

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; CI, confidence interval. *Values expressed as mean ± sd, †values expressed as median, ‡interferon treatment history was not known for three patients, §data for 62 patients are missing.

Table 3 Factors associated with SVR among patients who completed the treatment – multivariate analysis

Factor	Category	Odds ratio	95% CI	P-value
Age (years)	By 10	–	–	NS
HCV RNA (KIU/mL)	By 100 KIU/mL	–	–	NS
Platelets (×10^4/mm^3)	By 1 × 10^4/mm^3	1.068	1.002–1.139	0.045
Past IFN therapy	Naïve/experienced	–	–	NS
RVR	Yes/no	11.251	5.184–24.419	<0.0001

IFN, interferon; HCV, hepatitis C virus; CI, confidence interval.

8 mg/kg/day, from 8 to >10 mg/kg/day, from 10 to >12 mg/kg/day, from 12 mg/kg/day). SVR rates relative to the mean drug doses during the full treatment period and the timing of HCV RNA clearance are shown in Table 6. As also shown in Fig. 1a, the respective rates for SVR according to the timing of viral clearance were 92% in patients clear of HCV RNA until week 4, 64% from week 5 until week 8 and 14% from week 9 until week 24. On the contrary, according to mean drug doses, the respective rates for SVR were 89% (24/27), 73% (11/15), 79% (85/107) and 82% (40/49) in patients who received Peg-IFN up to 0.9 μg/kg/week, from 0.9 to >1.2 μg/kg/week, from 1.2 to >1.5 μg/kg/week and from 1.5 μg/kg/week, respectively, and 80% (24/30), 80% (40/50), 82% (68/83) and 79% (27/34) in patients who received ribavirin up to 8 mg/kg/day, from 8 to >10 mg/kg/day, from 10 to >12 mg/kg/day and from 12 mg/kg/day, respectively. If the category of the timing of viral clearance was the same, the respective rates for SVR attainment according to the mean doses of both Peg-IFN and ribavirin were similar. Furthermore, multivariate analysis by the Mantel–Haenszel chi-square test showed that neither the mean dose of Peg-IFN (P = 0.795) nor ribavirin (P = 0.649) affected SVR rates after stratification of the timing of viral clearance. Among the patients with RVR, SVR rates were as high as 88–100% regardless of Peg-IFN alpha-2b medication, and the least medicated group (<0.9 μg/kg/week, the mean dose with SD was 0.77 ± 0.10 μg/kg/week, 0.50–0.89) showed 100% of SVR rate (19/19). Similarly, SVR rates were as high as 91–94% regardless of ribavirin medication among the patients with RVR, and 17 of 18 patients (94%) in the least medicated group (<8 mg/kg/day, the mean dose with SD was 6.9 ± 0.90 mg/kg/day, 5.0–7.9)

Table 4 Factors associated with RVR among patients who completed the treatment – univariate analysis

Factor	RVR (n = 133)	Non-RVR (n = 80)	P-value
Age (years)*	51.9 ± 12.3	56.3 ± 11.3	0.010
Sex (male/female)	60/73	32/48	0.279
Body weight (kg)*	60.2 ± 11.6	58.6 ± 11.9	0.276
Body mass index (kg/m^2)*	22.9 ± 3.2	22.6 ± 3.1	0.369
HCV RNA (KIU/mL)†	1050	1800	0.001
Past IFN therapy (naive/experienced)‡	97/34	50/29	0.068
Fibrosis (F 0–2/3–4)§	86/8	50/7	0.315
Activity (A 0–1/2–3)§	51/43	31/26	1.000
White blood cells (per mm^3)*	5300 ± 1760	4850 ± 1400	0.205
Neutrophils (per mm^3)*	2740 ± 1290	2530 ± 1090	0.340
Red blood cells (×10^4/mm^3)*	440 ± 45	432 ± 49	0.628
Haemoglobin (g/dL)*	13.9 ± 1.4	13.9 ± 1.4	0.975
Platelets (×10^4/mm^3)*	18.9 ± 6.1	17.5 ± 6.1	0.170
ALT (IU/L)*	87 ± 93	69 ± 52	0.630
γ-GTP (U/L)*	57 ± 71	53 ± 53	0.658
Creatinine (mg/dL)*	0.7 ± 0.1	0.7 ± 0.1	0.203
Mean Peg-IFN dose (μg/kg/week)*	1.31 ± 0.27	1.31 ± 0.29	0.259
Mean ribavirin dose (mg/kg/day)*	10.1 ± 1.8	10.3 ± 2.1	0.637

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; CI, confidence interval. *Values expressed as mean ± SD, †values expressed as median, ‡interferon treatment history was not known for three patients, §data for 62 patients are missing.

Table 5 Factors associated with RVR among patients who completed the treatment – multivariate analysis

Factor	Category	Odds ratio	95% CI	P-value
Age (years)	By 10	0.648	0.494–0.850	0.002
HCV RNA (KIU/mL)	By 100 KIU/mL	0.964	0.944–0.984	<0.0001

HCV, hepatitis C virus; CI, confidence interval.

achieved SVR. In addition, we examined the drug impact on SVR in the patients with the least medication of both drugs (<0.9 μg/kg/week of Peg-IFN and <8 mg/kg/day of ribavirin). Nine patients were categorized into this group and six of these patients achieved SVR (67%); patients with RVR had a significantly higher SVR rate (100%, 5/5) than patients without RVR (25%, 1/4; P = 0.048). Thus, SVR attainment was dependent on time, not on drug dose.

DISCUSSION

In the present study, we found that having RVR and high platelet counts were statistically associated with reaching SVR according to multivariate analysis. The timing of viral clearance was closely related to the treatment effect in patients with genotype 2, similar to the case for those with genotype 1. Ninety-two per cent of SVR was observed for patients with RVR and, conversely, 96% of the patients with HCV RNA positivity at week 8 showed non-SVR. The predictability of SVR based on EVR, defined as a decline of at least 2-log from the baseline of the HCV RNA level at week 12, has been assessed, and genotype 1 patients who have failed to reach EVR are recommended to discontinue the treatment after 12 weeks, because the likelihood of SVR is 0–3% in the absence of EVR [5,13]. On the basis of our examination of patients with genotype 2, not EVR, but 8-week monitoring of the HCV RNA level can be used.

As a significant factor for SVR, not liver fibrosis, but the platelet count was selected. Everson *et al.* [14] reported that patients with low platelet counts (≤12.5 × 10^4/mm^3) achieved lower SVR rates than patients with normal platelet counts (>12.5 × 10^4/mm^3) even in the case of patients with the same category of liver fibrosis treated by Peg-IFN plus ribavirin combination therapy. Thus, independent of liver fibrosis, thrombocytopenia itself seems to participate in treatment failure, although the mechanism remains unknown.

Our study also demonstrated that younger age (OR 0.648, 95% CI 0.494–0.850, P = 0.002) and lower HCV RNA level (OR 0.964, 95% CI 0.944–0.984, P < 0.0001) were statistically associated with reaching an RVR. Zeuzem *et al.* [7] previously reported that pretreatment viral load was not

Table 6 SVR rates according to Peg-IFN alpha-2b and ribavirin exposure and the timing of viral clearance among patients with virological response during the treatment

Timing of viral clearance (week)	Peg-IFN dose (μg/kg/week)				Ribavirin dose (mg/kg/day)				Total
	<0.9	0.9–1.2	1.2–1.5	1.5≤	<8	8–10	10–12	12≤	
1–4	100% (19/19)	91% (10/11)	92% (65/71)	88% (28/32)	94% (17/18)	92% (33/36)	91% (51/56)	91% (20/22)	92% (122/133)
5–8	63% (5/8)	33% (1/3)	64% (19/30)	71% (12/17)	58% (7/12)	54% (7/13)	74% (17/23)	60% (6/10)	64% (37/58)
9–24	–	0% (0/1)	17% (1/6)	–	–	0% (0/1)	0% (0/4)	50% (1/2)	14% (1/7)
Total	89% (24/27)	73% (11/15)	79% (85/107)	82% (40/49)	80% (24/30)	80% (40/50)	82% (68/83)	79% (27/34)	81% (160/198)

*P = 0.795 for comparison of the four Peg-IFN groups after stratification of the timing of viral clearance. **P = 0.649 for comparison of the four ribavirin groups after stratification of the timing of viral clearance.

associated with reaching RVR in genotype 2 patients. In contrast, Dalgard *et al.* [15] reported that independent predictors of RVR in genotype 2 or 3 patients were male gender, younger age (≤40 years) and low viral load (≤400/KIU/mL). The influence of viral load on reaching RVR remains controversial in the Peg-IFN and ribavirin combination therapy in genotype 2 patients, but patients with lower viral load seem favoured to reach HCV RNA levels below the detection limit, that is, to attain RVR, if the virological response is the same.

Recently, because of substantial adverse effects and costs associated with this therapy, studies have been carried out to determine the possibility of further reducing the total amount of drug medication without compromising antiviral efficacy in HCV genotype 2 and 3 patients. There seem to be two ways to achieve. One is by shortening the treatment duration, and the other is by decreasing the doses of the treatment drugs. With respect to the former, several studies on genotype 2 patients have been reported. At first, some studies of small numbers of subjects demonstrated that cumulatively analysed genotype 2 and 3 patients had high SVR rates up to 12 to 16 weeks of therapy (82–94%), similar to patients subjected to 24-week therapy (76–95%) [16–19]. However, further prospective investigation of large numbers of subjects revealed that shortening the treatment duration was associated with an increase in the rate of relapse and that significantly higher relapse rates led to lower SVR rates (71–81.1%), even among those with RVR [15,20,21]. The latest study by Mangia *et al.* [22] showed that shortened therapy after RVR was acceptable only for patients who had no signs of advanced liver fibrosis and low BMI. Considering the results of these trials, shortened therapy is regarded as optional treatment for selected patients displaying favourable baseline characteristics. Therefore, shortening treatment duration from 24 weeks should not be generally recommended for patients who are infected genotype 2 or 3 and can tolerate 24-week Peg-IFN and ribavirin combination therapy.

Another attempt to improve the treatment tolerability for genotype 2 or 3 patients has focused on dose reduction of treatment drugs. Weiland *et al.* [23] examined low-dose Peg-IFN alpha-2a (135 μg weekly) with a weight-based standard-dose of ribavirin (11 mg/kg daily) for genotype 2 and 3 patients. They demonstrated that SVR rates of 86% were achieved, which is equal to those in previous representative randomized controlled studies of standard dose Peg-IFN therapy (76–84%) [4–6]. In contrast, Ferenci *et al.* [24] examined the efficacy of standard-dose Peg-IFN alpha-2a (180 μg weekly) with low-dose ribavirin (400 mg daily) in comparison with standard-dose Peg-IFN alpha-2a (180 μg weekly) and ribavirin (800 mg daily) for genotype 2 and 3 patients, and demonstrated that there was no difference between the two treatment groups with respect to SVR rates (64% with 400 mg/day compared with 69% with 800 mg/day) and relapse rates (20% with 400 mg/day compared

with 17% with 800 mg/day). These studies showed that either drug dose can be reduced for genotype 2 and 3 patients without compromising antiviral efficacy. In the present study, neither Peg-IFN nor ribavirin drug exposure participated in reaching RVR and SVR. In particular, more than 90% of patients having RVR achieved SVR regardless of the drug exposure level, as long as the mean Peg-IFN dose was over 0.5 μg/kg/week and ribavirin was over 5.0 mg/kg/day. The results of our study suggested that genotype 2 patients may receive reduced levels of both drug doses on the condition that they can complete the full 24-week course of combination therapy. Randomized, prospective trials that reduced both Peg-IFN and ribavirin should be conducted for CHC patients to clarify this.

In the present study, while the treatment outcome was independent of the individual ribavirin exposure in patients who had completed the 24-week treatment, the most common reason to withdraw the treatment was decreased haemoglobin because of ribavirin medication. Based on the results of randomized controlled trials [6], using a ribavirin dose of 800 mg/day is recommended for genotype 2/3 patients [1–3]. However, several studies have shown that some patients cannot tolerate even this suboptimal ribavirin dose. This is a serious problem for patients with the risk of anaemia, especially elderly patients. The ageing of patients is progressing around the world, requiring improvement in treatment tolerability. Recently, Andriulli *et al.* [25] examined the effect of ribavirin in a 12-week course of therapy on CHC genotype 2 patients with RVR in two groups, one continuing with ribavirin and the other receiving Peg-IFN alpha-2a alone after week 6. The relapse rates were higher (46% *vs* 17%; $P < 0.001$) and overall SVR rates were lower (54 *vs* 82%; $P < 0.001$) in patients who stopped receiving ribavirin at week 6. Thus, ribavirin medication throughout the treatment period is necessary to raise the SVR rate even in genotype 2 or 3 patients with RVR. In the present study, the ribavirin dose could be reduced without loss of efficacy for genotype 2 patients, as long as the patients were treated for 24 weeks. Therefore, in the patients with the risk of anaemia, it would be better to reduce the dose of ribavirin before anaemia arises rather than being forced to discontinue the combination therapy because of anaemia caused by ribavirin medication. We previously reported that in CHC patients treated by IFN or Peg-IFN in ribavirin combination therapy, a decline of haemoglobin concentration by 2 g/dL at the end of 2 weeks from the start of the treatment can be used to identify patients likely to develop severe anaemia [26,27]. This kind of predictive factor for the progression to severe anaemia can be of much help in reducing ribavirin with appropriate timing.

Our study has some limitations. First, it is a retrospective study, and we could not obtain complete information for all patients. However, this is the first study of Peg-IFN and ribavirin combination therapy in which the drug dose of Peg-IFN and ribavirin taken by each patient was assessed independently for HCV genotype 2 patients. Our results can be taken as an evidence offering suggestions for the treatment of CHC genotype 2 patients. Second, this cohort included patients with different histories of past IFN treatment. Patients who had failed to recover with previous IFN-based treatment were likely to experience treatment failure again [28]. Therefore, we examined the predictors of treatment response separately according to treatment history, and confirmed that in both naïve and treatment-experienced patients, the mean dose of Peg-IFN and ribavirin showed no correlation with SVR or RVR in both groups.

In conclusion, our study demonstrates that RVR is an important treatment predictor and more than 90% of patients having RVR achieve SVR with combination therapy of Peg-IFN and ribavirin for genotype 2 infected CHC patients regardless of the drug exposure. Further prospective, randomized studies are necessary to assess whether the standard or a reduced dose of each drug can produce equivalent outcomes.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Absence of invariant natural killer T cells deteriorates liver inflammation and fibrosis in mice fed high-fat diet

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Abstract
Background Invariant natural killer T (iNKT) cells have been suggested to play critical roles in a wide range of immune responses by acting in a proinflammatory or anti-inflammatory manner. Nonalcoholic steatohepatitis (NASH) is a chronic liver disease progressing to advanced cirrhosis and hepatocellular carcinoma. Despite the abundance of iNKT cells in the liver, their role in the pathogenesis of NASH remains obscure. Here, we investigated their role in the development of diet-induced steatosis/steatohepatitis.
Methods We used BALB/c wild-type mice and Jα18-deficient (KO) mice lacking iNKT cells fed either a normal diet or a high-fat diet (HFD). The liver and blood were collected from these mice to examine liver inflammation, steatosis, and fibrosis at the indicated time points.
Results KO mice fed the HFD, compared with control mice fed the HFD, exhibited a clearly higher serum alanine aminotransferase level and a greater number of hepatic inflammatory foci, although there was no significant difference in hepatic lipid retention between these groups of mice. The HFD enhanced hepatic messenger RNA expression of inflammatory cytokines and chemokines in KO but not in control mice. The HFD also increased the proportion of hepatic CD4 T cells and CD8 T cells that composed hepatic inflammatory foci in KO mice, but not in the controls. Prolonged feeding with the HFD augmented liver fibrosis in KO but not in control mice.
Conclusions These findings indicate that iNKT cells play a protective role against liver inflammation progressing to fibrosis, but not against steatosis, enhanced by dietary excess fat, suggesting a key role of these cells in NASH pathogenesis.

Keywords iNKT cells · Nonalcoholic fatty liver disease · Nonalcoholic steatohepatitis · Cytokine · Chemokine

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
iNKT	Invariant natural killer T
NK	Natural killer
TCR	T cell receptor
Th	T helper
IFN	Interferon
IL	Interleukin
WT	Wild type
ND	Normal diet
HFD	High-fat diet
KO	Jα18-deficient
ALT	Alanine aminotransferase
RT-PCR	Reverse transcription polymerase chain reaction
H&E	Hematoxylin–eosin
SEM	Standard error of the mean
TNF	Tumor necrosis factor
CCL	Chemokine (C–C motif) ligand
CXCL	Chemokine (C–X–C motif) ligand

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver disorders ranging from nonalcoholic steatosis to nonalcoholic steatohepatitis (NASH), which can develop to progressive disease including advanced liver fibrosis and hepatocellular carcinoma [1, 2]. Prolonged overnutrition causes accumulation of free fatty acid and triglycerides within the liver, which is referred to as steatosis. Simple steatosis leads to a predisposition for steatohepatitis, which exhibits inflammatory cell accumulation and fibrosis in the liver in addition to the steatosis [1, 2]. To transform from steatosis to steatohepatitis, several key biological responses such as oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, and abnormal cytokine properties have been reported to be required [1–4]. However, the immunological aspect, in particular, that is involved in the development of steatosis/steatohepatitis remains to be fully elucidated.

Invariant natural killer T (iNKT) cells are characterized by the expression of surface markers of natural killer (NK) cells together with a single invariant T cell receptor (TCR) encoded by Vα14-Jα18 in mice and Vα24-Jα18 in humans [5]. These cells are included within the population of T cells expressing NK cell markers, also known as NKT cells [5, 6]. iNKT cells recognize glycolipid antigens presented in association with the major histocompatibility complex class Ib molecule CD1d [5], which is expressed on a variety of cells including dendritic cells, B cells, and stellate cells, as well as hepatocytes in the liver [5, 7, 8]. Following the recognition of antigens via TCR, iNKT cells have the ability to produce the T-helper (Th) 1 cytokine, interferon (IFN)-γ, and the Th2 cytokines, interleukin (IL)-4, -5, and -13, modulating subsequent immune responses [5, 6, 9]. These cells have been shown to play a proinflammatory role in some immune responses and an anti-inflammatory role in other immune responses [5, 6, 9]. iNKT cells most frequently reside in the liver in mice [10, 11]. Although humans appear to have proportionally fewer iNKT cells than mice, human iNKT cells also preferentially reside in the liver [12, 13]. Several lines of evidence indicate that the number of NKT cells is dysregulated in the development of NAFLD. Hepatic iNKT cells or NK1.1+ CD3+/TCRβ+ NKT cells, for instance, have been reported to decrease with the development of steatosis in wild-type (WT) as well as leptin-deficient ob/ob mice [14–17]. A reduced level of peripheral Vα24+ NKT cells has been associated with human NAFLD [18]. On the other hand, CD56+ CD3+ NKT cells have been recently reported to be increased in the livers of patients with NAFLD [19]. Also, the adoptive transfer of NK1.1+ CD3+ NKT cells has been shown to alleviate hepatic steatosis in ob/ob mice [20]. However, the precise role of NKT cells in the pathogenesis of NAFLD has not been investigated in the presence of a deficiency of these cells.

In the present study, we used iNKT cell-deficient as well as WT mice fed either a normal diet (ND) or a high-fat diet (HFD), and examined the role of these cells in the development of HFD-induced steatosis/steatohepatitis. We found that the lack of iNKT cells, together with the HFD, led to liver inflammation, which was characterized by the enhanced gene expression of inflammatory cytokines and chemokines and by T cell accumulation. We also found that prolonged liver inflammation in the absence of iNKT cells developed to liver fibrosis which was strongly enhanced by the HFD. This study delineated an immunoregulatory function of iNKT cells and their key role against liver inflammation progressing to fibrosis exacerbated by an HFD, which might represent a clinical aspect of human progressive NAFLD.

Materials and methods

Animals and animal care

Specific pathogen-free BALB/c WT mice were purchased from CLEA Japan (Tokyo, Japan) as needed. Breeding pairs of BALB/c Jα18-deficient (KO) mice [21, 22] were provided by Drs. Masaru Taniguchi and Ken-ichiro Seino (RIKEN, Yokohama, Japan). The KO mice were confirmed to have no iNKT cells by the use of mouse-CD1d tetramers loaded with α-galactosylceramide in the flow cytometry procedure described below (data not shown). These mice were kept in isolation facilities at the Institute of Experimental Animal Science, Osaka University. They were housed in groups of five in filter cages and were maintained in a temperature-controlled, specific-pathogen-free room on 12-h light and dark cycles with ad libitum access to water and diet as indicated.

Experimental protocol

Male mice used in the experiments were fed an irradiated HFD consisting of 56.7% of the calories from fat (HFD32; CLEA Japan) or an irradiated ND consisting of 14% of the calories from fat (CRF-1; Oriental Yeast, Osaka, Japan), starting from when the mice were 6–8 weeks old. In preliminary experiments, we monitored the body weight of the WT mice and KO mice fed the ND or HFD every 2 weeks after the initiation of feeding, because a gain of body weight usually parallels the level of hepatic steatosis as well as obesity. We did not observe a gain of body weight of more than 25% until 4 weeks after the initiation of feeding. In mice fed the HFD, the body weight gain reached a plateau around 14–16 weeks after the initiation