Broadly neutralizing antibodies abrogate established hepatitis C virus infection
Ype P. de Jong et al.
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Editor's Summary

Neutralizing Antibodies Take Down the HCV Establishment

In most individuals infected with hepatitis C virus (HCV), the HCV sets up shop—establishing a long-term, chronic infection that damages the liver and can lead to cirrhosis or liver cancer. de Jong et al. now report that a trio of neutralizing antibodies not only can prevent infection but also can treat and maybe even cure already established infection in multiple animal models. The broadly neutralizing antibodies, which could block multiple genotypes of HCV, were delivered into the muscle by a virus—an adeno-associated vector that does not cause disease—resulting in prolonged expression of the antibodies. If these data hold true in people, this approach may provide a new tool for treating HCV infection.

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Broadly neutralizing antibodies abrogate established hepatitis C virus infection

Ype P. de Jong,¹,²* Marcus Dorner,²¹ Michiel C. Mommersteeg,² Jing W. Xiao,² Alejandro B. Balazs,³ Justin B. Robbins,⁴ Benjamin Y. Winer,⁵ Sherif Gerges,⁵ Kevin Vega,⁵ Rachael N. Labitt,² Bridget M. Donovan,² Erick Giang,⁴ Anuradha Krishnan,⁶ Luis Chiriboga,⁷ Michael R. Charlton,⁶ Dennis R. Burton,³⁴ David Baltimore,⁸ Mansun Law,⁴ Charles M. Rice,² Alexander Ploss²,⁵*}

In most exposed individuals, hepatitis C virus (HCV) establishes a chronic infection; this long-term infection in turn contributes to the development of liver diseases such as cirrhosis and hepatocellular carcinoma. The role of antibodies directed against HCV in disease progression is poorly understood. Neutralizing antibodies (nAbs) can prevent HCV infection in vitro and in animal models. However, the effects of nAbs on an established HCV infection are unclear. We demonstrate that three broadly nAbs—AR3A, AR3B, and AR4A—delivered with adenovirus-associated viral vectors can confer protection against viral challenge in humanized mice. Furthermore, we provide evidence that nAbs can abrogate an ongoing HCV infection in primary hepatocyte cultures and in a human liver chimeric mouse model. These results showcase a therapeutic approach to interfere with HCV infection by exploiting a previously unappreciated need for HCV to continuously infect new hepatocytes to sustain a chronic infection.

INTRODUCTION

Hepatitis C virus (HCV) chronically infects at least 170 million worldwide, and until recently, curative therapies were poorly tolerated and ineffective in most patients (1). HCV is among the few viruses that cause human pathology that can either establish a chronic infection or be spontaneously cleared. Although an essential function for T cells is widely accepted in HCV clearance, the role of antibodies in controlling HCV infection remains elusive. Individuals almost universally seroconvert to HCV infection may be further diminished by HCV’s remarkable genetic diversity and high proliferative rate, which readily yields mutations that allow the virus to escape from immune pressure (9). In vitro experiments in human hepatoma cell lines suggest that the effect of antibodies on ongoing infection may be further diminished by HCV’s ability to spread directly from cell to cell via routes that are inaccessible to nAbs (10-12). However, clinical reports using the B cell–depleting antibody rituximab in chronically infected patients showed that HCV viremia rose between 10- and 100-fold after rituximab treatment and returned to baseline after reappearance of B cells (13, 14). Similarly, agammaglobulinemic patients have been shown to progress more rapidly to cirrhosis (15), although there are case reports that such patients retain the ability to spontaneously clear HCV (16). These clinical observations suggest that B cells and antibodies play a role in virus control but are not essential for virus clearance.

To better define the role of nAbs in HCV infection in model systems that more reliably capture some aspects of human physiology, we used three different systems: primary hepatocyte cultures, mice expressing the human HCV entry factors, and human liver chimeric mice. We chose three potent nAbs and assessed their ability to prevent infection in all three systems. In addition, we tested their effects on established infection in primary hepatocyte cultures and liver chimeric mice.

RESULTS

Adeno-associated virus–delivered nAbs neutralize across HCV genotypes

We recently showed that recombinant adeno-associated viruses (AAVs) are highly efficient vectors for antibody delivery after intramuscular injection (17). We constructed AAV8 vectors expressing the three HCV nAbs: AR3A, AR3B (18), and AR4A (19). Injection of 10¹¹ genome copies of AAV-AR3A, AAV-AR3B, and AAV-AR4A or an anti-HIV monoclonal antibody (mAb) (B12) (20) into the gastrocnemius muscle of highly immunocompromised non-obese diabetic (NOD) Rag1⁻⁻ II2Rγcnull (NRG) mice or immunocompetent FVB mice resulted in stable, prolonged expression of human immunoglobulin G (IgG) expression for more than 4 months (Fig. 1, A and B). It was previously shown that AR3A, AR3B, and AR4A potently inhibit HCV entry in cell lines. To test the capacity of in vivo–expressed human nAb to inhibit HCV infection, we performed in vitro neutralization assays using a broad spectrum of intergenotypic chimeras harboring the structural proteins of diverse HCV genotypes (21-23). Serum containing anti-HCV nAbs efficiently neutralized most HCV genotypes, preventing infection of Huh-7.5 hepatoma cells. Of the three nAbs, AR4A was the most potent and...
showed IC₅₀₈ (median inhibitory concentrations) between 1 and 3 log₁₀ lower than the previously published nAb 3/11 (12) (Fig. 1C).

**Three nAbs protect genetically humanized mice from HCV infection**

Having established that the AAV-delivered nAbs could efficiently neutralize HCV in vitro, we set out to test their ability to block productive viral entry in vivo. We used a genetically humanized mouse model based on adenoviral delivery of human HCV entry factors into mice, in which expression of firefly luciferase is repressed under steady-state conditions via a loxP site-flanked transcriptional stop cassette (24). Rosa26-Fluc mice received nAb-expressing AAV vectors (n = 12 per group). Subsequent challenge with a bicistronic HCV genome expressing Cre recombinase (25) showed that each of the three nAbs alone or as a pool efficiently prevented HCV entry as determined by a lack of in vivo bioluminescence (Fig. 1D). We next examined the ability of the nAbs to prevent infection of human liver chimeric mice, the only small-animal model that robustly supports the entire HCV life cycle. We constructed a novel xenorecipient strain by crossing the fumaryl acetoacetate hydrolase (Fah) knockout allele (26) for 13 generations onto the NRG background. After transplantation of adult human hepatocytes into the resulting FNRG mice, mouse liver damage was induced by intermittent withdrawal of the protective drug nitisinone and engraftment levels were followed over time by measuring human albumin (hAlb) levels in the sera. In closely related Fah⁻/⁻ Rag2⁻/⁻ IL2Rγnull (FRG) mice, hAlb levels were previously shown to correlate with hepatocyte chimerism (27). Although engraftment varied by human donor, FNRG mice consistently engrafted to higher levels than did FRG mice (fig. S1A) (27, 28). The number of human cells could be quantified by flow cytometry for human CD81⁺ cells in mouse liver (fig. S1B) and confirmed by histological staining for Fah (fig. S1C). Having established this human liver chimeric FNRG model, we selected animals that were engrafted with adult human hepatocytes to hAlb levels >1 mg/ml (huFNRG), because this had previously been shown to correspond to the minimal engraftment level required for HCV permissiveness (27, 29, 30). To test whether the AAV-delivered nAbs could prevent infection, we inoculated huFNRG mice with a pool of the three nAb-expressing AAV vectors (AR3A/AR3B/AR4A) or the control AAV vector (B12). Sixteen days after AAV injection, the mice displayed serum IgG levels between 26 and 126 μg/ml and, 3 days later, were infected with a low dose (about 3000 copies) of HCV genotype 1a clone H77 (31). Whereas two of three control mice became viremic, none of the three mice that received the AR pool displayed viremia (Fig. 1E and table S1). Because AAV injection resulted in a decrease of hAlb serum levels (fig. S2A), which could affect the human graft’s ability to support infection, a separate group of huFNRG mice received three injections of a pool of the purified nAbs or b6 control nAb before challenge with low-dose H77. Similar to the AAV protection experiments, both mice that received the three nAbs remained aviremic, whereas three of three control nAb recipients rapidly became viremic. These data show that nAbs AR3A, AR3B, and AR4A delivered through an AAV vector can effectively neutralize HCV across most genotypes and that these nAbs can protect human liver chimeric mice from HCV infection with a low-dose inoculum.

**nAbs abrogate established HCV infection in primary hepatocyte cultures**

Considering their potent HCV neutralization in vitro and their ability to protect against virus challenge in vivo, we tested the ability of these three nAbs to interfere with an established HCV infection. HCVcc infection is most reproducibly studied in the HuH7 hepatoma cell line and its derivatives, which have two major limitations: Proliferation limits the time window of any studies, and their impaired innate immunity allows for high replication to supraphysiological levels. We therefore chose to study the role of these nAbs in primary human fetal
liver cultures (HFLCs), in which the hepatocytes have intact innate immunity, are nonproliferating, and can support HCVcc for several weeks (32). We first performed a neutralization assay and found that, similar to results in Huh7 cells, the three purified nAbs were able to prevent infection of a cell culture–produced HCV reporter virus that secretes Gaussia luciferase, termed HCVcc-Gluc (33) (Fig. 2A). We then used a “therapeutic” protocol in which HFLCs were infected with HCVcc-Gluc, and 3 days later, after replication had been established, purified nAbs were added and maintained in the medium for the remainder of the experiment. Longitudinal luminescence measurements (Fig. 2B) or combined endpoints from three of four livers that supported infection (Fig. 2C) showed that nAbs were able to interfere with established HCV infection in HFLCs, although not nearly as efficiently as the polymerase inhibitor 2′-C-methyl adenosine (2′CMA) (34). These data suggest that extracellular spread contributes to maintenance of infection in primary hepatocytes.

**HCV-infected liver chimeric mice can be cured with nAbs**

These in vitro results led us to test the therapeutic effect of nAbs on established HCV infection in huFNRG mice. After intramuscular injection of FVB mice with a firefly luciferase–expressing AAV8, we observed luminescence in both liver and muscle (fig. S2B). Intramuscular injection of nAb-expressing AAVs into huFNRG mice (n = 8 mice total) resulted in a consistent 5- to 10-fold drop in hAlb serum levels, suggesting that AAV8 vectors were also affecting the human xenograft (fig. S2A). We therefore used a passive immunization approach to investigate the role of these nAbs on established viremia. We infected huFNRG mice with a cell culture–derived HCVcc (clone J6/JFH) (23). HCVcc was previously shown to be infectious in liver chimeric mice (35) and in vitro, which allowed us not only to determine viral RNA titers but also to quantify the number of infectious particles in limiting dilution assays. Seventeen to 25 days after infection, mice (n = 3 for AR pool, n = 2 for b6 controls) were treated with 500 µg of each nAb (AR3A/AR3B/AR4A) or 1.5 mg of control IgG (b6) every 3 days, which resulted in high and stable IgG levels (Fig. 3A and table S1) without affecting serum hAlb levels (Fig. 3B and table S1). nAb treatment suppressed serum infectivity to below the limit of detection within 1 day (Fig. 3C and table S1). Notably, HCV (RNA copy numbers decreased in all mice that received the AR pool and fell below the limit of detection between 5 and 11 days after starting nAb treatment (Fig. 3D and table S1). These data are in line with previously published observations that administration of a single dose of a nAb reduced the HCV viral load below the limit of quantification in a chronically infected chimpanzee (36). We treated HCV-infected human liver chimeric mice for 30 days with nAbs and longitudinally monitored their IgG levels in the serum until they became undetectable, 55 to 69 days after the last injection (Fig. 3A and table S1). A repeat experiment using the same conditions (n = 3 for AR pool, n = 4 for b6 controls) again showed the rapid loss of J6/JFH viremia between 4 and 8 days after starting nAb injection (Fig. 3E and table S1). In contrast to a previous study performed in experimentally HCV-1a–infected chimpanzees (36), J6/JFH viremia did not reappear after the nAb levels had fallen below the limit of detection. These data suggest that either the graft was no longer permissive to HCV or the virus was indeed cleared and the remaining HCV reservoirs may have been eliminated. To prove that the human graft could still support HCV infection, mice were challenged with a heterologous HCV-1a (clone H77) at ~2 × 10^4 copies (Fig. 3D and table S1). Three of three mice that still had hAb levels >1 mg/ml became infected, illustrating that the lack of viremia was not due to graft loss or nonpermissiveness in these mice. These results show that three nAbs can efficiently abrogate serum infectivity, which leads to rapid loss of viremia that cannot be attributed to graft loss.

We next aimed to determine whether treatment with these three nAbs could abrogate viremia in mice infected with a different HCV genotype. Using a similar passive immunization strategy as before, H77 viremic huFNRG mice (n = 3 for AR pool, n = 3 for b6 controls) were treated with either the pool of three anti-HCV nAbs or an isotype control (b6). Similarly to J6/JFH-infected mice, all nAb-treated mice lost their viremia to below the limit of detection, although the difference in viremia with the control mice was less pronounced (Fig. 3F and table S1). However, and in contrast to the rapid disappearance in J6/JFH-infected animals, it took 15 to 30 days for H77-infected huFNRG mice to lose their viremia. When mice were followed, virus reappeared spontaneously, indicating that although viremia was suppressed, these mice were not cured by nAb treatment. Sequencing of H77 virus in the mice...
that relapsed did not reveal any escape mutations with the viral envelope associated with these nAbs (fig. S3).

DISCUSSION

Vectored immunoprophylaxis has recently been shown to protect mice efficiently against HIV or influenza A virus infection (17, 37, 38). Likewise, our results show that potent nAbs delivered by AAV vectors can prevent HCV infection, although this is generally much less efficient than in vitro neutralization assays may suggest (39, 40). Using a different nAb, others have shown that this approach may hold promise for protecting liver grafts from reinfection (41). Dosing of nAbs for this application will likely depend on their neutralization ability because AbXTL68 did not protect the graft from reinfection even at serum concentrations of 200 µg/ml (42).

Our findings are the first to show that nAbs can cure liver chimeric mice, even in the absence of an adaptive immune system. It is yet unclear if these findings are specific for J6/JFH or generalizable to all HCV isolates. Treatment of liver chimeric mice infected with HCV genotype 1a (strain H77) with the pool of antibody also lead to a decrease of viremia below the limit of detection, but HCV relapsed spontaneously, which could be explained by the shorter nAb treatment duration or by strain differences between J6/JFH and H77. Nevertheless, our data imply that HCV must continuously reinfect new hepatocytes to sustain viremia. We speculate that the mechanism by which nAbs have the ability to cure liver chimeric mice involves the protection of uninfected hepatocytes from becoming infected and thereby allowing for clearance of HCV in already infected hepatocytes. Given the distinct kinetics in viremia decline between H77 and J6/JFH, our findings suggest differences in the survival of HCV in hepatocytes, which could be due to cytopathic mechanisms or clearance by innate immune pathways. To better dissect the relative contributions of these mechanisms, it will be important to extend these observations to other

HCV isolates and genotypes, for example, HCV genotype 3. This genotype was recently shown to require prolonged suppression with a polymerase inhibitor (sofosbuvir) compared to genotype 2 isolates despite being equally interferon-sensitive (43).

Human primary hepatocyte cultures and humanized mice systems do not completely mimic the three-dimensional architecture of liver, but they are, apart from chimpanzees, arguably the most advanced systems put into question whether cell-to-cell spread is a dominant route for reinfection, although it is conceivable that this process is compromised and less efficient in these experimental systems than in humans.

Fig. 3. Combination treatment of human liver chimeric mice with broadly neutralizing anti-HCV antibodies clears established HCV infection. (A) Highly engrafted huFNRG mice were infected with HCVcc (J6/JFH) and, 25 days after infection, when all mice were viremic, were injected with either the pool of nAb AR3A, AR3B, and AR4A or control mAb b6 at time points indicated by the gray arrows. Human IgG levels in mouse serum were determined by ELISA longitudinally until they became undetectable. (B) In the same group of mice from (A), hAb levels in mouse serum were measured by ELISA for the duration of the experiment. (C) Starting 1 day after nAbs were injected, HCV serum infectivity was measured in a limiting dilution assay on Huh-7.5 hepatoma cells. TCID50, median tissue culture infectious dose. (D) HCV RNA was determined in mouse serum by qRT-PCR for the duration of the experiment. On day 163 after infection and 56 days after nAb levels had fallen below the limit of detection, the three surviving mice were challenged with HCV (H77) (red arrow) and their viremia since rechallenge is depicted by red circles. Each dot represents an individual liver chimeric animal. Lowest P value for individual time point is 0.01 on treatment day 13 by unpaired t test; P = 0.067 for experiment by two-way ANOVA. (E) Repeat experiment in which J6/JFH viremic huFNRG mice were treated with injections of nAb (gray arrows), and HCV RNA serum levels were determined by qRT-PCR. J6/JFH viremic huFNRG mice were treated with injections of nAb (gray arrows), and HCV RNA serum levels were determined by qRT-PCR. One surviving mouse was rechallenged with H77 (red arrow), and serum RNA is depicted in red circles. Lowest P value for individual time point is 0.016 on day 39 by unpaired t test. (F) Combined graph of two experiments with H77-infected huFNRG mice. Viremic mice received five (squares) or seven (circles) injections of nAb (arrows), and HCV RNA in mouse serum was determined by qRT-PCR.

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Recent findings demonstrate that broadly nAbs can also efficiently suppress viremia in humanized mice (17, 44) and Rhesus monkeys infected with HIV or SHIV (45, 46), respectively. Our data extend this work to hepatotropic viral infections.

Whether these nAbs will become useful in HCV-infected patients will largely depend on the ability of upcoming direct-acting antivirals (1) to eradicate this infection in every patient. Although we used high doses of nAb and have not determined the minimally effective dose at which we can cure liver chimeric mice, it is currently hard to speculate what nAb levels are required to prevent infection of hepatocytes in the human liver. Passive immunization with a nAb in chimpanzees (36) was slightly above the dose typically used clinically for mAb treatments of other conditions. It is therefore feasible that passive nAb transfer may be an adjunct therapeutic modality in a subset of HCV-infected individuals who cannot be cured by upcoming direct-acting antivirals.

MATERIALS AND METHODS

Hepatitis C virus
Plasmids encoding chimeric HCV genomes were linearized with Xba I and transcribed using MEGAscript T7 (Ambion). RNA was electroporated into Huh-7.5 cells using an ECM 830 electroporator (BTX Genetronics), and infectious virus was collected from supernatants 48 to 72 hours after transfection (23). Serum containing the H77 gt 1a isolate was obtained from infected chimpanzees (31).

RT-PCR quantification of HCV RNA
Total RNA was isolated from sera using the RNeasy kit (Qiagen). HCV genome copy number was quantified by one-step RT-PCR using Multi-Code-RTx HCV RNA Kit (EraGen) and a LightCycler LC480 (Roche Applied Science) according to the manufacturers’ instructions.

Human antibodies
Anti-HCV mAbs AR3A, AR3B (18), and AR4A (19) and the anti-HIV b6 (20) control antibody were generated as previously described.

Animals and cell lines
Mice with a targeted disruption in the fumaryl acetoacetate hydroxase gene (Fah−/−) (47) were provided by M. Grompe [Oregon Health & Science University (OHSU)] and intercrossed with Rag2−/− β2m−/− mice or crossed for 13 generations onto the NOD Rag1−/− β2m−/− (48) background. The presence and zygosity of the mutant alleles were confirmed by allele-specific PCR. Resulting FNRG mice were maintained on nitisinone (Yecuris). Gt(Rosa)26Sortm1(Luc)Kaelin(49) (Rosa26-Fluc) were obtained from The Jackson Laboratory. Rosa26-Fluc mice contain the firefly luciferase (luc) gene inserted into the Gt(Rosa)26Sor locus. Expression of the luciferase gene is blocked by a loxP-flanked STOP fragment placed between the luc sequence and the Gt(Rosa)26Sor promotor. Cre recombinase–mediated excision of the transcriptional stop cassette results in luciferase expression in Cre-expressing tissues. Mice were bred and maintained at the Comparative Bioscience Center of the Rockefeller University according to guidelines established by the Institutional Animal Committee. Huh-7.5 and Huh-7.5.1 were maintained in 5% fetal bovine serum (FBS), and 293T and human embryonic kidney (HEK) 293 were maintained in 10% FBS containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% nonessential amino acids.

Isolation and culture of HFLCs
HFLCs were prepared as described previously (32). Briefly, deidentified fetal livers (16 to 24 weeks of gestation) were procured through Advanced Bioscience Resources. Livers received on ice were washed with hepatocyte wash buffer (HWB) consisting of Williams’ E medium (WEM) plus 10 mM Hepes, gentamicin (50 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, Life Technologies). Tissue was minced and then resuspended in 20 to 40 ml of warm digestion buffer consisting of Hanks’ balanced salt solution plus 40 mM Hepes, 3.26 mM CaCl2, deoxyribonuclease (DNase) I (grade II) (2 U/ml) (Roche), and 0.2% collagenase type IV (Sigma). Tissue was digested for 30 min at 37°C, then diluted 1:1 with HWB, and gently pushed through 70-μm cell strainers (BD Biosciences). The suspension was centrifuged at 100g for 3 min, and the cell pellet containing large hepatocytes was washed twice by resuspension in 50 ml of HWB and centrifugation at 100g for 4 min. Hepatocytes were enriched by 1g sedimentation in 25 ml of HWB for 1 hour at room temperature, followed by additional washing. In some experiments, hepatocytes were further enriched by centrifugation through lymphocyte separation medium (CellGro) as described (49). Hepatocyte yields ranged from 0.5 × 107 to 4 × 107 cells per tissue, and cells were generally >80% viable as assessed by trypan blue exclusion and collagen attachment. Hepatocytes were plated at ~1 × 107/cm2 on 96-well collagen I–coated plates (BD Biosciences) in WEM containing 10% FBS (Omega Scientific), 2 mM L-glutamine (Invitrogen), 1× ITS Plus (BD Biosciences), and antibiotics. After overnight incubation, adherent cells were washed with WEM and maintained in Hepatocyte Defined Medium (BD Biosciences) plus L-glutamine and antibiotics. The culture medium was aspirated and replaced every 2 days.

Engraftment of human hepatocytes and nitisinone cycling
FRG and FNRG mice greater than 6 weeks of age were transplanted with 3 × 106 or 1 × 106 adult human hepatocytes, respectively, that were either freshly obtained from surgical specimens (50) or cryopreserved human hepatocytes or purchased from BioReclamationIVT. During isoflurane anesthesia, mice underwent skin and peritoneal incision, exposing the spleen. Using 28-gauge needle, hepatocytes were injected in the spleen and pressure was applied to injection site to prevent hemorrhage. The peritoneum was then approximated using 4.0 VICRYL sutures (Johnson & Johnson), and skin was closed using MikRon Autoclip surgical clips (Becton Dickinson). Mice were cycled off the drug nitisinone on the basis of weight loss and overall health. FRG required about 1 week of nitisinone per month, and FNRG mice required about 1 week per 3 weeks.

Enzyme-linked immunosorbent assays
Human hepatocyte engraftment was monitored by serial hAlb determination. Serum was obtained through tail vein bleeding and diluted for measurement by homemade enzyme-linked immunosorbent assays (ELISAs), using goat polyclonal capture (Bethyl), mouse monoclonal Alb1 (Abcam) primary detection, and goat anti-mouse horseradish peroxidase (HRP) secondary antibodies. Human IgG was measured in mouse serum using goat Fc-purified anti-human IgG capture and HRP-conjugated detection antibodies, both from Bethyl.

Determination of human hepatocyte chimerism
Mice were asphyxiated using CO2 and perfused with 0.05% collagenase type IV. The peritoneum was then approximated using 4.0 VICRYL sutures (Becton Dickinson). Mice were asphyxiated using CO2 and perfused with 0.05% collagenase type IV. The peritoneum was then approximated using 4.0 VICRYL sutures (Becton Dickinson).
cell strainer before fixing in 10% formalin. After permeabilization with 0.1% Triton X-100, cells were stained for human CD81 (clone JS-81, BD Pharmingen) and their frequency was quantified by flow cytometry. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded human chimeric murine liver tissues using mouse anti-human (clone 2) FAH (Abcam). In brief, sections were deparaffinized in xylene (three changes), rehydrated through graded alcohols (three changes 100% ethanol, three changes 95% ethanol), and rinsed in distilled water. Antibody incubation and detection were carried out on a NEXES instrument (Ventana Medical Systems) using Ventana’s reagent buffer and iVIEW detection kit unless otherwise noted. Endogenous peroxidase activity was blocked with hydrogen peroxide. Heat-induced epitope retrieval was performed in a 1200-W microwave oven at 100% power in 10 mM sodium citrate buffer (pH 6.0) for 20 min. Sections were allowed to cool for 30 min and then rinsed in distilled water. Mouse antihuman FAH was diluted 1:800 in Dulbecco’s phosphate-buffered saline (PBS) (Invitrogen, Life Technologies) and incubated overnight at room temperature. Primary antibody was detected with biotinylated goat antimouse followed by application of streptavidin-HRP conjugate. The complex was visualized with 3,3’-diaminobenzidine and enhanced with copper sulfate. Matched Ig isotype, at equivalent concentration and diluted in PBS, was used as a negative control. Upon completion of staining, all slides were washed in distilled water, counterstained with hematoxylin, dehydrated, and mounted with permanent medium. Stained slides were scanned at ×40 magnification using the Leica Microsystems SCN 400F Whole Slide Scanner. Images were viewed and captured using SlidePath’s Digital Image Hub (Leica Microsystems).

HCV generation and infections
Huh-7.5.1 or Huh-7.5 cells were electroporated with in vitro-transcribed full-length HCV RNA. Seventy-two hours after electroporation, the medium was replaced with DMEM containing 1.5% FBS and supernatants were harvested every 6 hours starting from 72 hours. Pooled supernatants were clarified by centrifugation at 1500 rpm, filtered through a 0.45-μm bottle top filter (Millipore), and concentrated using a stirred cell (Millipore). Viral titers (TCID50) were determined from the ELISA-based concentration of antibody and particle titers of AAV aliquots were confirmed in vitro by transducing 293T cells and quantifying antibody concentration in the cell supernatant by ELISA.

Production of recombinant adenoviruses
Adenovirus stocks encoding human and murine homologs of the four HCV entry factors (CD81, SCARB1, CLDN1, and OCLN) were generated as previously described (51). Briefly, adenovirus constructs were transfected into HEK293 cells (American Type Culture Collection) using the calcium phosphate method. Transfected cultures were maintained until cells exhibited complete cytopathic effect (CPE), then harvested, and freeze-thawed. Supernatants were serially passaged two more times with harvest at complete CPE and freeze-thaw. For virus purification, cell pellets were resuspended in 0.01 M sodium phosphate buffer (pH 7.2) and lysed in 5% sodium deoxycholate, followed by DNase I digestion. Lysates were centrifuged, and the supernatant was layered onto a CsCl gradient (1.2 to 1.46 g/ml) and then spun at 23,000 rpm on a Beckman Optima 100K Ultracentrifuge using an SW28 spinning bucket rotor (Beckman Coulter). Adenovirus bands were isolated and further purified on a second CsCl gradient using an SW41.1 Ti spinning bucket rotor. Resulting purified adenoviral bands were isolated using an 18.5-gauge needle and twice-dialyzed against 4% sucrose. Adenovirus concentrations were measured at 1012 times the dilution factor times the OD260 reading on a FLUOstar Omega plate reader (BMG Labtech). Adenovirus stocks were aliquoted and stored at −80°C.

Cloning of HCV nAbs into AAV vector
Sequences corresponding to the heavy and light chain variable regions of the AR antibodies were PCR-amplified from expression vectors and used in overlapping PCRs designed to fuse the variable regions into the IgG1 constant region framework present in the VIP expression vector as previously described (17).

Production of recombinant AAVs
AAV production and intramuscular injection were performed as previously described (17). Briefly, 1.2 × 108 293T cells were transfectioned with 80 μg of the vector encoding the antibody of interest, pHELP (Applied Viroemics), and pAAV 2/8 SEED (University of Pennsylvania Vector Core) at a ratio of 0.25:1:2. Supernatant was collected five times over the course of 120 hours. Virus was purified by polyethylene glycol precipitation and cesium chloride centrifugation before being diafiltered, concentrated, and buffer-exchanged through 100K MWCO centrifuge filters (Millipore) into buffer consisting of 100 mM sodium citrate and 10 mM tris (pH 8) before aliquoting and storage at −80°C. To quantify aliquots, virus was thawed, treated with DNase, and titered by qPCR as described previously (17). Briefly, virus titer was determined by quantitative PCR using a standard curve generated from previously titered, purified, AAV2/8 encoding 4E10 antibody. The infectivities of AAV aliquots were confirmed in vitro by transducing 293T cells and quantifying antibody concentration in the cell supernatant by ELISA.

In vitro neutralization assays
Six- to 8-week-old FVB/J or Rosa26-LSL-Fluc mice (n = 4) were injected with 1011 AAV particles encoding the HCV-specific antibodies AR3A, AR3B, and AR4A, either individually or in combination, intramuscularly in a total volume of 25 μl. The HIV-specific B12 antibody– and firefly luciferase–encoding AAV were used as controls. Levels of human serum IgG levels and in vivo bioluminescence were quantified longitudinally. Serum IgG levels of FVB/J mice, injected with AR3A-, AR3B-, AR4A-, AR3A/AR3B/AR4A-, or B12-expressing AAV, were quantified by ELISA. Limiting dilutions of serum were incubated with H77C(1a)/JFH (T2700C, A4080T), J4(1b)/JFH (T2996C, A4827T), J6/JFH, ED43(4a)/JFH1g (A2819G, A3269T), SA13(5a)/JFH1 (C3405G, A3696G), or QC69(7a)/JFH (T2985C, C8421T) for 1 hour at 4°C before infection of Huh-7.5 cells at a multiplicity of infection of 0.05. Forty-eight hours after infection, infected cells were detected by staining for NS5A with an AF633-conjugated anti-NS5A mAb (clone 9E10) and quantified by flow cytometry. The inhibitory concentration of tested antibodies was subsequently determined from the ELISA-based concentration of antibody and the relative infection frequency by three-parametric nonlinear regression analysis using GraphPad Prism.

Protection experiments in humanized mice
The genetically humanized mouse model for HCV infection has been described previously (25, 52). Briefly, Rosa26-Fluc mice containing the firefly luciferase gene, which is transcriptionally repressed by an upstream loxP-flanked STOP cassette, were injected intravenously with...
recombinant adenoviruses expressing the HCV entry factors human CD81, scavenger receptor class B type I (SCARB1), claudin 1 (CLDN1), and occludin (OCLN). Adenoviral gene delivery resulted in efficient transduction of murine hepatocytes in vivo. The animals were then injected intravenously with recombinant HCV expressing Cre recombinase, and infection was monitored by bioluminescence imaging of firefly luciferase activity at 3 days after challenge. To study passive antibody protection against HCV, the animals were injected intramuscularly with AAV-expressing antibodies as indicated before adenovirus infection. Similarly, highly engrafted FNRG mice were injected intramuscularly with $10^{11}$ AAV particles encoding the HCV-specific antibodies AR3A, AR3B, and AR4A or $3 \times 10^{11}$ AAV particles encoding the B12 control.

**HCV E2 sequencing**

HCV (H77) E2 complementary DNA (cDNA) was synthesized from total RNA extracted from mouse sera using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies) with the primer pair PU-O-1574/PU-O-1575. Resulting cDNA was PCR-amplified via Q5 High-Fidelity DNA Polymerase (New England Biolabs Inc.) using the primer pair PU-O-1576/PU-O-1577, with the exception of mouse number 4992 (AR, before treatment), which was amplified with the primer pair PU-O-1585/PU-O-1573. Cycling parameters for PCR amplification were 98°C for 2 min, denaturation at 98°C for 30 s, followed by 35 cycles of annealing for 30 s at 48.5°C and elongation at 72°C for 2 min before a final elongation step at 72°C for 10 min.

PCR amplicons were then cloned into either the pCR4 or pCR II TOPO vector using the Blunt TOPO TA Cloning Kit (Invitrogen, Life Technologies). Clones were screened by Eco R1 restriction digest, and sequence coverage was completed via M13F/M13R primers (Macrogen) as well as a single internal primer (5′-GGTCCTGGTAGTGCTGTCG-3′).

Sequence data from forward, reverse, and internal reads were then compiled using an H77 E2 reference sequence with either MacVector (MacVector Inc.) or SeqMan software of the DNASTar Lasergene suit. For visualization of escape mutations, comparison to the H77 E2 region was done and highlighter plots were generated using the Los Alamos Highlighter tool (http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter.html).

**Bioluminescence imaging**

Unless otherwise specified, mice were injected with $10^{11}$ adenovirus plaque-forming units 24 hours before intravenous injection with $2 \times 10^{2}$ TCID<sub>50</sub> HCV-Cre. At 72 hours after infection, mice were anesthetized using ketamine/xylazine and injected intraperitoneally with 1.5 mg of luciferin (Caliper Life Sciences). Bioluminescence was measured using an IVIS Lumina II platform (Caliper Life Sciences).

**Human subjects**

All protocols involving human tissue were reviewed and exempted by the Rockefeller University Institutional Review Board.

**Statistical analysis**

Statistical analyses were performed with GraphPad Prism Software. Statistics were calculated using t test or Kruskal-Wallis one-way ANOVA. P values less than 0.05 were considered statistically significant.

**SUPPLEMENTARY MATERIALS**

www.sciencetranslationalmedicine.org/cgi/content/full/6/254/254ra129/DC1

Fig. S1. Human hepatocyte engraftment in Fah<sup>−/−</sup> NOD Rag1<sup>−/−</sup> IL2R<sup>γc−/−</sup> mice. Fig. S2. AV8 vectors affect the liver.

Fig. S3. Comparison of synonymous and nonsynonymous mutations in AR- and b6-treated mice. Table S1. Source data.

**REFERENCES AND NOTES**


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Huh-7.5 cell line, HCV cell culture system, the use of OCLN to construct HCV animal models,
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AR3B, AR4A, and B12 are the intellectual property of M.L. Caltech has a patent pending on
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