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Successful Anti-Scavenger Receptor Class B Type I (SR-BI) Monoclonal Antibody Therapy in Humanized Mice After Challenge With HCV Variants With *In Vitro* Resistance to SR-BI-Targeting Agents

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Hepatitis C virus (HCV)-induced endstage liver disease is currently a major indication for liver transplantation. After transplantation the donor liver inevitably becomes infected with the circulating virus. Monoclonal antibodies (mAbs) against the HCV coreceptor scavenger receptor class B type I (SR-BI) inhibit HCV infection of different genotypes, both in cell culture and in humanized mice. Anti-SR-BI mAb therapy is successful even when initiated several days after HCV exposure, supporting its potential applicability to prevent HCV reinfection of liver allografts. However, HCV variants with reduced SR-BI dependency have been described in the literature, which could potentially limit the use of SR-BI targeting therapy. In this study we show, both in a preventative and postexposure setting, that humanized mice infected with HCV variants exhibiting increased *in vitro* resistance to SR-BI-targeting molecules remain responsive to anti-SR-BI mAb therapy *in vivo*. A 2-week antibody therapy readily cleared HCV RNA from the circulation of infected humanized mice. We found no evidence supporting increased SR-BI-receptor dependency of viral particles isolated from humanized mice compared to cell culture-produced virus. However, we observed that, unlike wild-type virus, the *in vitro* infectivity of the resistant variants was inhibited by both human high density lipoprotein (HDL) and very low density lipoprotein (VLDL). The combination of mAb1671 with these lipoproteins further increased the antiviral effect. *Conclusion*: HCV variants that are less dependent on SR-BI *in vitro* can still be efficiently blocked by an anti-SR-BI mAb in humanized mice. Since these variants are also more susceptible to neutralization by anti-HCV envelope antibodies, their chance of emerging during anti-SR-BI therapy is severely reduced. Our data indicate that anti-SR-BI receptor therapy could be an effective way to prevent HCV infection in a liver transplant setting. (Hepatology 2014;60:1508-1518)

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Approximately 3% of the world's population is chronically infected with the hepatitis C virus (HCV). Depending on the genotype of the infecting virus, 50 to 80% of chronically infected patients can clear the virus upon treatment with pegylated interferon combined with ribavirin.[1] Addition of one of the protease inhibitors, telaprevir or boceprevir,

Abbreviations: CD81, cluster of differentiation 81; DAA, direct-acting antiviral; E1/E2, envelope glycoprotein 1 and 2; EC_{50}, half maximal effective concentration; HCV, hepatitis C virus; HCVcc, cell culture derived HCV; HDL, high density lipoprotein; HVR1, hyper variable region 1; mAbs, monoclonal antibodies; mHCV, mouse passaged HCV; SCID, severe combined immunodeficient; SR-BI/Cla1, scavenger receptor class B type I; uPA, urokinase-type plasminogen activator; VLDL, very low density lipoprotein.

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significantly increases the response rate in genotype 1 patients.[2] Besides the existence and possible emergence of antiviral resistant mutants, side effects and drug-drug interactions severely complicate the use of double and triple therapy in chronically infected patients in need of liver transplantation.[3,4] Therefore, safer and more effective cocktails of direct-acting antivirals (DAA) without interferon or alternative novel antiviral strategies are needed to treat this expanding patient population. Chronic HCV infection can lead to liver fibrosis, cirrhosis, and hepatocellular carcinoma (50-76% of all liver cancers), which represent the major indications for liver transplantation. However, after transplantation graft reinfection occurs almost immediately and disease progression can be accelerated in these immune-suppressed patients.[5] This highlights the need for adequate measures to prevent reinfection after liver transplantation and for better and safer therapies to treat reinfection in case prevention fails.

Currently available data indicate that HCV entry into hepatocytes is a complex multistep process requiring an interplay between various host and viral factors, thereby offering multiple targets for antiviral intervention (reviewed[6]). The virus probably first interacts with cellular membrane proteins that concentrate the virus at the cell surface of the host cell. While this initial contact occurs in a rather nonspecific manner, it is followed by more specific interactions between the virus and the host thereby triggering viral entry. Besides cluster of differentiation 81 (CD81), claudin-1, and occludin, scavenger receptor class B type I (SR-BI) is one of these important HCV (co-)receptors.[7-10]

SR-BI is involved in HCV cell entry based on both its physiological lipid transfer function and its ability to interact with the HCV glycoprotein E2.[8,11] Molecules targeting this host factor may offer an innovating and promising strategy to prevent and/or treat HCV infections. Indeed, small-molecule inhibitors of SR-BI-mediated cholesteryl ester lipid uptake with anti-HCV activity *in vitro* have been described.[12,13] In addition, monoclonal antibodies (mAbs) against SR-BI are able to inhibit HCV infection of Huh7.5 cells in a dose-dependent manner.[14] Moreover, prophylactic administration of an anti-SR-BI mAb protects chimeric mice from infection by HCV of different genotypes,[15] and from a viral variant that became dominant after liver transplantation.[16] In some of these mice HCV RNA levels remained undetectable even when therapy was initiated 3 days after viral challenge, indicating an inhibitory effect on intrahepatic viral transmission. Therefore, this antibody may represent a novel therapeutic tool to prevent HCV reinfection of liver allografts.

However, different HCV variants have been described that carry changes in their envelope glycoproteins, which render them more resistant to SR-BI-blocking anti-HCV therapy in cell culture.[17-21] Here we investigate how these variants respond to an anti-SR-BI mAb therapy in humanized urokinase-type plasminogen activator (uPA) severe combined immunodeficient (SCID) mice.

Materials and Methods

A detailed description of all Materials and Methods can be found in the online supplement.

In Vitro *HCV Neutralization Assay.* Genotype 2a cell culture derived HCV (HCVcc) (Jc1wt, Jc1ΔHVR1, Jc1mtCD81, Jc1G451R, and J6/JFH1 Clone2) were generated as previously described.[18,22,23] The receptor-targeting neutralization assay and the cell-to-cell spread assay were performed as described.[15,16,24,25] To investigate the effect of human HDL and human VLDL on HCVcc infectivity, cells were preincubated with ∼230 μg HDL and 180 μg VLDL cholesterol/mL (BTI Biomedical Technologies, Stoughton, MA) either alone or in combination with 20 μg/mL mAb1671, JS81 (0.2 μg/mL), or ITX-5061 (2 μM).

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Potential conflict of interest: Dr. Wong-Staal owns stock in iTherX Pharma.

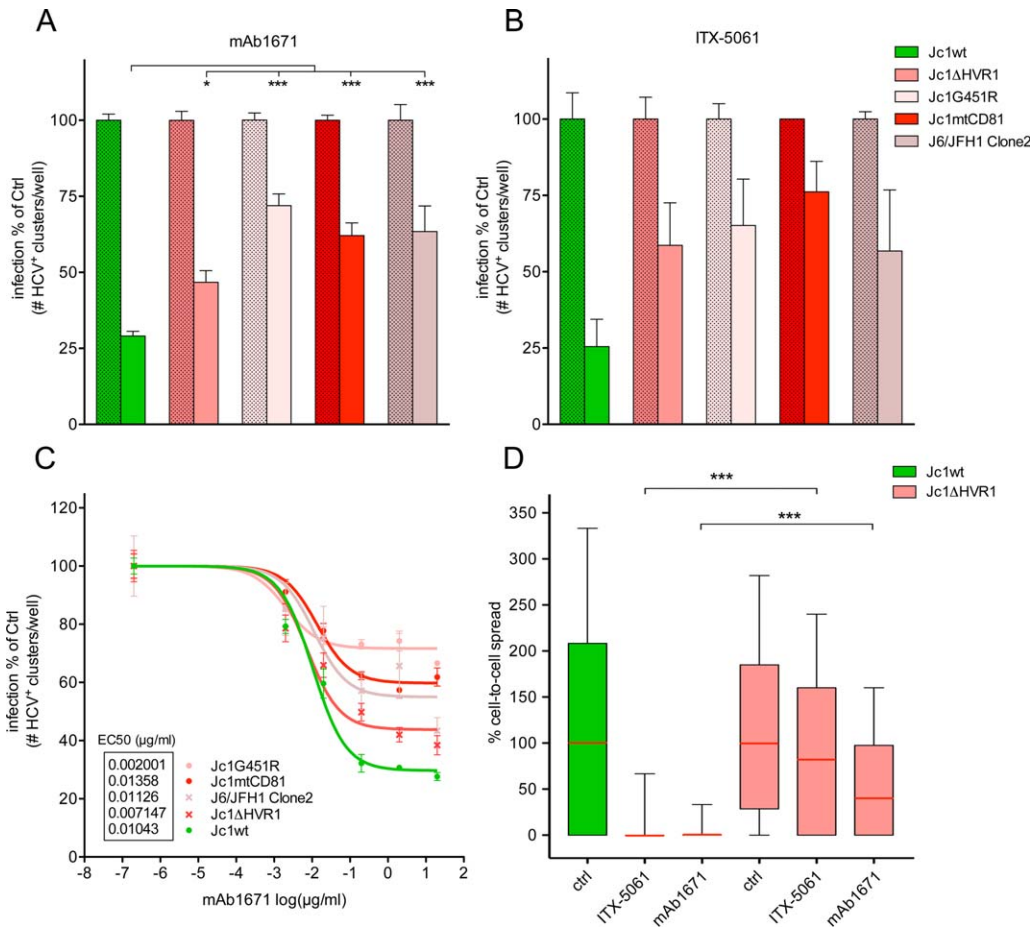

Fig. 1. *In vitro* neutralization assay. Huh7.5 cells were pretreated with 20 μg/mL mAb1671 (A) and 2 μM ITX-5061 (small molecule SR-BI antagonist) (B) before infection with Jc1wt, Jc1ΔHVR1, Jc1G451R, Jc1mtCD81, and J6/JFH1 Clone2. After 2 days the number of HCV-positive clusters was counted and normalized to control. The effect of mAb1671 on the infectivity of Jc1wt, ΔHVR1, and mtCD81 was evaluated in 10 separate wells over four different experiments, while the effect on Jc1G451R and J6/JFH1 Clone2 was assessed over eight separate wells in three different experiments. The data of these experiments were merged and the means are shown. The asterisks ($*P < 0.05$; and $***P < 0.001$) indicate that the effect of mAb1671 on Jc1ΔHVR1, Jc1G451R, Jc1mtCD81, and J6/JFH1 Clone2 differs significantly from its effect on Jc1wt infectivity. The effect of ITX-5061 was assessed in one experiment and the means of duplicates are shown (this limited sample size did not allow statistical analysis). (C) HCVcc infectivity under increasing concentrations of mAb1671. All conditions were tested in quadruplicate and the mean values are shown. (D) Box-and-whisker presentation of cell-to-cell spread. While mAb1671 (20 μg/mL) and ITX-5061 (2 μM) efficiently inhibit direct cell-to-cell transmission of Jc1wt, only a minor effect can be observed against Jc1ΔHVR1 ($***P < 0.001$). For each condition, the amount of infected target cells per cluster was determined in at least 100 clusters and normalized to the median of the control. The box extends from the 25th to the 75th percentile, while the whiskers indicate the 10th and 90th percentile. The red horizontal line indicates the median. Error bars in (A-C) represent the standard error of the mean.

In Vivo *HCV Neutralization Experiments.* Human liver-uPA-SCID mice (chimeric mice) were produced as previously described.[26,27] All mice were transplanted with primary human hepatocytes obtained from a single donor (donor HH223; BD Biosciences, Belgium). The effectiveness of mAb1671 was evaluated in a preventive and postexposure setting.[15,16] Infections for all the Jc1 variants were done with an equivalent virus inoculum. HCV RNA in plasma was quantified using the COBAS Ampliprep/COBAS TaqMan HCV test (Roche Diagnostics, Belgium).

Statistics. Statistical significance of experimental results was assessed by the Kruskal-Wallis test (nonparametric analysis of variance [ANOVA]) with Dunn's Multiple Comparisons posttest using GraphPad InStat v. 3.06 (GraphPad Software).

Results

Comparison of* In Vitro *Cell-Free and Cell-to-Cell Transmission of Wild-Type and Variant Viruses. To confirm that the variants used in this study

(Jc1ΔHVR1, Jc1G451R, Jc1mtCD81, and J6/JFH1 Clone2) are more resistant to anti-SR-BI therapy *in vitro*, their infectivity in the presence of SR-BI inhibitors was assessed and compared to that of the wild-type virus. As shown in Fig. 1A, a significantly less pronounced inhibition of the different variants was observed compared to the inhibition of the wild-type virus by mAb1671. A similar inhibition pattern was observed when using the SR-BI-blocking small molecule ITX-5061, but given the limited sample size in this experiment a statistical analysis could not be performed (Fig. 1B). A dose-response study showed that the half maximal effective concentration (EC_{50}) values were comparable between all the viruses, but the maximal inhibitory effect of the antibody was considerably lower against the variants compared to the wild-type virus (Fig. 1C). In addition, the effects of SR-BI inhibition (mAb1671 and ITX-5061) on cell-to-cell spread of Jc1ΔHVR1 and Jc1wt viruses were compared. Figure 1D shows that viral transmission of Jc1ΔHVR1 by way of cell-to-cell spread was significantly less responsive to SR-BI blockade than that of Jc1wt ($P < 0.001$). This indicates that in addition to cell-free infectivity, the direct cell-to-cell spread of Jc1ΔHVR1 is also less dependent on SR-BI.

In Vivo *HCV Neutralization Experiments.* Next, the antiviral efficacy of the anti-SR-BI mAb1671 against infections with wild-type and anti-SR-BI resistant variants was evaluated in humanized mice. Whereas viremia in nontreated mice rapidly increased to 10^6 IU/mL, prophylactic administration of the antibody was able to prevent infection with Jc1ΔHVR1 in two out of three mice (Fig. 2A). Viremia was controlled in the third Jc1ΔHVR1-injected mouse but a rebound was observed after cessation of therapy. Figure 2B-E represents the effect on HCV viremia during and after postexposure anti-SR-BI treatment of humanized mice injected with Jc1wt, Jc1ΔHVR1, Jc1mtCD81, and Jc1G451R. Already 3 days after injection of the virus, high levels of HCV RNA could be detected in the mouse plasma. In seven out of eight nontreated control mice HCV RNA remained detectable until at least 60 days after infection. One animal spontaneously cleared the virus 5 weeks after inoculation. In all 10 treated mice the viremia declined steeply during antibody administration and remained below the limit of detection for at least 2 months after infection.

HCVcc Mouse Passaging and Iodixanol Ultracentrifugation. The *in vivo* antiviral efficacy of anti-SR-BI mAb therapy turned out to be considerably higher than what had been observed in cell culture. One major difference between both experimental settings may be the lipoprotein composition of the viral particles. For the *in vitro* experiments we used HCVcc that were produced in hepatoma cells, which are known to have impaired VLDL biogenesis.[28,29] For the *in vivo* experiments the same HCVcc was used to initially infect the mice but new viral particles are rapidly produced, this time by fully functional primary hepatocytes. This is known to have an impact on the lipoprotein composition of these newly formed HCV and to change certain physicochemical and biological characteristics.[30,31] It might well be that their SR-BI receptor usage was also modified. However, density fractionation analysis did not show an increased proportion of infectious viral particles at lower densities for mouse-derived (mHCV) Jc1wt or Jc1ΔHVR1 particles compared to the respective cell culture-derived virions (Fig. 3). Although based on our analyses we cannot exclude potential discrepancies in apolipoprotein composition (or other alterations) between mHCV and HCVcc virions, our *in vitro* infection prevention experiments did not reveal increased sensitivity to anti-SR-BI mAb therapy of mHCV compared to HCVcc (Fig. 4A). Furthermore, prevention experiments using increasing concentrations of anti-SR-BI mAb did not show increased antiviral responses against specific mHCV subfractions (Fig. 4B-D). This indicates that the sensitivity of HCVcc to mAb1671 treatment in cell culture did not change after mouse passaging of the virus and that the increased *in vivo* activity of mAb1671 is not directly caused by potential *in vivo* adaptations of the virus.

Human Lipoproteins Influence HCVcc Infectivity and Improve mAb1671 Treatment Outcome. Human serum and especially its lipoprotein constituents are known to influence the infectivity of HCV, and therefore also the efficacy of anti-HCV neutralizing antibodies. Cell culture media contains considerably fewer lipoproteins than serum *in vivo* (data not shown). Therefore, the effects of *in vivo*-like concentrations of human lipoproteins HDL and VLDL on HCV infectivity and their effect on the mAb1671 therapy efficacy were evaluated in cell culture. Huh7.5 cells were pretreated with 230 μg human HDL cholesterol/mL or 180 μg human VLDL cholesterol/mL. These concentrations correspond with HDL and VLDL cholesterol levels detected in serum from humanized uPA-SCID mice. As shown in Fig. 5, human VLDL inhibited infectivity of all viruses and, in combination with mAb1671, it showed an additive antiviral effect to the activity of mAb1671 alone. In contrast, HDL only inhibited the infectivity of the anti-SR-BI resistant variants, whereas wild-type infectivity remained

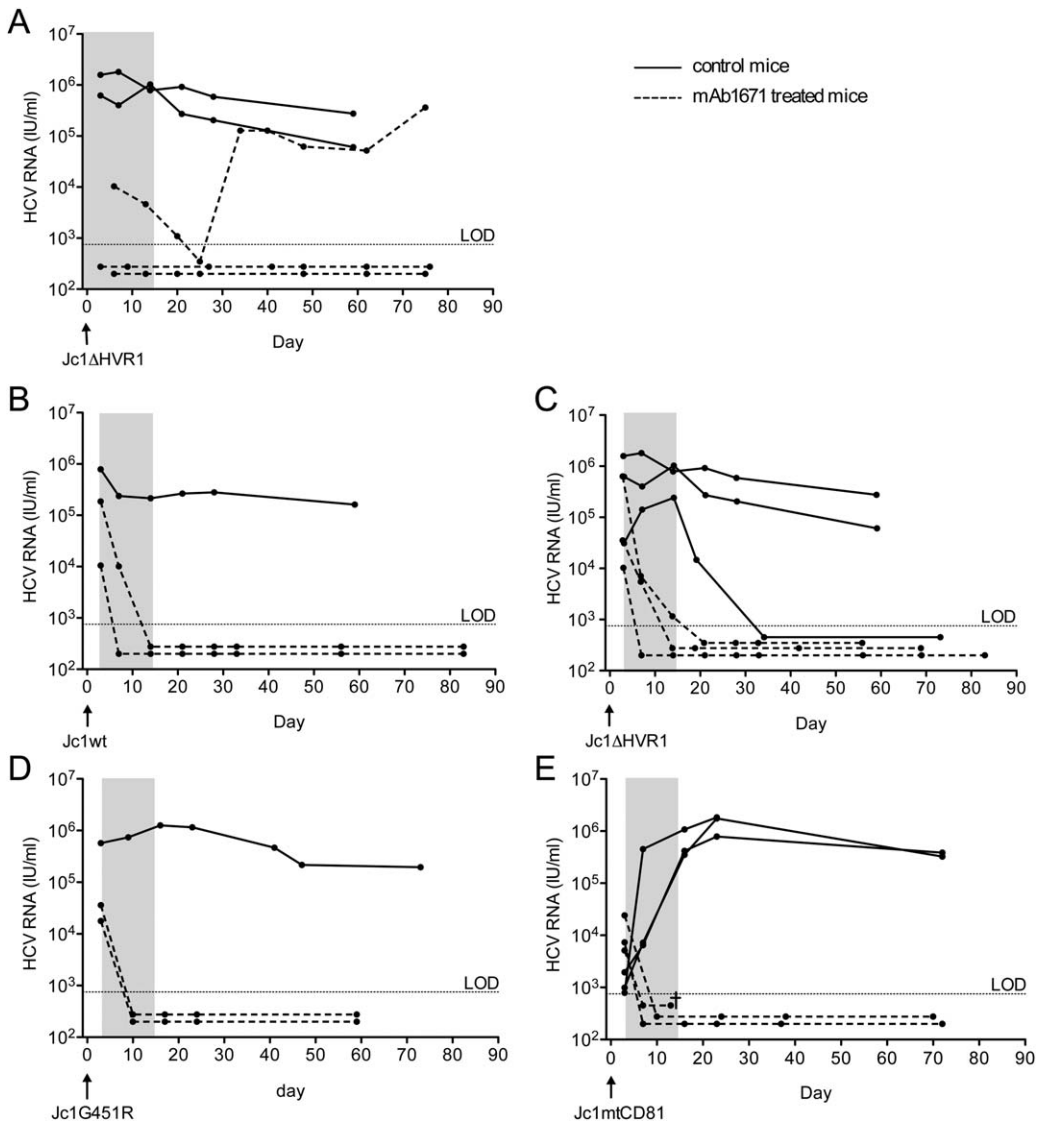

Fig. 2. Efficacy of the SR-BI-specific antibody mAb1671 in blocking HCV infection in humanized mice. Within a 2-week period (indicated by the gray area) the animals received six intraperitoneal injections, each containing 400 μg of the antibody. The antibody was tested in two different settings: (A) a prevention experiment where the first antibody dose was administered one day before viral challenge; and (B-E) a postexposure setup where the anti-SR-BI therapy was initiated 3 days postviral challenge. Antibody-treated mice are indicated with a dotted line, whereas nontreated control animals are represented by solid lines. Chimeric mice were challenged at day 0 with Jc1wt (B), Jc1ΔHVR1 (A,C), Jc1G451R (D), or Jc1mtCD81 (E). Each data point represents the plasma HCV RNA level (IU/mL) of an individual chimeric mouse at a given timepoint. The limit of detection (LOD) equals 750 IU/mL.

unchanged. Three different batches of human HDL were tested for their effect on Jc1wt infectivity; the first batch enhanced Jc1wt infectivity, the two others slightly inhibited infectivity of this virus. Merging of these datasets results in a seemingly unchanged infectivity of the wild-type virus in the presence of human HDL. Nevertheless, the combination of HDL and mAb1671 nearly completely inhibited infection of all viruses tested. Although HDL alone did not, on average, change Jc1wt infectivity, it is able to significantly enhance the antiviral efficacy of the mAb1671 against this virus ($P < 0.05$). While HDL had a clear synergistic effect on the activity of mAb1671 against the wild-type virus, due to the pronounced activity by HDL as such, it seemed to have a more additive effect on mAb1671's activity against the resistant viruses. None of mAb1671-lipoprotein combinations decreased Huh7.5 cell growth or metabolic activity (data not shown). To test whether the enhanced antiviral activity against the wild-type virus exerted by HDL is specific to mAb1671, we assessed the effect of HDL on the anti-CD81 mAb JS81 and the anti-SR-BI small molecule ITX-5061. Figure 6 shows that human HDL only improved the antiviral effect of mAb1671 and not that of JS81 or ITX-5061.

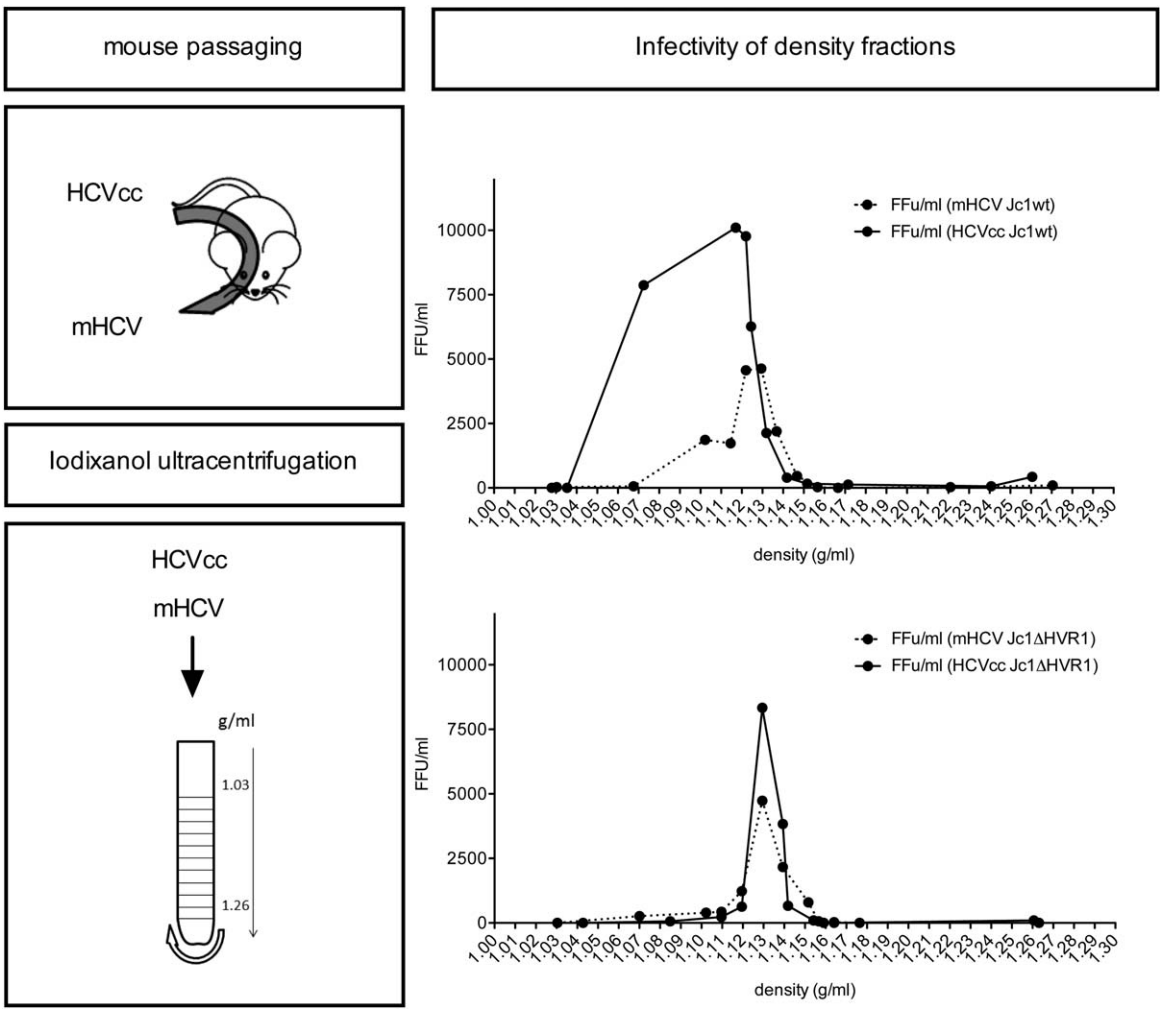

Fig. 3. Buoyant density gradient analysis of HCV produced in cell culture and in humanized mice. Serum was collected over a 2-month infection period from humanized mice inoculated with cell culture produced (HCVcc) Jc1wt and Jc1ΔHVR1. Pooled serum containing mouse-passaged HCV, designated mHCV, and culture supernatant containing HCVcc was ultracentrifuged over an iodixanol gradient. Twelve fractions were collected from the top of the gradient and analyzed for cell culture infectivity (in triplicate) expressed as FFU/mL.

Discussion

HCV entry into the hepatocyte is a crucial event in the infection process, which can be targeted by anti-HCV antibodies.[32-35] However, prevention of HCV recurrence after liver transplantation using antibodies that target HCV envelope proteins has not yet proven to be very successful,[36,37] probably due to the high heterogeneity of the viral envelope proteins. In addition, it was shown that HDL can reduce the neutralizing effect of anti-HCV antibodies,[38,39] raising additional concerns about the efficacy of anti-HCV antibodies for passive immunotherapy. Besides viral envelope proteins, different host factors are involved in the HCV life cycle. Mensa et al.[40] recently reported that early reinfection kinetics of HCV after liver transplantation are modulated by HCV receptor levels, such as SR-BI, at the time of transplantation. This suggests that blockade of this receptor may delay or prevent HCV reinfection of the graft. Accordingly, we have shown *in vivo* efficacy of anti-SR-BI mAbs in chimeric mice and their potential applicability in the liver transplantation context.[15,16] Evidence for the clinical safety of SR-BI inhibition has been established for the SR-BI small molecule inhibitor ITX-5061[41,42] and a monoclonal antibody similar to mAb1671.[43] Clinical efficacy of ITX-5061 in chronic HCV patients is lacking,[44] but phase 1 efficacy studies in liver transplant patients are currently ongoing.

The emergence of HCV variants, resulting from the lack of proofreading capacity of the viral polymerase, which are resistant to anti-envelope antibodies or DAA, is a major cause of immunologic and therapeutic failure. The more conserved nature of host factors

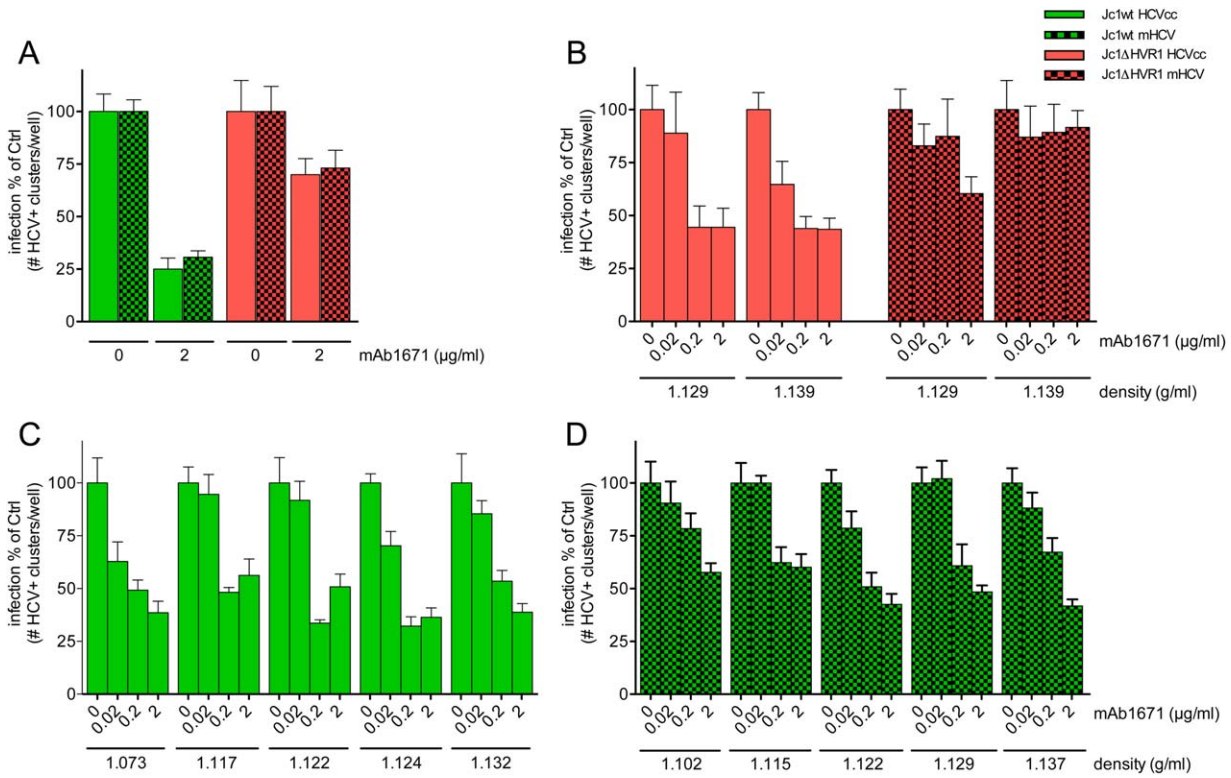

Fig. 4. *In vitro* anti-SR-BI mAb1671 sensitivity determination. (A) Huh7.5 cells were pretreated with 2 μg/mL mAb1671 before infection with cell culture and mouse-passaged Jc1wt (green) and Jc1ΔHVR1 (red). Two days after infection, HCV-positive clusters were enumerated. (B-D) The different density fractions with *in vitro* detectable infectivity were incubated with mAb1671-pretreated Huh7.5 cells. Two days later the number of HCV-positive cell clusters was determined. All conditions were tested in quadruplicate and the means are shown. Error bars represent standard error of the mean.

compared to viral proteins makes the former interesting therapeutic targets. However, HCV variants have been described that carry changes in their envelope glycoproteins which are less dependent on SR-BI for infection of hepatoma cells *in vitro*. Compared to wild-type virus, cell culture infectivity of these variants is less efficiently blocked by anti-SR-BI therapy.[17-21] The existence of partially anti-SR-BI resistant HCV variants indicates that the efficacy of the SR-BI-targeting approaches might be compromised by reduced susceptibility of some (re-)infecting virus, raising questions about its applicability *in vivo*.

Since viral mutations that render the virus less dependent on a specific cell entry factor may also affect its *in vivo* fitness, we first determined whether humanized mice could be infected with HCV variants that are less dependent on SR-BI and examined next whether these variant-infected mice would still respond to anti-SR-BI mAb therapy. The mutants investigated in this study were: E2ΔHVR1-deleted Jc1 (Jc1ΔHVR1),[18] E2 G451R substituted Jc1,[19] the mouse CD81-adapted Jc1 virus carrying amino acid changes in E1 (L216F) and E2 (V388G and M405T),[19] and the recently described J6/JFH1 Clone2 with mutations in E1 (I374L) and E2 (I411V).[20] Having confirmed the reduced sensitivity of these mutants to anti-SR-BI targeting agents (mAb1671 and ITX-5061) *in vitro*, we examined the effect of SR-BI-blockade *in vivo*. Administration of mAb1671 to mice not only inhibited infection with wild-type Jc1, but also suppressed HCV RNA in mice infected with Jc1ΔHVR1, Jc1G451R, and Jc1mtCD81 to undetectable levels. This suppression not only occurred in a prophylactic setting, as shown for Jc1ΔHVR1, but even when administration of the antibodies was initiated several days after the infection was established. Although a decrease in viremia was observed during mAb1671 administration in a mouse infected with J6/JFH1 Clone2, no clear-cut proof of its antiviral effect against this virus could be obtained due to unstable viremia in infected control animals (data not shown). This indicates that the J6/JFH1 Clone2 virus may have acquired *in vitro* adaptations that negatively affect its *in vivo* fitness.

Next, we addressed potential mechanisms that may contribute to the discrepancies in antiviral efficacy of

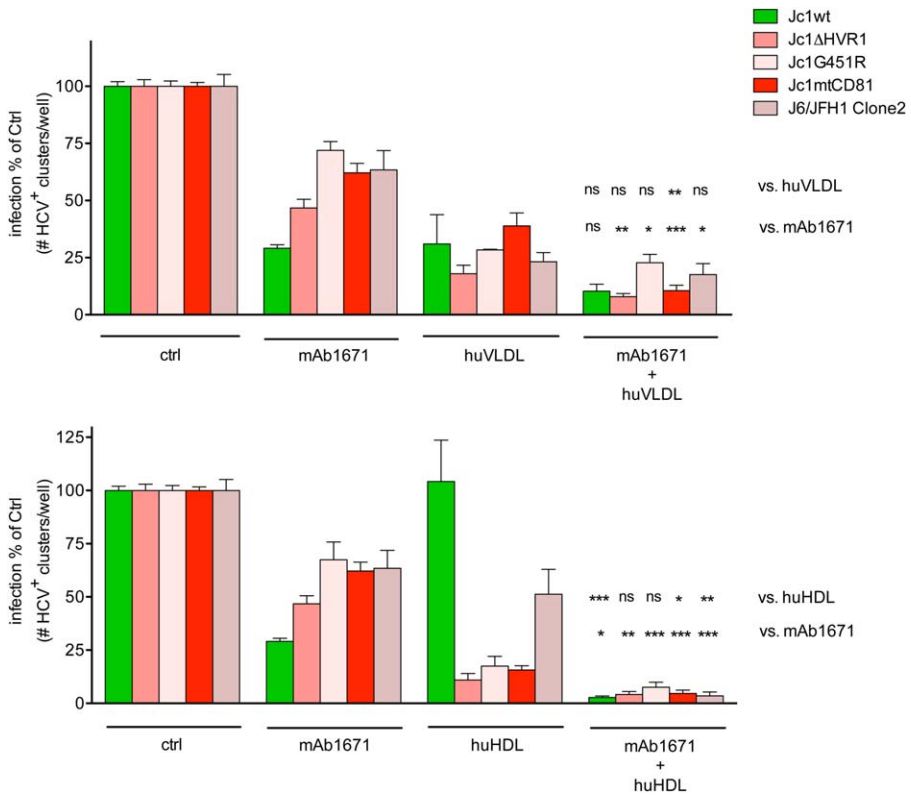

Fig. 5. Effect of HDL and VLDL on *in vitro* HCVcc neutralization. Huh7.5 cells were pretreated with 230 μg human HDL cholesterol/mL or 180 μg human VLDL cholesterol/mL alone or in combination with 20 μg/mL mAb1671 (HDL and VLDL concentrations correspond with levels detected in serum from humanized uPA-SCID mice) before infection with Jc1wt, Jc1ΔHVR1, Jc1G451R, Jc1mtCD81, and J6/JFH1 Clone2. After 2 days, the number of HCV-positive clusters was counted. The data shown for Jc1wt, Jc1ΔHVR1, and Jc1mtCD81 originates from three (HDL and combination with mAb1671) and two (VLDL and combination with mAb1671) individual experiments, whereas the effect on Jc1G451R and J6/JFH1 Clone2 was assessed over two experiments (HDL and combination with mAb1671) and in one individual experiment (VLDL and combination with mAb1671). In each experiment all conditions were tested in duplicate and different batches of VLDL or HDL were used. Error bars represent standard error of the mean. The asterisks ($*P < 0.05$, $**P < 0.01$ and $***P < 0.001$) indicate statistically significant differences, whereas "ns" stands for not significantly different.

mAb1671 observed in cell culture experiments (*in vitro*) versus studies performed in humanized mice (*in vivo*). Administration of mAb1671 to humanized mice completely prevented HCV infection with the wild-type and mutant viruses, whereas *in vitro* this was not the case. In addition, cell-to-cell spread of Jc1ΔHVR1 virus could not be completely inhibited *in vitro*, whereas *in vivo* even postexposure therapy was highly effective against this virus.

We have previously observed that chimpanzee- and humanized mouse-derived J6/JFH1 viral particles have a higher specific infectivity than cell culture derived virus, correlating with a decreased average buoyant density.[30] This suggests that differences in physical association of HCV with low-density factors in these HCV infection models influence viral infectivity. Hence, we hypothesized that humanized mouse-derived lower density viral particles, associated with lipoproteins, might be more dependent on the

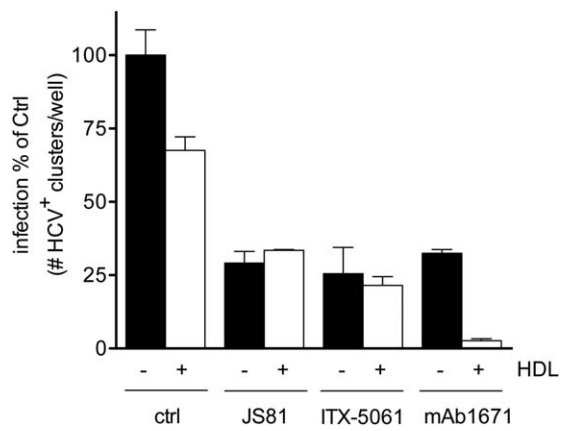

Fig. 6. *In vitro* effect of HDL in combination with different antireceptor therapies. Huh7.5 were pretreated with 4 μM ITX-5061, 0.2 μg/mL JS81, and 20 μg/mL mAb1671 alone or in combination with 230 μg human HDL cholesterol/mL before infection with Jc1wt. After 2 days the number of HCV-positive clusters was counted. All conditions were tested in duplicate and error bars represent standard error of the mean.

physiological HDL-binding and cholesterol transfer function of SR-BI and therefore more sensitive to anti-SR-BI therapy. However, the infectious virus particles used in this study did not show a shift to lower buoyant density fractions after passaging in the humanized mouse; in fact, for Jc1wt rather the opposite was observed. Whether this relates to a difference between Jc1 and J6/JFH1 or to batch-to-batch differences is not clear. Additionally, no increased SR-BI-receptor usage could be observed in mouse-derived HCV as compared to culture-derived HCV. This is consistent with the observation that *ex vivo* chimpanzee-derived HCVcc was equally sensitive to anti-SR-BI mAb than cell culture produced HCV.[14]

Although HDL lowers the neutralization efficacy of anti-HCV antibodies by enhancing HCV entry,[38,39] it does not negatively affect the anti-HCV effect of anti-SR-BI mAb C167 in culture.[14] Previously, it was reported that SR-BI blocking agents are able to inhibit the enhancement of HCV infectivity mediated by HDL.[45,46] While in these studies low amounts of HDL were used (range 1.6 to 6 μg HDL cholesterol/mL), we describe here that the addition of *in vivo*-like concentrations of human HDL (230 μg HDL cholesterol/mL) to mAb1671 is able to substantially enhance its *in vitro* efficacy against the wild-type virus. While HDL and VLDL by themselves already have an inhibitory influence on the infectivity of the resistant viruses, addition of mAb1671 seemed to further increase this effect, although not always statistically significantly. Both these observations may explain why *in vivo* higher protection rates can be achieved against wild-type and resistant viruses compared to what was previously observed *in vitro*. While HDL seemed to have a synergistic effect on the antiviral efficacy of mAb1671 against wild-type HCV, it did not alter the antiviral activity of JS81 or ITX-5061, indicating that this synergism might possibly be specific for mAb1671. Our study also confirms that human VLDL inhibits the infectivity of HCVcc and shows that mAb1671 further increases this inhibition.

In vivo-like concentrations of HDL clearly inhibited the anti-SR-BI resistant mutants and seemingly did not affect wild-type infectivity. However, different HDL batches behaved differently in such a way that one batch had an enhancing effect on Jc1wt infectivity while the two others were inhibiting. The observation that some HDL batches decreased rather than enhanced Jc1wt infectivity is consistent with another study that used increasing concentrations of HDL and observed reduced wild-type infectivity at the highest concentrations.[14] Overall, even when HDL enhanced Jc1wt infectivity, addition of HDL to mAb1671 was able to almost completely suppress both variant and wild-type infectivity.

HDL-mediated enhancement of infection depends both on the lipid transfer function of SR-BI and its ability to bind HCV E2.[11,47] The fact that mAb1671 interferes with both functions (data not shown) should enable this therapeutic approach to at least prevent the enhancement of infection, as was shown for other SR-BI inhibitors.[12,39,45] Moreover, we observe here that inhibitory effects of HDL start to dominate when it fails to support HCV infectivity, as is the case for viruses with particular envelope mutations that alter the SR-BI receptor usage or in case of antibody-, but not ITX-5061-, mediated SR-BI blockade. These particular factors that preclude the virus from using the SR-BI-mediated HCV entry route seem to convert HDL into an inhibitory particle. We hypothesize that HDL's effect on HCV infectivity is double-edged. In case the SR-BI-mediated entry pathway is fully operational, the HDL-mediated infection enhancement either dominates or at least conceals the HDL inhibitory effects. However, in situations leading to a partial redundancy of the SR-BI-mediated entry pathway, HDL's inhibitory effects prevail. Accordingly, while ApoCI, an exchangeable apolipoprotein that resides in HDL, was shown to be a key mediator of the HDL-mediated infection enhancement process, increasing its concentration resulted in decreased HCV infectivity by specifically disrupting the viral membrane.[46] Although ITX-5061 also inhibits the SR-BI receptor, it is interesting to note that HDL did not enhance the therapeutic efficacy of ITX-5061 *in vitro*. Possibly, this phenomenon may be specific for the antibody used in this study. Other signs for the inhibitory activity of HDL can be found in the work of Bartosch et al. and Dreux et al.[46,47] Their studies show that HCVpp containing the E2 point mutation L399R do not profit from HDL-mediated infection enhancement and that the presence of HDL (6 μg/mL) even reduced infectivity. In addition, Dao Thi et al.[47] mention that "the absence of HDL-mediated infection enhancement uncovered an inhibitory activity of lipoproteins," which they attribute to the possible presence of oxidized lipids.

The results from our study address possible concerns arising from the fact that antiviral pressure may select for therapy-resistant HCV variants, resulting in an increased likelihood of virologic failure during anti-SR-BI therapy. However, compared to the wild-type virus, all the investigated variants are more vulnerable to inhibition by HDL and envelope-targeting neutralizing antibodies.[18-20] Because of these characteristics, in addition to remaining sensitive *in vivo* to anti-SR-

BI mAb therapy, it is unlikely that such variants would emerge and propagate during the course of an anti-SR-BI therapy. In fact, to maximize virologic response, a therapy that combines anti-SR-BI with anti-envelope agents might be worth considering.

We demonstrate for the first time that, except for J6/JFH1 Clone2, HCV variants with altered SR-BI usage *in vitro* are fit in humanized uPA-SCID mice but can be successfully blocked by the SR-BI-targeting antibody mAb1671. The differences between *in vitro* and *in vivo* mAb1671 therapy outcomes may be explained by the presence *in vivo* of human lipoproteins. We show that VLDL by itself inhibits both wild-type and resistant virus infectivity, whereas HDL only has a direct negative impact on the infectivity of the resistant viruses but also potentiates mAb1671's antiviral effect against the wild-type virus. This study also highlights that the humanized mouse is a more appropriate HCV infection model than the Huh7.5 cell culture system, presumably by more physiologically relevant location and function of the receptors on polarized primary hepatocytes in the liver. Our findings implicate that novel (host-targeting) therapeutics should preferentially be evaluated in this model.

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