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Broadly neutralizing antibodies abrogate established hepatitis C virus infection

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Abstract

Hepatitis C virus (HCV) establishes a chronic infection in the majority of exposed individuals and can cause 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. Here, we demonstrate that three broadly nAbs, AR3A, AR3B and AR4A, delivered with adeno-associated viral (AAV) 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

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showcase a novel therapeutic approach to interfere with HCV infection exploiting a previously unappreciated need for HCV to continuously infect new hepatocytes in order to sustain chronicity.

Introduction

HCV chronically infects at least 170 million worldwide, and until recently curative therapies were poorly tolerated and ineffective in the majority of patients(1). HCV is among the few viruses causing human pathology that either establishes a chronic infection or is spontaneously cleared. Although an essential function for T cells in HCV clearance is widely accepted, the role of antibodies in controlling HCV infection remains elusive. Individuals almost universally seroconvert 2–10 months after infection(2) but it remains controversial if early development of neutralizing antibodies (nAb) predicts viral clearance(3–6). In addition, there are several case reports of seropositive patients who were successfully cured of their HCV and subsequently became re-infected(7). Moreover, chimpanzees that spontaneously resolved HCV infection remain susceptible to homologous re-challenge(8). These observations suggest that naturally arising immunity does not universally protect from reinfection. Failure of the immune system to protect from re-challenge can be explained in part by HCV's remarkable genetic diversity and high proliferative rate readily yielding 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 10–100 fold following 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), even though there are case reports that such patients retain the ability to spontaneously clear HCV(16). These clinical observations suggest 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 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 10^{11} genome copies of AAV-AR3A, -AR3B, AR4A or an anti-HIV control mAb (B12)(20) into the gastrocnemius muscle of highly immunocompromised NOD Rag1^{-/-} IL2R γ c^{null} (NRG) mice or

immunocompetent FVB mice resulted in stable, prolonged expression of human IgG expression for more than 4 months (Fig 1a & b). It was previously shown that AR3A, 3B and 4A 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 IC50s between 1–3 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 utilized a genetically humanized mouse model based on adenoviral delivery of human HCV entry factors into Gt(ROSA)26Sor^{tm1(Luc)Kaelin} (Rosa26-Fluc) 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 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) knock-out 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^{-/-} IL2R^γ^{null} (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 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), as this had previously been shown to correspond to the minimal engraftment level required for HCV permissiveness(27, 29, 30). To test if the AAV-delivered nAbs could prevent infection, we inoculated huFNRG mice with a pool of the three nAb-expressing AAV vectors (AR3A/3B/4A) or the control AAV-vector (B12). Sixteen days after AAV injection, the mice displayed serum IgG levels between 26–126 µg/ml and 3 days later were infected with a low dose (ca. 3000 copies) of HCV genotype 1a clone H77(31). Whereas 2 out of 3 control mice became viremic, none of the three mice that received the AR pool displayed viremia (Fig 1e). 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 3 injection 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 3 out of 3 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.

Abrogation of established HCV infection by nAbs in primary hepatocyte cultures

Given 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 supra-physiological 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 non-proliferating 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 three days later, after replication had been established, purified nAbs were added and maintained in the media for the remainder of the experiment. Longitudinal luminescence measurements (Fig 2b) or combined endpoints from 3 out of 4 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 (Suppl Fig 2b). Intramuscular injection of nAb expressing AAVs into huFNRG (n=8 mice total) resulted in a consistent 5–10 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/3B/4A) or 1.5 mg of control IgG (b6) every three days, which resulted in high and stable IgG levels (Fig 3a) without affecting serum hAlb levels (Fig 3b). nAb treatment suppressed serum infectivity to below the limit of detection within one day (Fig 3c). 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). These data are in line with previously published observations that administration of single dose of a neutralizing antibody 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, 55–69 days after the last injection (Fig 3a). 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). 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. In order to prove that the human graft could still support HCV infection, mice were challenged with a heterologous HCV-1a (clone H77) at $\sim 2 \times 10^4$ copies (Fig 3d). Three out of three mice that still had hAlb levels > 1 mg/ml became infected, illustrating that the lack of viremia was not due to graft loss or non-permissiveness 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 either with 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). However, and in contrast to the rapid disappearance in J6/JFH infected animals, it took 15–30 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 as AbXTL68 did not protect the graft from reinfection even at serum concentrations of 200 μ g/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 led to 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 in order

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, e.g. HCV genotype 3. This genotype was recently shown to require prolonged suppression with a polymerase inhibitor (sofosbuvir) as compared to genotype 2 isolates in spite of 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 to study HCV infection in its physiological environment. Our observations put into question whether cell-to-cell spread is a dominant route for re-infection, although it is conceivable that this process is compromised and less efficient in these experimental systems than in humans. 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. Even though 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 XbaI and transcribed using MEGAscript T7 (Ambion). RNA was electroporated into Huh-7.5 cells using a ECM 830 electroporator (BTX Genetronics) and infectious virus was collected from supernatants 48–72 h 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 RNAeasy kit (Qiagen). HCV genome copy number was quantified by one step RT-PCR using Multicode-RTx HCV RNA Kit (Eragen) and a light cycler LC480 (Roche Applied Science), according to manufacturers' instructions.

Human antibodies

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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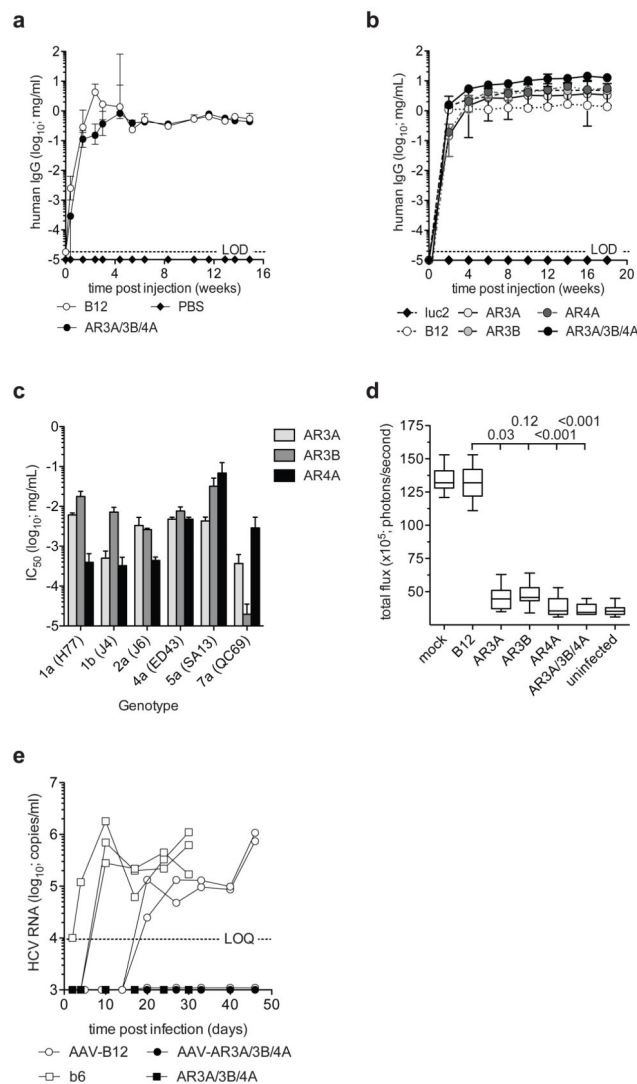


Figure 1. Prophylactic efficacy of broadly neutralizing anti-HCV antibodies

(a) A pool of AAV vectors expressing the three nAbs AR3A, 3B and 4A or control nAb B12 were injected intramuscularly in immunodeficient NRG mice and human IgG in mouse serum was measured by ELISA (b) FVB mice were injected with AAV vectors expressing the nAbs AR3A, 3B, 4A or control nAb B12 or a luciferase expressing AAV (luc2) and serum human IgG levels were measured by ELISA. (c) Sera from FVB mice that were injected with the AAV-nAb was used for *in vitro* neutralization assays of intergenotypic HCVcc on Huh-7.5 hepatoma cells. Indicated are the genotypes and origin of the structural proteins of the challenge strains. IC₅₀ values are depicted at mg/ml of human IgG in mouse serum. (d) R26-Fluc mice were administered AAV-nAbs. Once nAb reached peak titers, HCV entry factors were adenovirally delivered to the liver and challenged with HCVcc expressing Cre recombinase, after which bioluminescence was measured. P values comparing B12 to AR antibodies, one way ANOVA. (e) Highly engrafted human liver chimeric FNRG mice were either injected with the pool of three nAb expressing AAV vectors (n=3) or control B12 AAV (n=3), or received three injections of a pool of purified

nAbs (n=2) or b6 control nAb (n=3) and challenged with low dose H77. HCV RNA copies in mouse serum were measured by qRT-PCR. $P=0.012$, two way ANOVA for pooled experiments.

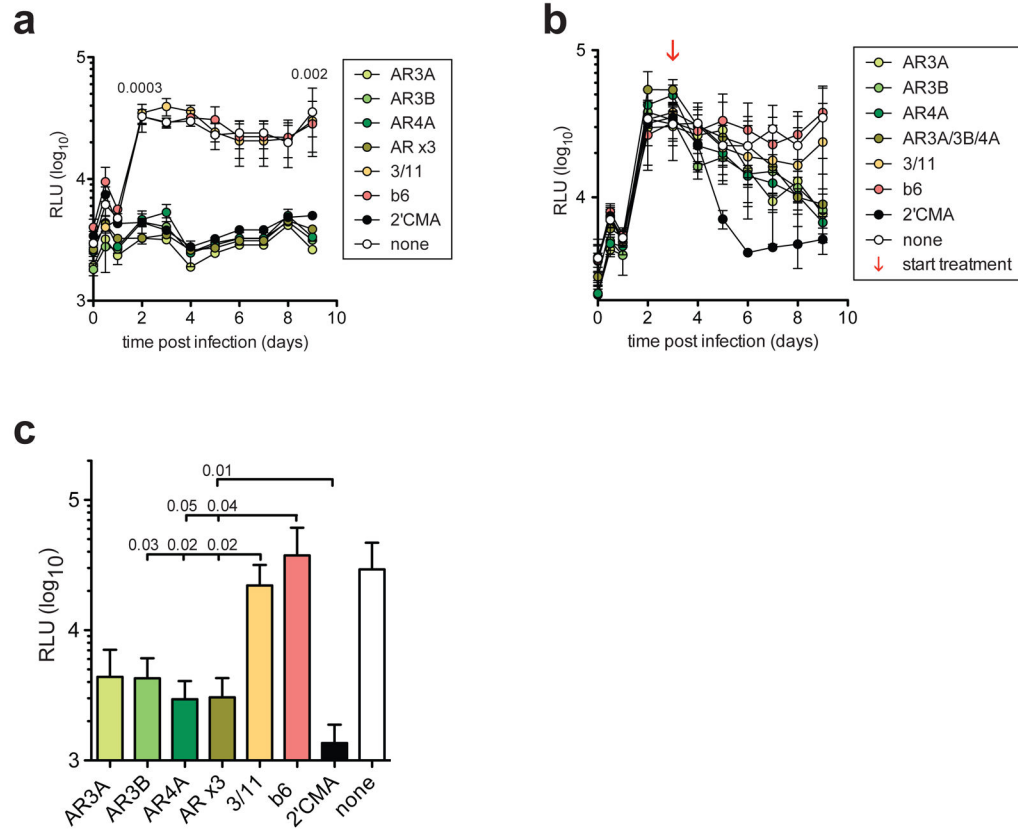


Figure 2. Combination treatment of primary human hepatocyte cultures with broadly neutralizing anti-HCV antibodies abrogate established HCV infection

(a) The three nAbs AR3A, 3B and 4A alone or combined (ARx3) were used for neutralization assays on human fetal liver cultures (HFLCs) and compared to the previously described nAb 3/11 or control mAb b6. Antibodies at 1 μ g/ml were added to cultures 1 hour prior to infection with HCVcc-GLuc and 6 hours later cultures were washed and maintained in media without nAbs for serial luminescence sampling. *P* values for ARx3 vs b6 on day 2 and day 9, unpaired *t*-test. (b) HFLC were infected with HCVcc-Gluc and after three days nAbs were added at 1 μ g/ml, which were maintained in the media for the duration of the experiment. (c) Pooled end point luminescence data from 3 out of 4 HFLCs that supported viral replication and received nAbs starting three days after infection with HCVcc-Gluc. *P* values by unpaired *t*-test.

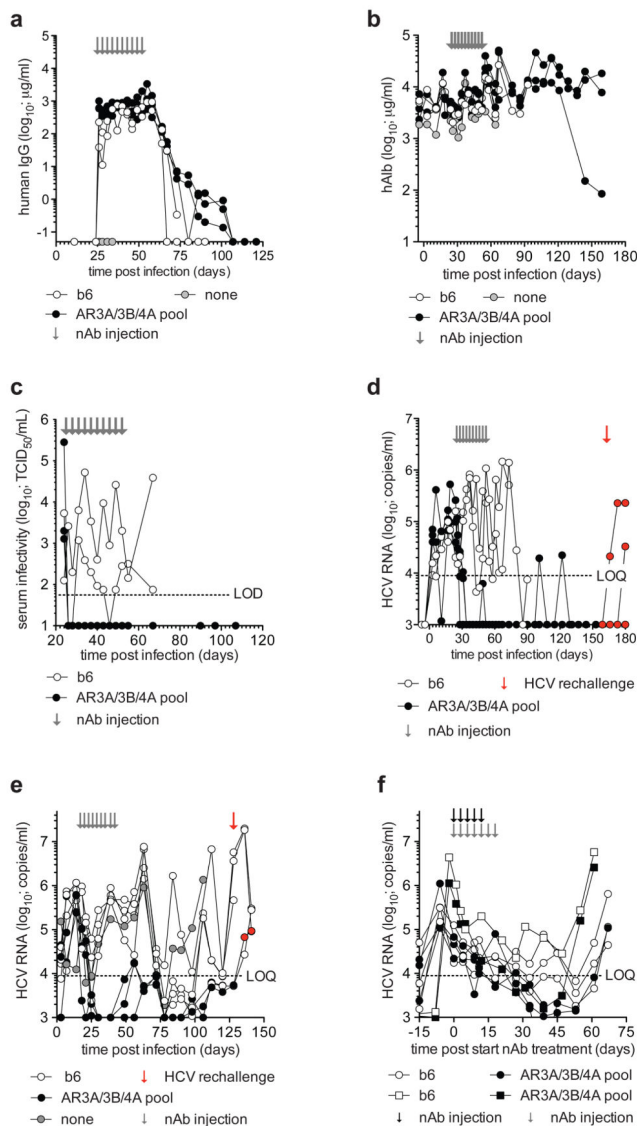


Figure 3. Combination treatment of human liver chimeric mice with broadly neutralizing anti-HCV antibodies clears established HCV infection

(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, 3B and 4A or control mAb b6 at time points indicated by the grey arrows. Human IgG levels in mouse serum were determined by ELISA longitudinally until they became undetectable. (b) In the same group of mice from figure (a) hAb levels in mouse serum were measured by ELISA for the duration of the experiment. (c) Starting one day after nAb were injected, HCV serum infectivity as measured in a limiting dilution assay on Huh-7.5 hepatoma cells. (d) HCV RNA was determined in mouse serum by qRT-PCR for the duration of the experiment. On day 163 post infection and 56 days after nAb levels had fallen below the LOD, 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 (grey 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 circle. 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 5 (squares) or 7 (circles) injections of nAb (arrows) and HCV RNA in mouse serum was determined by qRT-PCR.