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Functional and Biochemical Characterization of Hepatitis C Virus (HCV) Particles Produced in a Humanized Liver Mouse Model*

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Sara Calattini1–6, Floriane Fusil1–6, Jimmy Mancino1–6, Viet Loan Dao Th1–6, Christelle Granier1–6, Nicolas Gadot9, Jean-Yves Scoazec7, Mirjam B. Zeisel8, Thomas F. Baumert8,9, Dimitri Lavillette1–6,3, Marlène Dreux1–6,4,5, and François-Loïc Cosset1

From the 1CIRI-International Center for Infectology Research, Team EVIR (Enveloped Viruses, Vectors, and Innate Responses), Université de Lyon, 69007 Lyon, France, 2INSERM, U1111, 69007 Lyon, France, 3École Normale Supérieure de Lyon, 69007 Lyon, France, 4Université Lyon 1, Centre International de Recherche en Infectiologie, 69007 Lyon, France, 5CNRS, UMR5308, 69007 Lyon, France, 6LabEx Ecofect, Université de Lyon, 69007 Lyon, France, 7Structure Fédérative de Recherche (SFR) Lyon-Est, ANIPATH-Centre d’Histopathologie du Petit Animal de laboratoire, CNRS UMS3453-INSERM US7, 69372 Lyon, France, 8INSM, U1110, Institut des Maladies Virales et Hépatiques, 67000 Strasbourg, France, 9University of Strasbourg, 67000 Strasbourg, France, and 1Institut Hospitalo-Universitaire, Pôle Hépato-digestif, Hôpitaux Universitaires de Strasbourg, 67000 Strasbourg, France

Lipoprotein components are crucial factors for hepatitis C virus (HCV) assembly and entry. As hepatoma cells producing cell culture-derived HCV (HCVcc) particles are impaired in some aspects of lipoprotein metabolism, it is of upmost interest to biochemically and functionally characterize the in vivo produced viral particles, particularly regarding how lipoprotein components modulate HCV entry by lipid transfer receptors such as scavenger receptor BI (SR-BI). Sera from HCVcc-infected liver humanized FRG mice were separated by density gradients. Viral subpopulations, termed HCVfrg particles, were characterized for their physical properties, apolipoprotein association, and infectivity. We demonstrate that, in contrast to the widespread distribution of apolipoproteins across the different HCVcc subpopulations, the most infectious HCVfrg particles are highly enriched in apoE, suggesting that such apolipoprotein enrichment plays a role for entry of in vivo derived infectious particles likely via usage of apolipoprotein receptors. Consistent with this salient feature, we further reveal previously undefined functionalities of SR-BI in promoting entry of in vivo produced HCV. First, unlike HCVcc, SR-BI is a particularly limiting factor for entry of HCVfrg subpopulations of very low density. Second, HCVfrg entry involves SR-BI lipid transfer activity but not its capacity to bind to the viral glycoprotein E2. In conclusion, we demonstrate that composition and biophysical properties of the different subpopulations of in vivo produced HCVfrg particles modulate their levels of infectivity and receptor usage, hereby featuring divergences with in vitro produced HCVcc particles and highlighting the powerfulness of this in vivo model for the functional study of the interplay between HCV and liver components.

To complete its life cycle and to produce new infectious particles, HCV7 hijacks the host lipid metabolism. Indeed, it has been demonstrated that HCV assembly is tightly connected to the assembly pathway of very low density lipoprotein (VLDL). In the most considered assembly model, the nascent nucleocapsids fuse with VLDL precursors during the VLDL maturation process across the endoplasmic reticulum membranes. Before leaving the cells, these lipoproteins acquire lipids and

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1 Supported by a fellowship from the ANRS.
2 Present address: Laboratory of Virology and Infectious Diseases, The Rockefeller University, New York, NY 10065.
3 Present address: CNRS, UMR 5557 Microbial Ecology, Université Lyon 1, Microbial Dynamics and Viral Transmission, 69622 Villeurbanne, France.
4 Both authors contributed equally to this work.
5 To whom correspondence may be addressed: CIRI-International Center for Infectology Research, Team EVIR, Université de Lyon, 69007 Lyon, France. Tel.: 33-472-72-87-32; Fax: 33-472-72-81-37; E-mail: marlene.dreux@ens-lyon.fr.
6 To whom correspondence may be addressed: CIRI-International Center for Infectology Research, Team EVIR, Université de Lyon, 69007 Lyon, France. Tel.: 33-472-72-87-32; Fax: 33-472-72-81-37; E-mail: flcosset@ens-lyon.fr.

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apolipoproteins such as apoB, apoE, and apoC and are then secreted through the Golgi apparatus (1–3).

The association of HCV particles with lipoprotein components such as lipids and apolipoproteins was initially demonstrated for particles retrieved from the peripheral blood of infected patients (4–6). Such HCV particles, called lipoviroparticles (LVPs), resemble very low and low density lipoproteins and have a heterogeneous distribution in densities ranging from less than 1.06 to 1.25 g/ml (7). Cell culture-derived HCV (HCVcc) as well as LVPs can also be immunoprecipitated with antibodies against apoE and apoB (8–10). Moreover, a direct interaction between apoE and viral envelope glycoproteins has recently been demonstrated (11–13), further indicating a close relationship between viral particles and lipoprotein components.

Lipoproteins and their receptors as well as their associated lipid transfer activities are key factors for HCV entry into hepatocytes. Indeed, HCV entry is a multistep process involving the interactions of both viral and cellular components of the LVPs with several host cell receptors. The initial attachment of the virus to the cell is likely mediated by glycosaminoglycans (14), by the LDL receptor (15), and/or by the scavenger receptor BI (SR-BI) (16), which all bind apolipoproteins incorporated on the surface of viral particles. Then, a complex interaction between viral glycoproteins, SR-BI, and other cellular host factors such as the CD81 tetraspanin (17) and the tight junction protein Claudin-1 (18) and Occludin (19) allows entry and internalization of the viral particles (20). Moreover, other proteins implicated in cholesterol uptake such as Niemann Pick C1-like 1 (21), and host cell kinases such as the epidermal growth factor receptor and the ephrin receptor A2 (22) have been identified as cofactors of HCV entry. Using the HCVcc and HCV pseudoparticle models, our laboratory recently demonstrated that SR-BI mediates entry of particles independently of their density via its capacity to transfer lipids to and from different carriers (23, 24). Finally, presumably at a further step of entry, HCVcc particles of lower-intermediate density (i.e. 1.08–1.13 g/ml) and HCV pseudoparticles become able to bind SR-BI via a direct interaction with the E2 glycoprotein, which results in enhancement of their entry process (23–25).

HCV assembly and release have mainly been studied using the highly infectious molecular clone JFH1 or using JFH1-derived recombinant viruses produced in hepatoma cell lines (26–29). However, most of these cell lines have impaired production of VLDL because they express pre-VLDLs that are not fully lipidated and that do not fuse with apoE-containing luminal lipid droplets (30). The resulting HCVcc particles are thus poorly associated with apoB (9), and their buoyant density profile is significantly different compared with in vivo recovered viruses (31, 32). As lipid and apolipoprotein components are key factors influencing HCV biology, this highlights the need to better understand how they are distributed within the different forms of HCV particles circulating in vivo and how such distributions functionally influence their entry properties. Importantly, HCV particles from infected patients are not or not robustly infectious ex vivo, thus precluding their functional characterization.

A major advance in the study of HCV infection in vivo was made due to the development of immunodeficient mice that sustain human hepatocyte engraftment (33, 34). Using the Alb-uPA/SCID mouse model, Lindenbach et al. (31) showed that HCV physical particles, as assessed by viral RNA distribution, have a lower density compared with HCVcc particles; however, the infectivity of such viral populations remained undermined. Although this study suggested that viruses issued from infected mice may have a higher specific infectivity compared with in vitro cultured virus (31), to our knowledge, there is no study describing the composition (in terms of viral and cellular components) of HCV particles issued from these mice as well as their entry properties.

Here, we characterize HCV particles and viral subpopulations produced in humanized liver mice derived from the FRG mouse model (further designated HCVfrg), a triple mutant mouse knocked out for fumarylacetoacetate hydrolase (FAH), RAG2, and γc (35) that was previously shown to support HCV infection (36). We demonstrate that, unlike HCVcc, the most infectious in vivo recovered HCVfrg particles are highly enriched in apoE. Moreover, our work features further differences between HCVfrg and HCVcc because we show that HCVfrg particles exhibit a strongly increased dependence on SR-BI expression levels for entry of HCVfrg particles of very low density. Finally, we show that entry of HCVfrg particles of all densities is strongly dependent on the lipid transfer activity of SR-BI rather than on its binding to the viral glycoprotein E2. Thus, altogether, our results suggest that the lipoprotein components incorporated on viral particles play a crucial role in entry of infectious particles, and our results highlight the powerfulness of this in vivo model for the functional study of the interplay between HCV and liver components.

**Experimental Procedures**

**Cell Lines**—Huh-7.5 and 293T cells were grown in DMEM (Invitrogen) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum.

**Mouse Colony Maintenance,** Nitro-4-trifluoromethyl Benzo-cyclclohexanedione (NTBC) Cycling, and Primary Human Hepatocyte (PHH) Engraftment—FRG (Fah−/− Rag2−/− Il2rg−/−) mice (mixed background: C57BL/6 and 129Sv) were provided by M. Grompe (Oregon Health and Science University) and bred in our animal facility (Plateau de Biologie Expérimentale de la Souris, Lyon, France).

FRG mice are deficient for the FAH enzyme, which leads to an accumulation of a toxic tyrosine catabolite in the liver. To prevent this toxicity, mice are maintained on 8 mg/liter NTBC (Swedish Orphan Biovitrum) in the drinking water. NTBC acts to prevent this toxicity, mice are maintained on 8 mg/liter NTBC (Swedish Orphan Biovitrum) in the drinking water. NTBC acts by blocking the tyrosine catabolic pathway upstream of FAH and thus prevents the accumulation of toxic metabolites. FRG mice were kept in temperature- and humidity-controlled animal quarters with a 12-h light/12-h dark cycle.

PHHs were injected intrasplenically as described previously (34). Immediately after engraftment, the NTBC was progressively withdrawn as follow: 2 days at 10% of colony maintenance, then 2 days at 5%, and 2 days at 2.5%, and then the NTBC was completely removed. During the phase without NTBC, mice were weighed every 2 days. After 2–6 weeks, mice...
with clinical symptoms (lethargy and hunched posture) or severe weight loss (>15%) were again put on NTBC for 3 days before a second withdrawal (cycling). Cycling was repeated until clinical symptoms resolved. To prevent the development of a murine hepatocellular carcinoma, highly reconstituted mice selected for infectious experiments were subjected to further NTBC treatment (3–4 weeks without NTBC and 3 days with 100% NTBC). No other drugs were administered during the entire study. 48 h prior to engraftment, adult (6–10 weeks old) mice were injected intravenously with 2 × 10⁶ pfu of an adenoviral vector encoding the uPA transgene. 7 × 10⁵–1 × 10⁶ PHHs (BD Biosciences) were injected intrasplenically, and NTBC was progressively withdrawn. Experiments were performed in accordance with the European Union guidelines for approval of the protocols by the local ethics committee (Authorization Agreement C2EA-15, Comité Rhône-Alpes d’Ethique pour l’Expérimentation Animale, Lyon, France).

Immunohistochemistry Antibodies and Methods—For histological examination, liver tissues from FRG mice were fixed in 10% buffered formalin and embedded in paraffin. 4-μm-thick sections were prepared according to conventional procedures, stained with hematoxylin-phloxine-saffron, and observed with a light microscope. Immunohistochemistry was performed using a panel of primary antibodies against FAH (Aviva Systems Biology, San Diego, CA), carcinoembryonic antigen (Novus Biologicals, Littleton, CO), CD31, CD34, CK7, and CK18 (all from Dako, Glostrup, Denmark). These antibodies only reacted with human tissue.

An automated immunostainer (Ventana Discovery XT, Roche Applied Science) was used with the DABmap kit according to the manufacturer’s instructions. A standard horseradish peroxidase staining procedure was followed. All sections were counterstained with Gill’s hematoxylin. As negative controls, slides were stained without the primary antibody. Slides were analyzed in a Mirax slide scan (Zeiss, Jena, Germany).

The repopulation index was determined by using an ImageJ macro program. Images were split into their color components based on 3,3′-diaminobenzidine staining (color deconvolution), then automatically thresholded, and measured. Results were collected in a comma-separated value table. The repopulation index was calculated based on the surface ratio between the 3,3′-diaminobenzidine-positive surface and the total surface.

Serological and Immunohistochemical Analyses—Blood from transplanted mice and controls was collected every 2–4 weeks after engraftment by retro-orbital puncture. Sera were sent to a diagnostic laboratory for quantification of human serum albumin (Cobas C501 analyzer, Roche Applied Science). For lipoprotein analyses, sera were run on a Lipoprint Lipoprotein Subfractions Testing System (Quantimetrix) that quantified lipoprotein fractions (VLDL, LDL, and HDL) on the basis of their cholesterol amount. Human apoB and apoE concentrations were measured in FRG mouse sera and human samples by ELISA (Mabtech, Sweden) according to the manufacturer’s instructions.

HCVcc Production—In vitro transcribed RNA was electroporated into Huh-7.5 cells. 72 h postelectroporation, infectious titers were determined as focus-forming units (FFU)/ml estimated by NS5A immunostaining and colony counting as described previously (37). Infectious titers were between 1 and 2 × 10⁵ FFU/ml.

Virus Inoculation and Blood Analysis—Highly reconstituted mice (human serum albumin (HSA) >15 mg/ml) were infected with 1 × 10⁹ FFU of HCV of genotype 2a of the Jc1 chimera (29) by the intraperitoneal route. 100 μl of total blood were collected every week, and HCV RNA and viral infectivity were measured. RNA was extracted with a guanidine thiocyanate method, reverse transcribed (iScript cDNA synthesis kit, Bio-Rad), and quantified with the FastStart Universal SYBR Green Master kit (Roche Applied Science) on an Applied StepOne Real-Time PCR apparatus using HCV-specific primers (5′-TCTGGCGA-ACCGGTGAGTA and 5′-TCAGCAGTACCAACGACCC). As an internal control of extraction, an exogenous RNA from the linearized Triplescpt plasmid pTRI-Xef (Invitrogen) was added into the sera prior to extraction and quantified with specific primers (5′-CGACGTGTTGACCCGACCG and 5′-ACCAGCATTGTTAATGTC).

Iodixanol Gradient Separation of HCV Particles—800 μl of serum from infected mice or HCVcc virus-containing supernatants were loaded onto a 3–40% continuous iodixanol gradient (Optiprep, Axis Shield). Gradients were centrifuged for 16 h at 4 °C in an Optima L-90 centrifuge (Beckman).

Co-Immunoprecipitation (Co-IP) of Apolipoproteins and Viral RNA—Co-IPs of apolipoprotein-HCV RNA from HCVfrg and HCVcc were performed on 100 μl of non-fractionated samples or on iodixanol-separated fractions. Samples were run in a 3–40% iodixanol gradient. Seven fractions of 1500 μl were collected from the top of the gradient, and 1100 μl were used for co-IP experiments. Samples were preclearred with 40 μl of protein G magnetic beads (Millipore) for 1 h at 4 °C. For preabsorption of antibodies to protein G magnetic beads, 6 μl of polyclonal goat anti-apoE (AB947, Millipore) or anti-apoB (AB742, Millipore) or goat immunoglobulins (IP control) were mixed with 40 μl of beads in a total volume of 100 μl and incubated 1 h at 4 °C. The precleared samples were added into the antibody-coated beads and incubated for 3 h at 4 °C under continuous rotation. The immune complexes were washed three times in PBS, and beads were resuspended in guanidine thiocyanate buffer for HCV RNA extraction and quantification or alternatively in lysis buffer for Western blot analysis.

Establishment of Cell Lines Expressing WT or Mutant SR-BI—Retroviral vectors expressing human SRBI (hSR-BI), mouse SR-BI (mSR-BI), or the hSR-BI point mutant G420H/G424H were generated and expressed in Huh-7.5 cells as described previously (24). Down-regulation of hSR-BI was achieved using two small interfering RNAs (5′-GCAGCAGG-UCCUUAAGAAC and 5′-GGCACCTGTCCTGGAACCTT) as described previously (38). SR-BI overexpression was verified by FACS analysis (CLA-1 antibody, BD Pharmingen) or by Western blot analysis (NB400-104 antibody, Novus).

Neutralization Assays—Huh-7.5 cells overexpressing hSR-BI were preincubated with the anti-SR-BI antibody NK-8H5-E3 (39) or with control IgGs for 1 h at 37 °C at a final concentration of 10 or 100 μg/ml. Cells were then infected with dilutions of non-fractionated viruses or with gradient density-separated fractions of HCVfrg and HCVcc particles. Infectivity was measured 72 h postinfection by immunostaining the viral glycopro-
tein E2 using the human recombinant AR3A antibody (Ref. 40; a kind gift from M. Law).

Results

FRG Mice with Highly Reconstituted Human Liver Allow Productive HCV Infections—We first sought to validate that, in our experimental conditions, engraftment of FRG mice with primary human hepatocytes allows a robust liver reconstitution and thus a sustained HCV infection as described previously (36). 11–16 weeks postengraftment, HSA levels were higher than 15 mg/ml (range, 16–29 mg/ml) in 65% of the engrafted mice, i.e. close to the human physiologic concentrations (Fig. 1D). Human FAH immunostaining in liver sections confirmed a high and homogeneous repopulation with PHHs (Fig. 1A) with a good correlation between HSA and the repopulation index that represents the percentage of human FAH-positive cells in liver sections from PHH-engrafted FRG mice. h, human; w, week; HuHep, human hepatocytes.

FIGURE 1. Highly repopulated liver chimeric mice sustain HCV infection. A, human FAH staining of a moderately liver-reconstituted mouse, a highly liver-reconstituted mouse, and non-engrafted mouse. R.I., repopulation index. Original magnifications, ×15 and ×400 (insets). B, lipoprotein distribution in the sera from FRG mice reconstituted at different levels as indicated by HSA levels in mg/ml and human sera or non-engrafted FRG mouse sera as control. Sera were run on a Lipoprint system, and cholesterol content was measured within each lipoprotein fraction. Results represent the percentage of cholesterol in each subpopulation compared with total cholesterol. C, quantification of human apoB and apoE in the serum of human liver-reconstituted mice, human individuals, and supernatant from confluent HuH-7.5 cells. Statistical analyses were performed by a Mann-Whitney test. N.S., not statistically significant. D, follow up of HSA in human liver engrafted FRG mice (id 1–12). As a comparison, HSA was measured in plasma or sera from three healthy individuals (Donors 1–3). To note, HSA levels in engrafted mice are in accordance with a recently published study on humanized liver in NOD FRG mice (65). E, correlation between HSA and the repopulation index that represents the percentage of human FAH-positive cells in liver sections from PHH-engrafted FRG mice.
are the preponderant cholesterol-supplying lipoproteins. Accordingly, the determination of the LDL versus HDL proportion revealed that although sera from mice with low levels of human liver reconstitution are enriched with high density lipoproteins the profiles of highly human liver-reconstituted mice, as assessed by HSA levels, approached the human profiles, i.e., characterized by a preponderance of low density lipoproteins (Fig. 1B). Finally, highly liver-reconstituted FRG mice (HSA >15 mg/ml) secreted high levels of human lipoproteins close to human physiological concentrations (Fig. 1C). The histological analyses of human liver-reconstituted mice further confirmed that highly repopulated chimeric livers had a normal and functional liver architecture in which human hepatocytes of different degrees of maturation as well as a human bile canalicular network and sinusoid vessels were observed (Fig. 2).

Engrafted mice with HSA levels over 15 mg/ml were infected with $10^5$ FFU of HCVcc of genotype 2a (Jc1 chimera). The amount of HCV RNA detected in the sera increased steadily over time after infection to reach a plateau level at approximately 7 days postinoculation as described previously (36), and HCV infection was robust and sustained in all of the inoculated mice with both viral RNA (range, $10^5–10^6$ genome equivalents (GE)/ml) and infectious particles (range, $10^3–10^5$ FFU/ml) being detected for at least 4 weeks (Fig. 3A). Importantly, HCVfrg viral loads were comparable with HCV RNA titers measured in infected individuals in acute phase (range, $5 \times 10^5–5 \times 10^6$ GE/ml) (41).

**HCVfrg Infectious Particles Have a Lower Density as Compared with HCVcc**—Four weeks after infection, animals were sacrificed, and total blood was collected. Infected sera were loaded on continuous density gradients and separated in 15 fractions. Viral RNA and infectious virus levels were determined for each fraction. Buoyant density analysis revealed the distribution of HCVfrg RNA within two major RNA populations (Fig. 3B), the first in the 1.06–1.11 g/ml density range and the second in the 1.11–1.16 g/ml density range. Interestingly, the viral RNA population of lower density overlapped with the infectious population (Fig. 3B), showing that most of the infectious virus (~90%; range, 80–100%) had a density between 1.06 and 1.11 g/ml, whereas a large amount of physical particles, i.e., those of higher density, was not or very poorly infectious.

As a comparison, similar fractionation experiments were performed using HCVcc, and they raised important differences compared with HCVfrg particles. Indeed, we found that the buoyant density of the HCVcc infectious viral population was more heterogeneous as compared with HCVfrg particles (Fig. 3C). Indeed, 90% of HCVcc infectious particles were distributed among density fractions of 1.03–1.13 g/ml in contrast to the more restricted range of density of infectious HCVfrg (i.e., 90% in density fractions of 1.06–1.11 g/ml). Furthermore, the highest infectivity of HCVcc infectious particles was shifted among higher density fractions (1.08–1.13 g/ml) with a peak around 1.10 g/ml (Fig. 3C), whereas most physical particles were detected within fractions of 1.10–1.15 g/ml with a peak around 1.12 g/ml (Fig. 3C). Interestingly, viruses recovered in vivo had a approximately 100-fold augmentation in their specific infectivity (HCVfrg, $5.3 \times 10^{-2}$ FFU/GE; HCVcc, $4.2 \times 10^{-3}$ FFU/GE) as calculated by the median of the specific infectivity of each HCVfrg and HCVcc sample (Fig. 3, B and C, insets). Altogether, these results indicated that in vivo produced infectious HCVfrg particles are highly infectious and have a lower and more homogenous density as compared with cell culture-derived virus. These differences of biophysical properties imply that HCVfrg and HCVcc likely differ in terms of lipid and/or protein composition.

**Characterization of in Vivo Derived HCV Particles**

**Highly Infectious HCVfrg Populations Are Enriched with ApoE**—Therefore, we sought to determine whether the difference in density profiles between HCVfrg and HCVcc infectious particles was due to a differential association of the virus with lipoprotein components. To separate viral particles from lipoproteins, we performed co-IP assays using antibodies against apolipoproteins apoE and apoB, and we quantified the captured particles by HCV RNA-specific RT-quantitative PCR. We decided to use this approach because previous papers showed that the initial capture of viral particles with anti-E2 antibodies was inefficient probably because of poor epitope accessibility (9, 10). We first performed co-IP assays on non-fractionated samples using apoE or apoB antibodies. We could immunoprecipitate 57% (mean ± 29%; n = 7) and 30% (mean ± 20%; n = 9) of HCVcc viral RNA using anti-apoE- and anti-apoB-coated beads, respectively. In contrast, we could co-immunoprecipitate 23% of total HCV RNA of HCVfrg particles with apoE antibodies (mean ± 20%; n = 10) but less than 5% with apoB (mean ± 5%; n = 8) antibodies (data not shown). The weak apoB capture of HCV particles from HCV-infected human liver-reconstituted FRG mouse samples is likely to depend on the high concentration of apoB in the sera of human liver-reconstituted FRG mice (Fig. 1C), which may compete with apoB present on viral particles for the binding to the antibody. Indeed, addition of serum from non-infected human liver-reconstituted FRG mice to HCVcc particles decreased the co-IP rates by about 10-fold (data not shown). Moreover, the dissimilarities in co-IPs results of HCVfrg versus HCVcc particles could be due to a different lipidation of either type of viral particle and thus to a differential exposure of apoE and apoB proteins on their surface.

We then performed co-IPs of apoE-HCV RNA on density gradient-separated viral subpopulations. First, we sought to assess the distribution of apoE along the different buoyant density-separated fractions. Although the distribution of apoE in HCVcc samples was homogeneous among the different subpopulations (Fig. 4B), apoE was predominantly present in low density fractions of HCVfrg samples (i.e., <1.06 g/ml) (Fig. 4A). Nevertheless, apoE could be readily immunoprecipitated all along the gradient for both HCVcc and HCVfrg samples (Fig. 4, A and B).

To verify that this difference was due to a different lipid composition of mouse sera versus cell culture supernatants and not to viral components, uninfected mouse sera were subjected to gradient density separation, and apoE distribution was analyzed by Western blot analysis. The results showed a similar distribution of apoE among fraction samples derived from HCV-infected or uninfected mouse sera (Fig. 4, A and C), thus confirming a different composition of lipoproteins between the HCVfrg and HCVcc models.

Interestingly, when comparing apoE-HCV RNA co-immunoprecipitation in HCVfrg versus HCVcc samples sepa-
rated on density gradients, we found that although for HCVcc particles (Fig. 4E) apoE-enriched particles were distributed throughout the gradient without marked overlap with infectious populations the distribution of apoE-co-immunoprecipitated HCVfrg particles coincided with the highly infectious population (i.e. at a density range of 1.06 – 1.11 g/ml) (Fig. 4D). To rule out that the quantity of antibody was limiting for the immunoprecipitation of highly apoE-enriched fractions, we performed co-IP assays with a 10 times greater quantity of antibody than we otherwise used.

FIGURE 2. Highly repopulated chimeric livers maintain a normal and functional liver architecture. A, hematoxylin-phloxine-saffron staining reveals that liver parenchyma is repopulated by pale human hepatocytes. Human hepatocyte aggregates are easily discerned after hematoxylin-eosin staining because of their large size, abundant cytoplasm, and large nuclei containing dispersed chromatin with well formed nucleoli. B, immunodetection of human-specific carcinoembryonic antigen confirms the capacity for human hepatocytes to form bile canaliculi; the bile canalicular network is as dense and organized as in normal human liver. C, human hepatocytes are also detected with anti-human CK18. Mature human hepatocytes present a pale staining, whereas human hepatic progenitor cells are strongly immunoreactive for CK18. The black arrow indicates human hepatic progenitors. D, staining with anti-human CK7 confirms the presence of immature hepatocytes (white arrow), which are surrounded by mature hepatocytes that do not stain for CK7. After immunodetection of CK18 and CK7 (C and D), it is possible to identify human bile ducts cells (CK7 +) and CK7+/CK18 + small to intermediate sized cells with moderately abundant cytoplasm and excentered nuclei; they are similar to the so-called intermediate cells observed during human liver regeneration after injury or cell loss. Anti-human CK7 also stains human bile ducts cells (black arrow). E, within human hepatocyte aggregates, cells are arranged in plates of variable thickness separated by well formed sinusoid vessels lined by CD31 + or CD34 + (inset) endothelial cells. F, the presence of a few Ki67-positive cells, preferentially at the junction between human and murine hepatocytes, indicates proliferation of human hepatocytes in close proximity to dying murine hepatocytes. Original magnifications, ×400 (∗×200 for A and ×400 for inset). Scale bar, 100 μm (200 μm for A and 100 μm for inset). H, human hepatocytes; M, murine hepatocytes; VCL, centrolobular vein; P, portal track.
for such experiments (as in Fig. 4D), and we found that the distribution of the co-immunoprecipitated apoE-HCV RNA complexes had the same profile regardless of the apoE antibody concentration (data not shown).

Thus, in sharp contrast with HCVcc, apoE was reproducibly enriched in specific HCVfrg populations representing the most infectious particles. This reflected the homogeneity of HCVfrg infectious particles. In accordance with the in vitro studies of...
apoE function, our findings suggested that apoE is an essential factor for entry of infectious in vivo produced virus (23, 42–46).

**HCVfgr Show an Uneven Dependence on SR-BI for Entry of Viral Subpopulations**—Previous results from our laboratory indicated that specific functions of SR-BI are implicated in entry of HCVcc subpopulations in a manner dependent on the physicochemical properties of the viral particles (23). Importantly, the comparison of HCVfgr and HCVcc particles highlighted substantial dissimilarities in the apoE content of the different viral particle subpopulations (Fig. 4). Because apoE incorporated on virions acts as a ligand of SR-BI for HCV entry into cells (23, 42), we first sought to investigate the reliance on SR-BI for entry of HCVfgr subpopulations.

Thus, we modulated expression of human SR-BI in Huh-7.5 cells (Fig. 5A) to determine infectious titers and SR-BI dependence of HCVfgr subpopulations. We found that infectivity of non-fractionated HCVfgr as well as of HCVcc (Fig. 5, B and C) particles was decreased and increased by about 10 times in SR-BI down- and up-regulated cells, respectively. This indicated that, similarly to HCVcc, HCVfgr particles rely on SR-BI for entry into cells.

Consistently, in fractionated HCVfgr and HCVcc samples, SR-BI down-regulation resulted in a pronounced and uniform diminution of infectivity (Fig. 5, D and E) regardless of their density, thus confirming the importance of this receptor for entry. Moreover, SR-BI overexpression stimulated entry of both HCVfgr and HCVcc subpopulations (Fig. 5, D–F), particularly those that exhibited the highest infectivity (i.e. in fractions 1.06–1.11 and 1.08–1.13 g/ml, respectively). Interestingly, in sharp contrast to HCVcc, gradient density-separated HCVfgr viral subpopulations showed an uneven dependence on SR-BI for entry. Indeed, the effect of SR-BI overexpression on HCVfgr infectivity was specifically marked in the very low density fractions (Fig. 5F). This was particularly salient for HCVfgr particles of density <1.05 g/ml for which at least 10–100-fold higher infectious titers could be detected (Fig. 5, D and F). This indicated that SR-BI expression is limiting for entry of low density HCVfgr particles, which highlighted a further difference between HCVfgr and HCVcc.

To confirm SR-BI dependence for entry of HCVfgr viral subpopulations, cells were incubated prior to infection with the SR-BI antibody NK-8H5-E3 that has been shown previously to block
FIGURE 5. SR-BI expression differentially modulates entry of HCVfrg and HCVcc viral subpopulations. A, flow cytometry analysis of Huh-7.5 cells overexpressing (OE) or down-regulating (DR) SR-BI at the time of HCV infection. Huh-7.5 cells were transduced with two bicistronic lentiviral vectors coding small interfering RNAs (SR-BI/siRNA-2 and SR-BI/siRNA-24) together with the GFP protein (DR SR-BI, down-regulation of SR-BI) or with a lentiviral vector coding human SR-BI (OE SR-BI, overexpression of SR-BI). CTRL, cells transduced with the backbone vector expressing the GFP; NT CTRL, non-transduced cells; STN CTRL, staining without the primary antibody. Transduced cells were infected with serial dilutions of sera from non-fractionated HCVcc-infected mice (B) or of supernatants from HCVcc-infected Huh-7.5 cells (C). As a control, cells were transduced with the backbone vector expressing the GFP. Infection levels were within a range of 1.3 × 10²–3.9 × 10⁴ FFU/ml and 2.2 × 10²–5.3 × 10⁴ FFU/ml for HCVfrg and HCVcc, respectively. Statistical analysis was performed by Student’s t test (n = 3). SR-BI down- or up-regulated cells were infected with HCVfrg (D) or HCVcc (E) using serial dilutions of gradient density-separated fractions. Examples of distribution of infectivity from two humanized liver FRG mice (D) and from two HCVcc independent experiments (E) are shown. F, -fold increase of infectivity of HCVfrg and HCVcc subpopulations in hSR-BI-overexpressing cells compared with the controls. Results are the average of seven HCVfrg and six HCVcc independent experiments and are shown for each density fraction (F1–F15). Statistical analyses were performed by a Mann-Whitney test. N.S., not statistically significant. Error bars represent S.D.
HCVcc entry (39). In non-fractionated samples, blocking of SR-BI caused a marked decrease of infectivity of both HCVfrg and HCVcc samples in a dose-dependent manner (Fig. 6, A and B). Moreover, the SR-BI antibody strongly reduced infectivity of all viral subpopulations for both HCVfrg and HCVcc viruses (Fig. 6, C and D), confirming their usage of SR-BI for entry into cells.

**HCVfrg Entry Depends Mostly on the Lipid Transfer Activity of SR-BI**—Next, to investigate how SR-BI allows HCVfrg entry, we determined the infectivity of HCVfrg subpopulations (and of HCVcc for comparison) on Huh-7.5 cells expressing, in addition to the endogenous SR-BI, the murine ortholog mSR-BI (Fig. 7A), which does not bind HCV E2 (23, 47). Overexpression of either human or murine SR-BI significantly increased infectivity of both HCVcc and HCVfrg particles (Fig. 7, C and D), which indicated that SR-BI promotes HCV entry independently of E2 binding. Consistently, despite similar levels of overexpression of human and murine SR-BI (Fig. 7A), mSR-BI overexpression led to an augmentation of infectivity for all HCVfrg and HCVcc fractions (Fig. 7, E and F). Moreover, similarly to hSR-BI, overexpression of mSR-BI markedly increased infectivity of HCVfrg particles of very low density (<1.05 g/ml) (Fig. 7, E and F). Thus, altogether, these results highlighted the role of SR-BI for entry of in vivo derived particles and indicated that modulation of entry by SR-BI is independent of its capacity to bind to HCV E2.

The main physiological function of SR-BI is to transfer lipids between lipoproteins and cells (48), a function that is pivotal for HCVcc cell entry (23, 24, 47). We thus investigated the impact of the overexpression of the G420H/G424H hSR-BI mutant, which has impaired capacity to transfer lipids despite normal HDL binding capacity (49), on infectivity of HCVfrg and HCVcc subpopulations. Interestingly, we found that, despite comparable levels of cell surface expression of mutant and wild-type hSR-BI (Fig. 7, A and B), the overexpression of the G420H/G424H mutant in Huh-7.5 cells still expressing the endogenous SR-BI did not induce increased cell entry of either HCVfrg or HCVcc particles regardless of their density (Fig. 7, C–G). Altogether, these results highlighted that, among the different functions of SR-BI in HCV entry, the principal factor that promotes entry of HCVfrg particles is linked to its capacity to transfer lipids.

**Discussion**

Many aspects of HCV biology are not completely resolved because of the lack of convenient animal models that could reproduce HCV infection and replication. An early study performed on
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chimpanzees demonstrated that the low density viral subpopulations recovered in vivo were the most infectious when reinoculated in other animals (50). However, the interpretation of these results was limited by the fact that viral particles issued from chimpanzees contained anti-HCV antibodies that could have altered the intrinsic biophysical properties and infectivity of these particles. The development of immunodeficient mouse models with humanized livers that could sustain HCV infection was a great step forward to the understanding of HCV biology. Here, by using the FRG mouse model, we characterized the physicochemical and infectious properties of viral particles issued from humanized mouse liver. We showed that infectious particles produced in vivo have a lower density compared with HCVcc particles and that a high amount of non-infectious particles with higher density circulate in the blood of these animals. Because we and others (51) have demonstrated that FRG mice have a humanized lipoprotein profile, we can speculate that the low density profile of the infectious particles is due to a different lipid and/or protein content compared with the HCVcc model.

To better characterize HCV particles issued from the FRG model, we investigated their apolipoprotein composition. Viral particle capture with apoB antibodies resulted in co-immunoprecipitation of 30% of HCVcc RNA and of less than 5% of HCVfrg RNA. The presence of apoB in viral particles issued from HuH-7.5 cells is still a matter of debate. Although some studies have indicated that apoB is dispensable for production of infectious particles (52) and is barely detected in viral particles derived from HuH-7.5 cells (9, 53), other studies have revealed its importance in HCV assembly and maturation (1, 11, 54).

Moreover, the presence of apoB on secreted HCV particles has been demonstrated in viruses derived from cultured primary hepatocytes (32) or from long term cultures of HuH-7.5 cells differentiated with human serum-containing culture media (55) and in viruses recovered from patients (7, 8). However, in most studies, rather than direct apoB–HCV RNA co-immunoprecipitation, other methods such as affinity grid immunolabeling of apoB in precaptured tagged viruses or capture by antibody of immunogold-LVP complexes followed by apoB quantification by ELISA were used (7, 8, 10). Our results using non-fractionated as well as density gradient-fractionated sera (data not shown) from infected mice suggest that HCV particles produced in vivo are not as accessible as HCVcc for capture with anti-apoB antibodies. As mentioned above, our co-IP experiments with HCVcc alone or mixed with sera from non-infected human liver-reconstituted FRG mice suggested that the high amount of human apoB present in the sera of these mice could compete with apoB incorporated on HCVfrg particles for the binding to the antibody. In accordance, human apoE and apoB concentrations were 2 logs higher in sera from human liver-reconstituted FRG mice as compared with supernatant from the in vitro HCV culture cell model (Fig. 1C).

Moreover, we can speculate that HCVfrg particles, unlike HCVcc, are fully lipidated and that their lipid layer would mask apoB epitopes, making them inaccessible to anti-apoB antibodies. This concept is congruent with previous findings from Nielsen et al. (8) showing that treatment of purified LVPs in mild detergent conditions changes the apolipoprotein composition of viral particles.

In HCVfrg subpopulations, the distribution of the apoE-enriched viral particles clearly overlapped with the peak of infectivity, suggesting that this apolipoprotein plays a major role for entry of in vivo derived particles. On the contrary, for HCVcc particles, apoE co-immunoprecipitated with HCV RNA in all fractions irrespective of their infectious properties, indicating that a high proportion of apoE-containing particles issued from in vitro cultures are poorly infectious. This could reflect an improper maturation of lipoproteins (and thus of viral particles) in HuH-7.5 cells (30), which would not allow a correct incorporation and/or folding of apolipoproteins into lipoproteins and thus HCV particles. Alternatively, we could surmise that the physicochemical composition of HCVfrg particles retrieved from peripheral blood could be modified by peripheral lipoprotein lipases that influence VLDL lipid and apolipoprotein composition. Indeed, Shimizu et al. (56) showed that lipoprotein lipase treatment of HCVcc particles resulted in a decreased infectivity, a shift toward higher density, and a loss of apoE in viral particles. Finally and in a more general way, it has been demonstrated that in recombinant HDL complexes the lipid content modulates the stability of apoA-I (57), which indicates that lipid content modifications influence the apolipoprotein composition of lipoproteins. Thus, modifications of the lipid content of viral particles may influence the stability of their apolipoproteins.

HCV entry is a complex mechanism involving multiple cellular receptors. In a previous work, we demonstrated that among them SR-BI modulates entry of HCVcc subpopulations depending on their physicochemical properties (23). Because the initial capture of intermediate density HCVcc particles involves binding of SR-BI to apoE incorporated on viral particles and because we have observed a different distribution of this apolipoprotein in HCVfrg versus HCVcc subpopulations, we investigated the reliance on SR-BI for entry of HCVfrg subpopulations. First, we show that HCV particles recovered from sera of infected mice are dependent on SR-BI for entry because its down- or up-regulation modulated infectivity by about 1 log compared with control cells. These data are in accordance with Grove et al. (58) who showed that SR-BI up-regulation...
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In conclusion, our results highlight previously uncharacterized divergences between in vivo and in vitro produced HCV particles with respect to their composition and biophysical properties, which imprint their infectivity and receptor usage. Thus, humanized liver mouse models represent a powerful tool to investigate the interplay between HCV and liver components and to understand how the hepatic environment influences HCV biology.

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Author Contributions—S. C., M. D., and F.-L. C. coordinated the project, designed experiments, analyzed the data, and wrote the manuscript. S. C., F. F., J. M., V. L. D. T., N. G., and C. G. designed and performed experiments and analyzed the data. J.-Y. S. and D. L. provided critical discussion and technical advice. M. B. Z. and T. F. B. provided critical reagents and analyzed the data.

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