A role for B cells to transmit hepatitis C virus infection

Isabelle Desombere

Sciensano

Freya Van Houtte

Ghent University

Ali Farhoudi

Ghent University

Lieven Verhoye

Laboratory of Liver Infectious Diseases, Ghent University

Caroline Buysschaert

Ghent University

Yvonne Gijbels

Ghent University Hospital

Sibyl Couvent

Ghent University

Wilfried Swinnen

MSOC

Hans Van Vlierberghe

Ghent University Hospital

André Elewaut

Ghent University

Zania Stamatakis

University of Birmingham <https://orcid.org/0000-0003-3823-4497>

Philip Meuleman

Ghent University

Jane McKeating

University of Oxford <https://orcid.org/0000-0002-7229-5886>

Geert Leroux-Roels (✉ geert.lerouxroels@ugent.be)

Ghent University

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Abstract

Hepatitis C virus (HCV) is highly variable and transmits through infected blood to establish a chronic liver infection in the majority of patients. Our knowledge of the infectivity of clinical HCV strains is hampered by the lack of in vitro cell culture systems that support efficient viral replication. We previously reported that laboratory strains of HCV associated with non-permissive B cells could trans-infect hepatocytes and thereby evade host neutralizing antibody responses, suggesting a role for B cells in HCV transmission. To evaluate this hypothesis, we assessed the ability of B cells and sera from recent (<2 years) or chronic ($\geq$ 2 years) infections to infect humanized liver chimeric mice. HCV was efficiently transmitted by B cells from chronically infected patients whereas the sera were non-infectious. In contrast, we noted that B cells from recently infected patients failed to transmit HCV to the mice, whereas all serum samples were infectious. Only patients with circulating anti-glycoprotein antibodies harbored genomic HCV-RNA in B cells. Taken together, our studies provide direct in vivo evidence for HCV transmission by B cells and these findings may have clinical implications for prophylactic and therapeutic antibody-based vaccine design.

Introduction

Hepatitis C virus (HCV) is a positive-strand RNA virus in the hepacivirus genus of the *Flaviviridae* family and an important blood-borne pathogen. HCV infects an estimated 2–3% of the world's population and is a major cause of hepatitis, cirrhosis, and hepatocellular carcinoma (1). HCV replicates with an error-prone RNA-dependent RNA polymerase and persists as a diverse quasispecies (QS) in infected individuals (2). The development of highly effective antiviral agents has dramatically changed the treatment of this chronic disease (3–5), enabling curative therapies for many patients (6). However, the cost of such treatments limits accessibility in endemic regions and HCV continues to pose a major public health burden. Worldwide the number of hepatitis-related deaths is increasing and the WHO published strategies for global eradication of hepatitis viruses by 2030 (7). Successful elimination will rely on the curing of infected patients and preventive measures to reduce the rate of new infections, highlighting the urgent need for a vaccine. The high incidence of new infections in injecting drug users and those accidentally exposed to infected blood, justifies the continued search for a prophylactic vaccine (8–10).

Our understanding of the correlates that associate with a protective immune response is limited (11, 12). Approximately 25% of individuals acutely infected with HCV will spontaneously clear their infection (13) and several studies have reported an association between clearance and the early development of neutralizing activity in the plasma (14, 15). Broadly neutralizing HCV-specific antibodies (bnAbs) targeting the envelope E1E2 glycoproteins can be generated during natural infection (16–19), however, they often develop late in infection, after envelope glycoprotein mutations have given rise to new viruses that escape immune detection (20, 21). Our limited ability to propagate clinical isolates of HCV has compromised our understanding of the role antibodies play in neutralizing virus infection (22–24). HCV RNA levels are routinely measured in the plasma as a surrogate marker of viremia and yet the infectivity of these viral genomes is unknown. The majority of studies measuring bnAbs quantify the infectivity of

laboratory HCV strains for human liver hepatoma cells that lack Fc receptor expression and our knowledge of antibody-dependent enhancement (ADE) of infection is limited. Virus-specific antibodies that facilitate virus entry into target cells have been described for dengue virus, a related virus member of the *Flaviviridae* (25, 26), raising questions on the role of antibodies and infectivity of HCV immune complexes. In the lymphocytic choriomeningitis virus (LCMV) model it has been shown that B cells displaying glycoprotein-specific antibodies on their surface are selectively infected by the virus. The elimination of these B-cells by virus-specific cytotoxic cells leads to a delay in neutralizing-antibody production that may contribute to the establishment of persistent virus infections (27).

The major reservoir supporting HCV replication is hepatocytes, however, extra-hepatic manifestations of the disease have been reported (28). There is ample epidemiologic evidence for an association of chronic HCV infection with B cell lymphoproliferative disorders such as mixed cryoglobulinemia (MC) and B cell non-Hodgkin's lymphoma, both characterized by clonal expansions of B cells. The finding that HCV eradication by successful antiviral therapy often induces clinical resolution/regression of these B cell dysfunctions, highlights a pathogenic role for HCV (29–31). HCV RNA was reported to associate with B cells (32–34), however, there is limited evidence of productive viral replication in these immune cells. We previously reported (35) that the laboratory JFH-1 strain of HCV could associate with CD40L-stimulated B cells and although there was no evidence of virus replication in the B cells, the complex was infectious for human hepatoma cells. This led us to conclude that B cells can capture HCV and trans-infect liver cells, however, the relevance of these observations for the infectivity of clinical HCV strains has not been studied. However, this capacity of B cells to facilitate infection without active replication has also been shown in other conditions. Although HIV-1 cannot replicate in B cells, it can bind to the CD21 receptor expressed on mature B cells thus facilitating cell-to-cell spread (36) .

Since there are no fully immunocompetent small-animal models that support HCV infection, we used a well characterized immunodeficient murine xenograft uPA model (37–39) to investigate the role of B cells in defining HCV infectivity. Herein we carried out genetic profiling of HCV from a cohort of recent and chronic infected patients and evaluated the ability of peripheral blood-derived B cells and sera to infect this mouse model (40). Early, monophyletic viral isolates from human serum were infectious in this mouse model, but B cells from the same donors were not. Surprisingly, polyphyletic sera from chronic patients with demonstrable HCV-specific Abs were not infectious whereas B cells from the same patients transmitted HCV. Our experiments highlight a protective role for Abs in controlling cell-free chronic HCV transmission, and reveal a previously unrecognized role for B cells to transmit HCV infection.

Results

Differential infectivity of HCV in recent and chronic HCV infection. To evaluate and compare the infectivity of HCV in recent and chronic infection we identified a cohort of well characterized patients where clinical material was available. Subjects with a history of injection drug use (IDU) or blood transfusion, where the duration of infection was known, are summarized in **Table 1**. Patients with infections of < 2 years were classified as recently infected and those with infections of ≥ 2 years as

chronic. Patient P15, who was initially infected with a genotype 3a virus (gt3a) and was super-infected with gt1a, donated two biological specimens with a 3-year interval; one is considered recent, the second chronic (**Supplementary Fig. 1a and 1b**). HCV is an RNA virus and exists as a quasispecies (QS) where the genetic complexity associates with the duration of infection (2, 20, 41). Sequencing the viral encoded first and second envelope (E1E2) genes in viral RNA isolated from serum (**Supplementary Fig. 2**) enabled us to classify subjects into low, medium or high QS-complexity groups (Fig. 1a). Apart from P06, an atypical chronic carrier infected for > 10 years without developing nAbs, all subjects with low QS-complexity were recently infected. All subjects with medium or high QS-complexity were chronic. Pie charts representing the genetic variation in the five major variable E2 regions are depicted (Fig. 1a, upper panel) and show an association with duration of infection.

We assessed the infectivity of sera or B cells from all subjects listed in Table 1 in human liver uPA-SCID-mice. Sera were injected via the peritoneal cavity (IP, containing $\pm 3 \times 10^5$ IU HCV RNA) or spleen (IS, containing 7×10^4 IU HCV RNA) (Fig. 1b, upper panel) and infection assessed by measuring peripheral viral RNA in sequential samples collected over the 8-week duration of the study. Sera from 8 of 9 patients classified with low or monophyletic QS-complexity (8 recent and 1 atypical chronic) induced an early and high-titered viremia in the mice (Fig. 1b, left side of upper panel). The failure of P07 serum to infect the mice may be due to the lower viral loads (1.8×10^4) of this inoculum (uPA-SCID mouse B674L). In contrast, sera from 8 of 10 chronic infected patients with polyphyletic QS diversity failed to establish an infection in the mice (Fig. 1b, right side of upper panel). Patients P05 and P08 with a high QS-complexity behaved atypically as their serum was infectious in 1 of 3 mice tested for P05 and in 2 of 3 mice for P08. From both patients, serum was harvested at different time points (P05_in 2006 and 2011; P08_in 2010 and 2011). Serum from patients with intermediate QS-complexity (P04, P15_2009 and P16) showed negligible infectivity in the mice inducing undetectable or low-titered viremia (Fig. 1b). The monophyletic QS pattern of donor patient sera was mirrored in the plasma of the infected mice. Despite comparable peripheral HCV RNA loads in the mono- and polyphyletic QS patient groups (1.32×10^5 - 2.29×10^7 and 3.74×10^5 - 3.70×10^6) sera from recently infected patients were infectious, whereas sera from chronic infected patients with polyphyletic QS showed no or limited infectivity ($p = 0.006$).

We hypothesize that the infectious nature of sera from early phase or acutely infected patients reflects the presence of non-neutralized virions, whereas in chronic infection virions circulate as non-infectious immune-complexed forms (42, 43). To evaluate this model, we measured serum antibody responses to the laboratory H77 strain of HCV genotype (gt) 1a E1E2 glycoproteins and evaluated their ability to neutralize lentiviral pseudotypes expressing the cognate H77 glycoproteins (**Table 1**). Six of eight recently infected patients and atypical chronic patient P06 had no detectable nAbs whereas all polyphyletic chronic patients had nAbs. These data support our model and show that serum infectivity associates with the presence of circulating nAbs and is genotype-independent. For reasons unknown, P05 and P08 sera were infectious despite the presence of nAbs (**Table 1**). To the best of our knowledge this is the first study to evaluate the infectivity of sera from recent and chronic HCV infection in uPA-SCID mice, a phenomenon that was previously only reported in chimpanzees (44, 45). Our data is consistent with a

model where virus particles in chronic infection circulate in the periphery as non-infectious immune-complexed forms (42, 43), which may explain their negligible infectivity in the uPA-SCID mice.

To examine the infectivity of B cells from the same patients, purified CD19⁺ B cells ($\pm 10^6$) were injected into the spleen of human liver chimeric uPA-SCID mice. B cells from 7 of 10 polyphyletic chronic infected patients were infectious whereas those from the atypical chronic patient P06 and 7 of 8 recently infected patients (patient P07 was the exception) failed to establish an infection in the mice ($p = 0.019$). Mice inoculated with B cells from 7 different chronic infected patients (6 patients and P15_2009) showed evidence of HCV RNA in the periphery after 2 weeks of inoculation (Fig. 1b, lower panel). In 4 animals (K693R, K1196R, K1019 and K628R), the low viremia measured at week 2 increased over time and by week 3 was clearly positive. In contrast, mice receiving B cells from 8 patients with low QS-complexity (7 patients and P15_2006) showed no evidence of infection and remained HCV RNA negative until the end of follow-up (week 8). Importantly, we demonstrated the infectivity of viruses in the blood of chimeric mice following B cell-delivered infection by inoculating plasma from these infected mice into naive chimeric mice (10^4 IU HCV RNA, IP)(human→mouse →mouse). The latter became HCV RNA positive within 1 week after injection, demonstrating that B cell mediated HCV transmission results in the genesis of fully infectious viral particles in a new host (**Supplementary Fig. 3a**). To extend these studies we attempted to recapitulate these observations by challenging mice with B cells (10^6) derived from a non-infected individual pre-incubated for 2 h with infectious mouse-passaged virus (mK983, 5×10^4 IU) alone or immune-complexed with IgG purified from B cell donor P12 (100 μ g). This *ex vivo* HCV-B cell challenge was not infectious, suggesting that successful B cell mediated infection requires more than just attachment of HCV to the B cell surface (**Supplementary Fig. 3b**). In summary, these studies demonstrate the infectivity of B cells from chronic HCV patients to establish a productive infection, whereas B cells from recently infected patients failed to transmit HCV in this mouse model.

A role for anti-E1E2 IgG in defining HCV association with B cells. To better understand the infectious potential of B cells, we quantified genomic HCV RNA associated with B cells and serum from matched samples (Fig. 2a). HCV RNA ($\text{LOQ} \geq 46 \text{ IU}/10^6 \text{ B cells}$) was detected in purified B cells from 5 of 8 recently infected patients and we noted undetectable levels in three samples (P01, P02 and P13) that may explain their non-infectious nature. HCV RNA levels in the serum of P01, P02 and P13 (6.7×10^6 , 1.4×10^6 , and 5.4×10^5 , respectively) were comparable to those measured in other patients, hence it is unlikely that B cell mediated infections are solely explained by virion attachment to B cell surface in a non-specific manner. HCV RNA was detected in purified B cells isolated from all chronically infected polyphyletic patients. Importantly, we demonstrate a significant positive association between the presence of anti-E1E2 antibodies and B cell-associated genomic HCV RNA (Fig. 2b)

Infectious HCV originates from the intracellular B cell compartment. To elucidate the origin of the B cell transmitted virus, we compared the HCV QS in the human serum and B cell inocula along with the plasma from the infected mice (Fig. 3). We assessed QS distribution in five well defined immunogenic regions: hypervariable region 1 (HVR1), hypervariable region 2 (HVR2), Epitope I and II and antigenic domain B,

representing the discontinuous CD81 binding site (Fig. 3a) (46). Viral QS diversity is shown for chronic patients P09, P10 and P12 (Fig. 3b). The QS associated with the B cells reflects the viral heterogeneity found in serum, albeit less complex, and we assume it represents both extra- and intracellular variants. As reported by others, B cells harbour occult HCV strains that are not detected in the plasma (47). This is most pronounced in P12, where a diverse group of E1E2-variants (marked in green) was identified in the B cell fraction. Since P12 is a former IDU, highly divergent variants could have been acquired through super-infection. However, this seems unlikely since phylogenetic analyses demonstrate that all variants cluster within the same patient. Treating B cells with trypsin and RNaseA to digest extracellular bound virus (validation of the protocol in **Supplementary Fig. 4**), identified a unique population of viral variants. Unfortunately, insufficient amounts of patient blood were available to inoculate mice with trypsin/RNaseA digested B cells (B_tr). After successful B cell mediated infection of the mice, QS analyses of the plasma showed a limited viral diversity. Since immune pressure is absent in the chimeric uPA-SCID mice, these variants most likely reflect the infecting inoculum and support a model where infection is initiated by a limited number of viral variants. The intracellular origin of infectious HCV is supported by the observation with P08 at two consecutive time-points (P08_2010 and P08_2011, **Supplementary Fig. 5**). With time, different populations of HCV-variants accumulate in the B_tr compartments that closely resemble the B cell-transmitted variant. QS analyses of human and mouse compartments suggest that the transmitted virus originates from the intracellular B cell compartment. This conclusion is supported by: (i) the homogeneity of E1E2 sequences found in mouse and B_tr compartments; (ii) the clustering of representative reference sequences in phylogenetic trees, and (iii) the failure of viral transmission of virion- or immune-complex-loaded non-infected B cells (**Supplementary Fig. 3b**).

Circulating IgM predominantly recognizes HCV variants associated with B cells. To evaluate the potential role of viral specific IgM and IgG in defining the infectivity of B cell-associated HCV, we measured antibody responses against endogenous viral glycoprotein variants cloned from serum, total B cell and intracellular compartments from selected patients P09, P10 and P12. The majority of HCV envelope clones conferred lentiviral infectivity for human hepatocyte derived Huh-7 cells (data not shown), demonstrating functional E1E2 glycoproteins. With these variant-specific E1E2-glycoproteins at hand, we evaluated the presence in patient serum of IgG and IgM recognizing these variants. IgG-recognition of viral variants was diverse and not limited to a specific originating compartment. However, IgM-recognition, was limited to variants originating from the B cells (P12) and intracellular B cell compartment (P09 and P10) (Fig. 4), suggesting a role for variant-specific IgM in B cell association of HCV virions.

Discussion

Our study shows the differential infectivity of HCV for humanized uPA-SCID mice in clinical samples from acute and chronic stages of infection. Inoculating mice with comparable viral RNA loads showed that peripheral circulating virus collected from recently infected patients was infectious, whereas sera from chronic infected patients was non-infectious for the mice. In contrast, CD19⁺ B cells from chronic infected patients established infection in the mice whereas B cells from recently infected patients were non-

infectious; highlighting a dichotomy in the infectious sources of HCV. Comparing HCV QS-diversity in the sera and B cells of these infected subjects confirmed previous reports of viral compartmentalization (nonrandom phylogenetic distribution) in B cells (48, 49). The intracellular B cell compartment carried a unique population of pauci- or monoclonal viral variants that closely resembled replicating virus isolated from mice infected via B cell transfer. Furthermore, B cells harboring genomic HCV RNA were only found in patients with circulating antibodies targeting the viral E1E2 glycoproteins, suggesting a role for antibodies to mediate HCV association with B cells.

Our understanding of the parameters defining infectious HCV in man is incomplete due to the limited replication of clinical or primary HCV strains in *in vitro* model systems (50, 51). Previous studies have reported on the infectivity of patient-derived sera in chimpanzees (32) and humanized uPA-SCID mice (39, 44, 52), however, these studies were limited by the small number of clinical samples studied. Here we demonstrate that serum infectivity associates with the duration of infection and presence of circulating nAbs. We hypothesize that the infectious nature of sera from early phase infected patients reflects the presence of non-neutralized virions, whereas in chronic infection virus particles circulate as non-infectious immune-complexed forms (42, 43). These results, together with reports that HCV infection of chimeric liver mouse models can be neutralized by the passive transfer of monoclonal antibodies targeting conserved epitopes within the HCV-E2 glycoprotein, provides optimism on the feasibility to develop effective HCV vaccines (16, 18, 53, 54).

Our observation that B cells harboring HCV RNA are only observed in patients with circulating α -E1E2 IgG suggests a role for either the envelope-specific Abs or the B cells expressing such antibodies in B cell mediated transmission of HCV. Three recently infected patients (P01, P02, P13) had no detectable circulating anti-envelope Abs and the non-infectious nature of their B cells is most likely explained by the absence of intracellular genomic HCV RNA. However, for five other recently infected patients, in whom circulating anti-envelope IgG were readily detected, we demonstrated the presence of genomic HCV RNA in the B cell compartment and yet these B cells were non-infectious in chimeric uPA-SCID mice. These data suggest that the mere presence of envelope-specific IgG and B cell-associated HCV RNA is not sufficient for B cell mediated infection. This conclusion is supported by an *in vitro* mixing experiment where chimeric mice were challenged with B cells derived from a non-infected individual, pre-incubated with infectious virions alone or immune-complexed with autologous IgG. Although these observations do not definitely rule out antibody-dependent enhancement of infection, they are clearly not in favor of this mechanism.

Since IgM-recognition, but not IgG-recognition, was restricted to variant-specific E1E2-glycoproteins originating from the B cell compartment, we propose an infection model whereby novel viral variants, recently generated in the liver, associate with the surface IgM-B cell receptor (BCR). In human peripheral blood, the predominant B cell populations are naive $\text{IgM}^+\text{CD27}^-$ B cells, with non-mutated immunoglobulin genes, and class-switched memory $\text{IgG}^+\text{CD27}^+$ B cells. Recently, a population of $\text{IgM}^+\text{CD27}^+$ memory B cells, has been identified (55–57). Although this subset can re-enter germinal center reactions and differentiate into antibody-secreting cells, the generation and immune function of

these cells are poorly understood. Kong *et al.* (58) reported a significantly higher frequency of the IgM⁺CD27⁺ memory B cell subset in chronic hepatitis C compared to healthy controls. Tucci *et al.* (59) reported the presence of large B cell clones in the IgM⁺CD27⁺ B cell compartment of chronic patients. The expression of CXCR3, an inflamed tissue homing receptor, and CD11c on B cells will enable these cells to home to the inflamed liver, where CXCR3-ligands are expressed (60). Recent reports showing the increased expression of CXCR3⁺ on CD27⁺ B cells in chronic hepatitis C (61–63) support a role for these B cells in transferring infectious HCV to permissive hepatocytes in the liver. Despite several attempts we were unable to isolate sufficient IgM⁺-CD27⁺ B cells from our cohort of patients to demonstrate an enrichment of HCV RNA in this subpopulation.

Chronic hepatitis C has been associated with B cell lymphoproliferative disorders, ranging from mixed cryoglobulinemia to aggressive lymphomas (28–31, 64). Our findings may help to elucidate the role of HCV in the etiology of these HCV-associated diseases. The chronic B cell activation by ever-changing HCV variants may play a role in the increased occurrence of lymphomas in chronic HCV patients – predominantly marginal zone lymphomas (CD27⁺, IgM⁺) (65). For MC, HCV was reported to drive the clonal expansion of IgM⁺ + CD27⁺ B cells (66, 67). In summary, our study supports a role for nAbs to inhibit cell-free HCV transmission and provide optimism for the development of a HCV vaccine. However, our observation that envelope-specific antibodies are associated with B cell mediated viral transmission sheds a new light on HCV immunology. The occurrence of gp-specific, even nAbs is a hallmark of chronicity and evolution of the B cell repertoire. Gp-specific IgM, in particular directed against novel variants, may provide a biomarker of the presence of HCV transmitting B cells rather than being itself the mediator of transmission. A potential role for B cells in chronic HCV infection warrants further research to understand the etiology of B cell lymphoproliferative disorders and viral persistence. It cannot be excluded that a similar cell-mediated mechanism also applies to other error-prone RNA viruses.

Methods

Patients. Eighteen patients with ongoing HCV-infection were selected for inoculation experiments in chimeric uPA-SCID mice. Samples were taken early after initial HCV-infection or during persistent infection (ranging from 3 months to 30 years post exposure). HCV-genotyping of the human sera was performed using a Line Probe Assay developed by Innogenetics (Versant® HCV genotype Assay). All patients were vaccinated with HBsAg and non-infected with HIV.

Purification of polyclonal IgG from human plasma samples. Plasma samples, obtained from HCV-infected patients, were heat inactivation (56 °C for 30 minutes) and IgG was purified with a HiTrap Protein G column (GE Healthcare), as described previously (68).

Generation and inoculation of Hu-liv-uPA-SCID mice. Breeding and genotyping of *a/b*/uPA-CBySmn.CB-17 Prkdc^{scid} (uPA-SCID) mice have been described previously (38, 69). SCID mice homozygous for the uPA-transgene were transplanted with cryopreserved human hepatocytes from two HCV-uninfected donors as described previously (38). Only animals with a high repopulation grade, defined by a plasma human

albumin (hu-alb) content of 1 mg/mL or greater, were used. Hu-alb levels were quantified with the Human Albumin ELISA Quantitation kit (Bethyl Laboratories Inc.). Human liver-uPA^{+/+}SCID mice were injected intra-peritoneal (IP) or intra-splenic (IS; injection volume technically limited to 100 à 150 µl) with serum/plasma or human B cells, respectively. Animals were sampled weekly and followed until 8 weeks after infection. Mouse plasma samples were stored at -80 °C until analysis. Repopulation characteristics of chimeric mice and quality data of the injected B cell populations can be obtained on request.

Isolation and treatment of human B cells. PBMC were isolated from whole heparinized blood by density gradient centrifugation. Human B cells were positively selected from PBMC using the MACS® Column Technology (Miltenyi Biotec). In brief, anti-CD19 coated microbeads were incubated with PBMC for 15 min at 4 °C. After washing, the cell suspension was loaded onto the prepared MS Column. The unlabeled cells run through while the magnetically labeled cells are retained on the column. After washing, the column is removed from the magnetic field and the magnetically retained cells can be flushed out as the positively selected cell fraction. The purity ($\geq 98\%$ required) and viability ($\geq 85\%$ required) of the isolated CD19⁺ cell population was analyzed using flow cytometry. Purified B cells were either left untreated (B) or treated (B_tr) with trypsin and RNase A (70, 71). In brief, B cells were washed with cold PBS and incubated with trypsin (final concentration 0,25%; 37 °C for 15 min). After washing and trypsin-inactivation with soybean trypsin inhibitor (final concentration 0,05%; 37 °C for 10 min), cells were further treated with RNase A (final concentration 0,10%; 37 °C for 15 min) to remove residual viral RNA. After washing, RNase A is inactivated using Protector RNase inhibitor (37 °C for 15 min). Trypsin and trypsin-inhibitor are purchased from Invitrogen; RNase A and protector RNase Inhibitor are purchased from Roche. Method validation is shown in Supplementary Fig. 4.

RNA extraction for in-house RT-PCR amplifications. RNA extraction from serum was done using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics). For total RNA-extraction from non-treated or treated B cells we used the RNeasy Mini Kit (Qiagen). Since B cells could be lost due to the additional wash-steps in the treated preparations, the number of B cells was normalized to the amount of total RNA after extraction (NanoDrop, Thermo Fisher Scientific).

HCV RNA quantification. HCV RNA levels from human serum or mouse plasma were quantified using the COBAS Ampliprep TaqMan HCV Assay (Roche Diagnostics). Mouse plasma samples were tested at a 1/50 dilution, resulting in a limit of quantification (LOQ) of 750 IU/ml. The HCV RNA content of B cells was measured after total RNA extraction from 5×10^5 B cells. cDNA was synthesized using Superscript™ III Reverse Transcriptase (Invitrogen, Life Technologies) with random primers according to standard procedures. Five microliters of cDNA were used for HCV RNA quantification with the COBAS TaqMan HCV Assay, resulting in a LOQ of 46 IU HCV RNA/10⁶ B cells. All extractions and amplifications were run according to universally adopted precautions, such as the use of different rooms for pre-PCR experiments, in order to avoid cross-contamination.

Quasispecies analysis: E1E2 amplification, plasmid generation and sequencing. Quasispecies analysis based on sequencing of the envelope proteins was performed on HCV recovered from human serum, B

cells preparations and uPA-SCID plasma. After RNA-extraction, cDNA was synthesized as described and the HCV envelope region was amplified by means of a nested PCR with envelope-specific primers (72). Full-length E1E2 PCR products (containing EcoRV cleavage sites) were purified and cloned in the bacterial pCR®-Blunt vector (Zero Blunt® PCR Cloning Kit, Invitrogen). After plating of transformed cells and plasmid isolation (QIA prep Spin Miniprep Kit, Qiagen), the E1E2-region of the different clones was sequenced using the SANGER technique (ABI3730XL DNA Sequencing System; Applied Biosystems). Amino acid (AA) sequences were deduced and aligned using CLC-DNA workbench (v5.7) and BioEdit Sequence Alignment Editor. Visual inspection of the chromatograms confirmed AA diversity. Maximum-Likelihood phylogenetic trees showing the relationship between E1E2₁₉₂₋₇₄₅ sequences were inferred under the LG model (73) of AA substitution with estimated γ -distribution parameter, using PhyML software (74) (v3.0; South of France bioinformatics platform; ATGC). The tree topology (starting with a Neighbor-Joining tree) was optimized using the Nearest Neighbor Interchange (NNI) approach. Bootstrapping with 1000 replicates tested the statistical robustness of the phylogenetic trees. Only branching with a bootstrap value of $\geq 70\%$ was defined as robust clustering.

Generation of expression plasmids coding for patient-derived viral glycoproteins. Expression plasmids coding for E1E2 from viral variants most prevalent in the patient and mouse compartments were generated. After EcoRV-digestion of the bacterial vector, the isolated DNA fragment was cloned into expression vector pcDNA3.1/Hygro (Invitrogen)(ligation using T4 DNA ligase, standard procedures). After transferring the ligation mixture into competent cells, the transformed cells were plated and the correct insert-size and orientation were confirmed by colony PCR. Positive clones were cultivated for plasmid extraction and inserts were sequenced to confirm identity.

EIA to measure human anti-E1E2 antibody responses. To measure E1E2-antibodies recognizing H77 and variant-specific glycoproteins, HCV_{H77} and variant-specific envelope proteins were generated and used as coating antigens in EIA. To produce glycoproteins, 293-T cells were transfected (CaCl₂-method) with expression plasmids and the ensuing cell-lysates were used as source of E1E2-proteins. For EIA, immunoplates were coated overnight with Galanthus nivalis lectin (GNA; Sigma) and blocked with 5% Bovine Serum Albumin (BSA; Sigma). Cell-lysates were allowed to bind for 2 h at room temperature. After washing, human sera were incubated and bound antibodies were visualized with anti-human IgG (Fc)-HRP (goat, Bethyl Laboratories) or anti-human IgM-HRP (goat, Sigma) conjugates and tetramethyl-benzidine (TMB) substrate. Absorbance was measured at 450 nm. For H77-specific IgG detection, sera were serially diluted to determine end-point titers, defined as the highest serum dilution giving a signal above cut-off. The EIA cut-off value ($OD \leq 0,1$) was calculated by using serially diluted anti-HCV-negative sera in each assay. A positive control (HCV-positive serum with known anti-E1E2 end-point titer) was included in each assay to test the reproducibility of the assay procedure. The specificity of the EIA was further tested using anti-HCV negative controls, anti-HBsAg positive and anti-EBV positive sera. These sera never showed any reactivity. For IgG-detection to patient-derived variants, sera were assayed at a 1/100 dilution. For IgM-detection to patient-derived variants, sera were assayed at a 1/50 dilution.

Generation of retroviral pseudoparticles (HCVpp), infection and neutralization assay. The production of HCVpp expressing firefly luciferase and their use in neutralization assays were previously described (75–77). Briefly, 293T cells were co-transfected with an envelope-deficient human immunodeficiency virus (HIV) proviral genome expressing luciferase (pNL4.3.Luc.R-E-, NIH) and expression vectors encoding E1E2 glycoproteins (consensus H77c or patient-derived HCV strains), Murine Leukemia Virus (MLV) envelope or an empty vector ('No-ENV'). The culture supernatant was collected 48 h post transfection, filtered and aliquots were stored at -80 °C. If required, the successful generation of HCVpp was monitored by quantitation of lentiviral associated HIV p24 core protein using a commercially available EIA (QuickTiter™ Lentivirus Titer Kit, Cell Biolabs, San Diego, CA). Prior to neutralisation assay, mixing serial dilutions of HCVpp with Hep3B cells tested the infectivity of the produced HCVpp-stock. At 72 h post infection, cells were lysed and the luciferase activity of the cells was assayed using the addition of 50 µl Luciferase Assay Substrate (Promega). Relative light units (RLU) were measured in a luminometer (Berthold Centro LB 960). HCVpp stock dilutions resulting in $\pm 2 \times 10^5$ RLU were used as HCVpp dilution in neutralization assays. MLVpp and No-ENVpp served as positive and negative control in infection assays, respectively. For neutralization assays, serial two- to five-fold dilutions of sera were mixed with HCVpp, pre-incubated at 37 °C for 1 hour, and added to Hep3B cells at 37 °C. After 72 hours, luciferase-positive cells were quantified by measuring RLU. Results are reported as IC₅₀ neutralization titer, defined as the sample concentration or dilution conveying 50% reduction in the number of luciferase-positive cells.

Study approvals.

Human study. Participating subjects gave written informed consent and consented to unit blood donation. The Ethical Board of the Ghent University Hospital approved the study protocol (EC # 1994/137).

Animal study. The Animal Ethics Committee of the Faculty of Medicine and Health Sciences of the Ghent University approved the study protocol.

Declarations

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AUTHOR CONTRIBUTIONS

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Conflict of interest statement

The authors have declared that no conflict of interest exists.

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Tables

TABLE 1

Demographic, virological and immunological characteristics of HCV-infected patients.

Patient		HCV-infection		HCV-RNA		HCV-specific IgG		
ID	age, gender	route ^a	duration ^b	serum (IU/ml)	B-cells (IU/10 ⁶ B-cells)	anti-E1E2 _{H77} ^c	neutralization to HCVpp-H77 (IC ₅₀) ^d	
gt1a	P01	30, M	IDU	1y	6,74 10 ⁶	<46	< 10	< 50%
	P02	28, F	IDU	≤1y	1,42 10 ⁶	<46	< 10	< 50%
	P32	36, F	IDU	5m	1,29 10 ⁷	3,51 10 ⁴	559	< 50%
	P03	34, M	IDU	1y 4m	5,30 10 ⁶	9,54 10 ⁴	4898	4065
	P04	31, M	IDU	4y	1,77 10 ⁶	3,25 10 ⁴	35156	2860
	P05 ^e	47, F	IDU	25y	3,70 10 ⁶	2,15 10 ⁴	90991	3311
	P15_2009 ^f	28, M	IDU	3y	5,00 10 ⁶	1,11 10 ⁵	30440	21616
gt1b	P06	64, M	transfusion	>10y	1,25 10 ⁷	5,60 10 ⁴	259	< 50%
	P07	30, M	IDU	1y	1,89 10 ⁵	3,30 10 ³	6309	771
	P08 ^e	68, F	transfusion	30y	2,18 10 ⁶	8,28 10 ⁴	30339	14286
	P09	64, M	transfusion	15y	8,91 10 ⁵	1,91 10 ⁴	3040	1858
	P10	47, M	transfusion	28y	1,40 10 ⁶	6,52 10 ³	35727	6250
	P11	66, M	transfusion	20y	2,02 10 ⁶	6,46 10 ³	4046	922
	P12 ^e	28, F	IDU	≥ 5y	3,74 10 ⁵	4,31 10 ³	16218	6361
	P18	39, F	not known	not known	1,32 10 ⁵	1,12 10 ³	6854	3540
gt3a	P13	26, M	IDU	1y	5,41 10 ⁵	<46	< 10	< 50%
	P14	33, M	IDU	3m	2,88 10 ⁶	2,42 10 ⁴	109	< 50%
	P15_2006 ^f	25, M	IDU	4m	2,29 10 ⁷	1,46 10 ⁵	165	< 50%
	P16	31, M	IDU	not known	9,76 10 ⁵	3,38 10 ⁴	3396	513
	P17	44, M	IDU	≥ 5y	1,73 10 ⁶	3,63 10 ⁴	85113	10495

^aIDU, injecting drug use. ^bTime elapsed between initial HCV-infection/diagnosis and biological sampling for the present study (y, year; m, month). ^cAnti-E1E2_{H77} IgG levels are expressed as reciprocal endpoint dilution titer of autologous serum in EIA; <10, IgG was not detected at serum-dilution 1/10. ^dNeutralising activities of autologous serum to HCV-pseudoviral particles expressing envelope proteins of gt1a (HCVpp_H77) are expressed as IC₅₀ (reciprocal titer); <50%, neutralization did not reach 50%. ^eSerum and B cells from patients P05, P08 and P12 were sampled at multiple time points, only 1 time-point is shown. ^fPatient P15, initially infected with gt3a, experienced [a](#) HCV-super-infection with gt1a and has donated biological specimens twice with a 3-year interval.

Figures

Figure 1

Complexity of HCV quasiespecies in patient sera and viral outcome in hu-liver-uPA-SCID mice inoculated with serum or B cells from HCV-infected patients. (a) Grouping of HCV-infected patients (gt1a, gt1b and gt3a) according to E1E2-quasispecies (QS) complexity in serum. Pie charts represent the genetic variation in E2-regions of major variability and/or immunogenicity: HVR1 (AA_384-410); HVR2 (AA_474-480); Epitope I (AA_412-423); HVR3 (AA_434-446), Epitope II and Antigenic Domain B (domain B; AA_523-535). After amplification and cloning of E1E2, vector-inserts were sequenced and at least 20 clones were analysed per patient. Pie charts represent the percentage of each clone detected in serum based on diversity in the five E2-regions. Individual viral isolates are indicated by grey shading, and are unique for each patient. To assess E1E2-QS complexity, Maximum-Likelihood phylogenetic trees based on the full E1E2 region were inferred for each patient (Supplementary Figure 2). Based on the genetic distance between QS-variants, patients were classified in 3 groups: genetic distance < 0.05 (light grey), 0.05-0.1 (medium grey) and >0.1 (dark grey). (b) Viral outcome in hu-liver-uPA-SCID mice after inoculation of serum or B cells from HCV-infected patients. Most hu-liver-uPA-SCID mice were injected intra-peritoneal

(IP) with serum containing 3x10⁵ IU HCV RNA. Three animals (B674L, B528 and B528R) were injected with serum via the intra-splenic route (IS). Mice were injected IS with 10⁶ B cells isolated from HCV-infected patients. Mice were bled 1, 2, 3, 4, 6 and 8 weeks post-inoculation and plasma screened for HCV RNA level (color codes). '+, <750' means that HCV RNA was detectable but <750 IU/ml (LOQ). Repeated experiments are indicated by individual hu-liver-uPA-SCID mice numbers. Patient P15 experienced a HCV-super-infection and has donated blood in 2006 and 2009. SNA, sample not available. Δ, not done (colour code extrapolated). †, animal died before week 8.

Figure 2

Genomic HCV-RNA content of patient-derived B cells associates with the presence of circulating α-E1E2 IgG. (a) Genomic HCV RNA levels in serum and B cells from HCV infected patients. For each patient, total RNA was extracted from purified CD19+ B cells. After reverse transcription, HCV RNA content was quantified by qPCR and expressed as IU HCV RNA/106 B cells. The viral load in serum, measured with the same assay and expressed as IU HCV RNA/ml, and the α-E1E2H77 IgG levels, expressed as reciprocal end-point dilution in EIA, are shown on the left and right axis, respectively. Genomic HCV RNA content was compared for serum and B-cell fractions. Limit of HCV RNA quantification (LOQ) is 46 IU/106 B cells (dotted line) and 15 IU/ml for serum. (b) The presence of B-cell associated genomic HCV-RNA correlates with α-E1E2 IgG positivity in circulation. The RNA load was compared for serum and B-cell fractions using additional HCV patients: 4 α-E1E2H77 IgG-negative (3 gt1a and 1 gt3a) and 26 α-E1E2H77 IgG-positive (6 gt1a, 14 gt1b and 6 gt3a) patients.

Figure 3

HCV variants in human serum and B cells and in uPA-SCID mice plasma following inoculation with patient-derived serum or B cells. (a) Schematic representation of the HCV-E1E2 region showing the position of 5 major variable E2-regions: HVR 1; HVR2; HVR3; epitope I and domain B. (b) Pie charts represent the E1E2 viral quasispecies (QS) distribution in human serum and B cells (B, non-treated B cells; and B_tr, B cells after trypsin/RNase A treatment) at the time of injection and in uPA-SCID plasma for

gt1b-patients P09, P10 and P12 (additional patients in Supplementary Figure 5). RNA was extracted from each source and the envelope region amplified. HCV E1E2 amplicons were cloned and vector-inserts sequenced. The number of clones analysed per compartment is shown on top of the pie charts that represent the fraction of each clone/compartment based on diversity in single variable regions or combinations thereof. Viral isolates are indicated by different colours or grey-shades and are unique for each patient. The AA sequences of the variable regions and their corresponding colour and letter codes are shown. The consensus sequence HCV-J4 is used for E1E2-alignments and shown in the top row. For HVR 1, closely related viral variants (based on individual AA-similarity in HVR and clustering in phylogenetic analysis) are depicted in different intensities of the same colour. Mouse identification is shown above the circle graphs in the mouse plasma compartment. For each patient a phylogenetic tree based on the full E1E2 region and composed of the major viral variants found in the different compartments is shown on the right. Maximum-Likelihood phylogenetic trees are rooted on HCV-J4. Robust clustering (bootstrap values $\geq 70\%$) is depicted in red at the tree nodes. Black numbers (tree-leaves) are clone-codes, each corresponding to a unique HVR 1 AA sequence (coloured dot). For each clone, the corresponding variability in the other regions, the originating compartment and the % E1E2-similarity (554AA) with viral variants found in infected mouse plasma, are shown on the right. QS and phylogenetic analyses show that variants found in the mouse-plasma after B cell transfer closely resemble variants found in the intracellular B cell compartment.

Figure 4

HCV variants in human serum and B cells and in uPA-SCID mice plasma following inoculation with patient-derived serum or B cells. (a) Schematic representation of the HCV-E1E2 region showing the position of 5 major variable E2-regions: HVR 1; HVR2; HVR3; epitope I and domain B. (b) Pie charts represent the E1E2 viral quasispecies (QS) distribution in human serum and B cells (B, non-treated B cells; and B_tr, B cells after trypsin/RNase A treatment) at the time of injection and in uPA-SCID plasma for

gt1b-patients P09, P10 and P12 (additional patients in Supplementary Figure 5). RNA was extracted from each source and the envelope region amplified. HCV E1E2 amplicons were cloned and vector-inserts sequenced. The number of clones analysed per compartment is shown on top of the pie charts that represent the fraction of each clone/compartment based on diversity in single variable regions or combinations thereof. Viral isolates are indicated by different colours or grey-shades and are unique for each patient. The AA sequences of the variable regions and their corresponding colour and letter codes are shown. The consensus sequence HCV-J4 is used for E1E2-alignments and shown in the top row. For HVR 1, closely related viral variants (based on individual AA-similarity in HVR and clustering in phylogenetic analysis) are depicted in different intensities of the same colour. Mouse identification is shown above the circle graphs in the mouse plasma compartment. For each patient a phylogenetic tree based on the full E1E2 region and composed of the major viral variants found in the different compartments is shown on the right. Maximum-Likelihood phylogenetic trees are rooted on HCV-J4. Robust clustering (bootstrap values $\geq 70\%$) is depicted in red at the tree nodes. Black numbers (tree-leaves) are clone-codes, each corresponding to a unique HVR 1 AA sequence (coloured dot). For each clone, the corresponding variability in the other regions, the originating compartment and the % E1E2-similarity (554AA) with viral variants found in infected mouse plasma, are shown on the right. QS and phylogenetic analyses show that variants found in the mouse-plasma after B cell transfer closely resemble variants found in the intracellular B cell compartment.

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