Successful anti-SR-BI mAb therapy in humanized mice after challenge with HCV variants with *in vitro* resistance to SR-BI-targeting agents.

Detailed Materials and Methods.

In vitro HCV neutralization assay.

Genotype 2a HCVcc (Jc1wt, Jc1ΔHVR1, Jc1mtCD81, Jc1G451R and J6/JFH1 Clone2) were generated as previously described (1-3). For the receptor-targeting neutralization assay, 1.3×10^4 Huh-7.5 cells were seeded per well in a 96-well imaging plate (BD Biosciences, Erembodegem, Belgium) (4). The following day, cells were pre-incubated for 2 hours with either 2 µM anti-SR-BI small molecule inhibitor ITX-5061 (stock: 6 mM in DMSO, diluted in culture medium containing 0.1% DMSO), 0.2 µg/ml anti-CD81 mAb JS81 (BD Biosciences) or different concentrations of anti-SR-BI mAb1671 (from 0.002 µg/ml to 20 µg/ml) in 100 µL of culture medium. To investigate the effect of human HDL and human VLDL on HCVcc infectivity, cells were pre-incubated with 100 µl of approximately 230 µg HDL and 180 µg VLDL cholesterol/ml (BTI Biomedical Technologies, Stoughton, USA) either alone or in combination with 20 µg/ml mAb1671. Human HDL (230 µg HDL cholesterol/ml) was also combined with JS81 (0.2 µg/ml) and ITX-5061 (2µM). Then, 100 µl of HCVcc-containing supernatant (MOI of approximately 0.004) was added to the pre-incubation medium. After 48 hours the cells were fixed with paraformaldehyde (PFA) and HCV infected cells were visualized with the anti-NS5A monoclonal antibody 9E10 in combination with an Alexa647-conjugated goat-anti-mouse antibody (Invitrogen). Images were acquired using a BD Pathway 435 High Content Bioimager (BD Biosciences) with a 10x objective.

In vitro cell-to-cell spread assay.

$1.4\text{x}10^4$ Huh-7.5-RFP-NLS-IPS cells/well were seeded in a 96-well imaging plate (BD Biosciences, Erembodegem, Belgium) and served as acceptor cells (4). One day later, HCV Jc1wt- or Jc1ΔHVR1-transfected Huh-7.5-EGFP-IPS cells were added as donor cells in the presence of adequate amounts of a neutralizing anti-HCV antibody preventing cell-free transmission. The concentration of anti-HCV mAb during co-cultivation was selected such that it maximally inhibited cell free infectivity with minimal inhibition of cell-to-cell spread (150 µg/ml and 1 µg/ml for Jc1wt and Jc1ΔHVR1 co-cultures respectively). One hour prior to addition of the donor cells, Huh-7.5-RFP-NLS-IPS acceptor cells were treated with 20 µg/ml mAb1671 or 2 µM ITX-5061 in 100µl culture medium. Then, 100 µl of donor cell suspension ($3\text{x}10^3$ cells/ml) was added on top of the pre-incubation medium. All conditions were performed in duplicate. After two days of co-cultivation, the cells were fixed with PFA and HCV-infected cells were visualized with the anti-NS5A monoclonal antibody 9E10 in combination with an Alexa647-conjugated goat-anti-mouse antibody. The number of HCV-infected acceptor cells (Alexa647^{pos} and RFP^{pos}) per HCV-infected donor cell (Alexa647^{pos} and

EGFP^{pos}) was counted in at least 100 separate HCV-positive clusters. Images were acquired using a BD Pathway 435 High Content Bioimager with a 10x objective.

In vivo HCV neutralization experiments.

Human liver-uPA-SCID mice (chimeric mice) were produced as previously described (5, 6). All mice were transplanted with primary human hepatocytes obtained from a single donor (donor HH223; BD Biosciences, Erembodegem, Belgium). The effectiveness of the anti-SR-BI mAb1671 (400 µg per intraperitoneal injection) was evaluated in a preventive and post-exposure setting. Three mice were treated in a prophylactic manner, during which the antibody was administered one day prior to inoculation with Jc1ΔHVR1 ($3.43x10^5$ IU/mouse, a dose previously shown to be 100% infectious), followed by additional antibody injections at day 1, 5, 8, 12 and 15. In the post-exposure protocol, chimeric mice were challenged with genotype 2a HCVcc (Jc1wt, Jc1ΔHVR1, mtCD81 and Jc1G451R) on day 0 and were given mAb1671 on day 3, 5, 7, 10, 12 and 14. HCV RNA in plasma was quantified using the COBAS Ampliprep/COBAS TaqMan HCV test (Roche Diagnostics, Belgium). Due to dilution of the mouse plasma, the limit of detection (LOD) equalled 750 IU/ml. The study protocol was approved by the animal ethics committee of the Faculty of Medicine and Health Sciences of the Ghent University.

HCVcc mouse passaging and Iodixanol ultracentrifugation.

Human liver-uPA-SCID mice were infected with cell culture produced (HCVcc) Jc1wt and Jc1ΔHVR1 (4 and 5 mice respectively). Serum was collected over a two-month infection period and pooled as humanized-mouse passaged virus stock, designated mHCV.

Gradients were formed with 10–40% iodixanol. HCVcc and mHCV were loaded on top of a preformed continuous gradient. Gradients were run to equilibrium for 16 h at 4°C and 36,000 rpm (160,000xg). Twelve fractions were collected from the top of the gradient. Buoyant densities were determined by refractometry.

Graphs and statistics.

All graphs were prepared using Prism v4.01 (GraphPad Software Inc., La Jolla, CA). To analyze whether the difference between treatment groups was statistically significant, the data obtained from *in vitro* neutralization and cell-to-cell transmission experiments were analyzed using the Kruskal-Wallis test (Nonparametric ANOVA) with Dunn's Multiple Comparisons post-test. Data was analyzed using GraphPad InStat v3.06 (GraphPad Software Inc.).

References.

1. Lindenbach BD, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, Maruyama T, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623.
2. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.

3. Bankwitz D, Steinmann E, Bitzegeio J, Ciesek S, Friesland M, Herrmann E, Zeisel MB, et al. Hepatitis C virus hypervariable region 1 modulates receptor interactions, conceals the CD81 binding site, and protects conserved neutralizing epitopes. J Virol 2010;84:5751-5763.
4. Jones CT, Catanese MT, Law LM, Khetani SR, Syder AJ, Ploss A, Oh TS, et al. Real-time imaging of hepatitis C virus infection using a fluorescent cell-based reporter system. Nat Biotechnol 2010;28:167-171.
5. Meuleman P, Vanlandschoot P, Leroux-Roels G. A simple and rapid method to determine the zygosity of uPA-transgenic SCID mice. Biochem Biophys Res Commun 2003;308:375-378.
6. Meuleman P, Libbrecht L, De Vos R, de Hemptinne B, Gevaert K, Vandekerckhove J, Roskams T, et al. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. Hepatology 2005;41:847-856.