Monoclonal anti-envelope antibody AP33 protects humanized mice against a patient-derived hepatitis C virus challenge.

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Abbreviations: AA – amino acids, CC – chronic carrier, ctrl - negative control, EC_{50} – half maximal effective concentration, HCVcc – cell culture-derived HCV, HCVpp - HCV pseudoparticles, HVR1 – hypervariable region 1, IC_{50} – half maximal inhibitory concentration, LOQ – limit of quantification, LT – liver transplant, mAb - monoclonal antibody, mP05 - mouse passaged P05 virus, NI – non-infected, P - patient, QS – quasispecies, V - viral variant.

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Abstract

End-stage liver disease caused by hepatitis C virus (HCV) infection is a major indication for liver transplantation. However, immediately after transplantation the liver graft of viremic patients universally becomes infected by circulating virus, resulting in accelerated liver disease progression. Currently available direct-acting antiviral therapies have reduced efficacy in patients with end-stage liver disease and prophylactic strategies to prevent HCV recurrence are still highly needed.

In this study we compared the ability of two broadly reactive monoclonal antibodies (mAbs), designated 3/11 and AP33, recognizing a distinct but overlapping epitope in the viral E2 glycoprotein to protect humanized mice from a patient-derived HCV challenge. Their neutralizing activity was assessed using the HCVpp and HCVcc systems expressing multiple patient-derived envelopes and a human-liver chimeric mouse model.

HCV RNA was readily detected in all control mice challenged with a patient-derived HCV genotype 1b isolate, while three out of four AP33-treated mice were completely protected. In contrast, only one out of four 3/11-treated mice remained HCV RNA negative throughout the observation period, while the other three had a viral load that was indistinguishable from that in the control group. The increased *in vivo* efficacy of AP33 was in line with its higher affinity and neutralizing capacity observed *in vitro*.

Conclusion: Although mAbs AP33 and 3/11 target the same region in E2, only mAb AP33 can efficiently protect from challenge with a heterologous HCV population *in vivo*. Since mAb AP33 efficiently neutralizes viral variants that escaped the humoral immune response and re-infected the liver graft of transplant patients, it may be a valuable candidate to prevent HCV recurrence. In addition our data is valuable for the design of a prophylactic vaccine.

Introduction.

It is estimated that worldwide more than 185 million people are infected with the hepatitis C virus (HCV) (1). HCV is a major cause of liver fibrosis and a substantial fraction of chronically infected individuals will develop liver cirrhosis. Patients suffering from HCV-induced liver cirrhosis have a marked risk of developing hepatocellular carcinoma and developing hepatic decompensation.

Recently clinically licenced direct-acting antivirals (DAA) cure the majority of infected patients without major adverse effects. However, several challenges remain: high costs limit access to therapy even in high resources settings and certain subgroups of difficult-to-treat patients may need adjunctive therapeutic approaches (2). Vaccine development is hampered by viral evasion of host immune responses and a vaccine is not available (3). After liver transplantation (LT) in patients with detectable serum HCV RNA, the donor liver inevitably becomes infected and between 20 and 30% of these patients develop cirrhosis within 5 years after LT. Recently it was demonstrated that Sofosbuvir/Ribavirin combination treatment in a pre-transplant setting may successfully prevent post-transplant HCV recurrence, with recurrence inversely related to the number of consecutive days of undetectable HCV RNA before LT (4). The same combination treatment in a post-transplant setting resulted in a sustained virological response in about 60 to 70% of patients with recurrent HCV infection (5, 6).

Using a human liver chimeric mouse model (7), we have recently shown that blocking cellular receptors could provide a strategy to prevent HCV recurrence after orthotopic LT (8-12). A small molecule designated ITX-5061 (iTherX), which blocks the HCV co-receptor SR-BI, is currently under clinical investigation in LT patients (13, 14). An alternative approach is to neutralize the circulating virus with molecules that target the viral particle (15-20), one could hypothesize this approach would have limited off-target effects.

Previous studies using chimpanzees and humanized mice showed that HCV infection could be prevented using polyclonal and monoclonal antibodies (mAbs) (15, 17, 21, 22). Nevertheless, the first clinical trials using human polyclonal or monoclonal anti-HCV antibodies were disappointing. Although the antibody therapy was safe and well tolerated it had no or little effect

on viremia or HCV recurrence (23-26). However, these discouraging results were most likely explained by the limited neutralizing capacity or cross-reactive nature of the antibodies used leading to viral escape. Meanwhile, several new mAbs have been developed that show broader and more potent antiviral activity (22, 27-34). Recently, Chung and co-workers could demonstrate that the human mAb MBL-HCV1 delayed the median time to viral rebound compared to placebo treatment in HCV-patients undergoing LT (35). Although the *in vivo* neutralizing potential of MBL-HCV1 was clearly demonstrated, the primary endpoint of the study – the prevention of allograft HCV infection – was not met, justifying the quest for more potent mAbs.

In the present study we compared the sensitivity of patient-derived HCV strains to two non-human mAbs (AP33 and 3/11) targeting the same conserved region immediately downstream of HVR1 (hypervariable region 1) in E2 (36-41). Given our encouraging i*n vitro* results with AP33, its superior neutralizing potential was validated *in vivo* using chimeric uPA$^{+/+}$-SCID mice and LT-escape viral variants. Our *in vivo* results and the fact that a humanized version of AP33 is currently available warrant further exploration of this antibody in a human LT-setting. In addition our data is relevant for future rational vaccine design.

Materials and Methods

Ethical approval. Participating subjects gave written informed consent and the study protocol was approved by the ethical committees of the Ghent University Hospital (EC # 1994/137) and the University of Strasbourg Hospitals (CPP 10-17). The Animal Ethics Committee of the UGent Faculty of Medicine and Health Sciences approved all animal experiments.

Primary human hepatocytes, cell lines and monoclonal antibodies. Primary human hepatocytes (PHH) were obtained from patients undergoing partial liver resection. Huh7.5.1, HEK293FT and HEK293T cell lines have been described previously (42, 43). The isolation and production of the anti-HCV E2 mouse mAb AP33 and the rat anti-E2 mAb 3/11 have been described previously (36, 38-40).

***In vitro* experiments.** To measure the relative binding affinity of mAbs AP33 and 3/11 to HCV E1E2 glycoproteins, a cell-lysate based ELISA was performed. HCVpp and HCVcc neutralization assays were performed as described (42, 44, 45). Clonal sequence analysis was performed on all HCV-positive mouse samples and the inoculum.

A detailed description of all *in vitro* experiments and patient-derived viral variants can be found in the online supplement.

***In vivo* efficacy study.** Mice with a humanized liver were generated as described before (46, 47). Briefly, within 2 weeks after birth homozygous uPA$^{+/+}$-SCID mice (48) were transplanted intrasplenically with approximately 1 million cryopreserved PHH from a commercial source (BD Gentest, Erembodegem, Belgium; donor HH223). The extent of liver humanization was assessed by quantifying the concentration of human albumin in the mouse plasma by ELISA (Bethyl Laboratories, Montgomery, TX). All animals used in this study had human albumin levels ranging from 3.2 to 10.0 mg/mL. Passive immunization studies were performed according to a previously described protocol (15, 17). In brief, three days before viral challenge with 10^4 IU of mP05 virus, the animals were injected (intra-peritoneal route) with 1 mg of purified mAbs. As

previously demonstrated, 3 days after intra-peritoneal injection only a small fraction of the IgG load remained in the peritoneum, whereas plasma IgG levels were still high. The bioavailability of circulating mAbs in immunized mice is presented in Supplementary Figure 1. Challenge virus (mLT_P05) was originally isolated from an HCV-infected patient before LT (patient 5; (45)) and passaged in humanized mice (hence the prefix 'm') to eliminate any of the patient's neutralizing antibodies. Injection of 10^4 IU of mLT_P05 virus results in HCV-infection in all tested chimeric uPA$^{+/+}$-SCID mice so far (100% infectious dose). The animals were bled at weekly intervals and followed until 8 weeks after infection. Mouse plasma samples were stored at -80°C until analysis. The Animal Ethics Committee of the Faculty of Medicine and Health Sciences of the Ghent University approved the study protocol. Viral RNA was quantified using the Roche COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test (v2.0). Due to dilution of the mouse plasma, the limit of detection and limit of quantification were 750 IU/mL.

Statistics. Statistical significance of experimental results was assessed by the Wilcoxon matched-pairs signed-ranks test (Fig 1, Fig 2A+B) and the Kruskal-Wallis test (non-parametric analysis of variance [ANOVA]) with Dunn's Multiple Comparisons posttest (Fig 2C) using GraphPad InStat version 3.06 (GraphPad Software).

Results

mAb AP33, but not 3/11, efficiently binds and neutralizes envelopes of patient-derived HCV escape variants. Using PHH and HCVpp carrying viral envelope glycoproteins derived from HCV-infected patients undergoing LT, we previously demonstrated that in the early phase post-transplantation, viral variants characterized by enhanced viral entry and escape from autologous antibody-mediated neutralization were able to re-infect the liver graft (45). Here we compared the binding and neutralizing activity of two broadly neutralizing anti-E2 mAbs, AP33 and 3/11, using the envelopes of infectious HCV variants isolated from: (i) patients after LT (HCV variants that originate from the re-infected liver graft and are resistant to neutralization by autologous serum); and (ii) chimeric mice after challenge with HCV_gt1b from chronically infected patients (HCV variants that are selected during transmission to humanized mice). The detailed origin of the patient-derived viral variants can be found in the online supplement. While mAbs AP33 and 3/11 both recognize a linear, highly conserved region of E2 spanning amino acid (AA)-residues 412 to 423, their epitopes are distinct. The relative affinity of these antibodies for E2 derived from prototype HCV genotype 1a, 1b and 2a strains or those derived from the LT- and CC-patient variants, was determined using the GNA-capture ELISA. Dose-response experiments showed that the half maximal effective concentration (EC_{50}) values of both antibodies were significantly different (P<0,005), with mAb AP33 having a 3- to 50-fold higher apparent affinity depending on the isolate (Figure 1).

HCVpp expressing envelope glycoproteins from 3 LT_P05-derived viral variants were incubated with either mAbs AP33, 3/11 or isotype control IgG before infecting PHHs (Figure 2A). HCVpp expressing envelope glycoproteins from prototype gt1a and 2a, and 4 CC-patient-derived gt1b viral variants were pre-incubated with the same antibodies before infecting Huh7.5.1 hepatoma cells (Figure 2B). AP33 inhibited the entry of patient-derived HCVpp in PHH and Huh7.5.1 cells in a dose-dependent manner (Figure 2A-B, Supplementary Table 1). Compared to AP33, the neutralizing potential of mAb 3/11 was significantly reduced (P<0.005) and higher antibody concentrations were needed to reach comparable levels of inhibition. Similar results were obtained using the HCVcc model system using a well-characterized chimera LT_P01_VL/JFH1

expressing the structural genes of the HCV-VL strain (P01 patient) (43, 45) and the nonstructural genes of JFH1 (Figure 2C). Using this model, which was previously used to demonstrate the neutralizing potential of AP33 in hepatocytes and Huh7.5.1 cells (43, 45), the reduced neutralizing potential of 3/11 was confirmed ($P<0.005$). The AA-diversity in the E2-region spanning AA384-427 (HVR1+minimal epitope) of the patient-derived isolates used in the binding and neutralization experiments is illustrated in Figure 2D. Overall, HCVcc-data confirm HCVpp-data and suggest that mAb AP33, but not 3/11, efficiently inhibits entry of HCV escape variants that are resistant to autologous host responses.

mAb AP33, but not 3/11, efficiently inhibits cell entry of HCVpp expressing envelopes from patient variants isolated during the pre-transplantation period. Chronic HCV infection is associated with viral quasispecies that contain neutralization escape variants (44, 45). Using neutralizing antibodies immediately in the pre-transplantation period as an immunoprophylactic approach may reduce the pool of circulating viruses and ultimately prevent HCV recurrence after LT. The potential of AP33 to inhibit infection of HCV strains present during the pre-transplantation period was demonstrated previously (45). Here, the neutralizing potential of AP33 and 3/11 was compared using the HCVpp model system and normalized to isotype control IgG. All antibodies were pre-incubated at 10 µg/mL with a panel of 20 different HCVpp that express envelopes derived from variants isolated from pre-transplant serum of 5 LT-patients. Eleven out of these 20 patient-derived variants re-infected the liver graft and were resistant to autologous antibody neutralization. While AP33 efficiently inhibited infection of all HCVpp expressing envelopes of viral variants by up to 100%, 3/11 only partially neutralized infection of patient-derived HCVpp. Except for control H77c HCVpp, which was neutralized for 68%, the level of neutralization of patient-derived HCVpp by 3/11 ranged from 3 to 62%, with 50% neutralization achieved only for 2 out of 20 variants (P06_VF by 62% and P06_VI by 54%)(Figure 3). These data demonstrate that AP33, but not 3/11, efficiently inhibits the infectivity of HCVpp bearing envelope glycoproteins isolated from different patient-derived viral

variants that exist before and after LT. Therefore mAb AP33 is a relevant candidate for prevention of liver graft infection.

Lysine-residues at AA-positions 408 and 410 are important for mAb AP33 binding. To explain the superior binding and *in vitro* neutralization potential of mAb AP33 compared to 3/11, we investigated crucial binding residues for both mAbs(41). Although both mAbs recognize a linear region of E2 spanning AA-residues 412 to 423, their epitopes are distinct. The major determinants of AP33 are E2 residues L413, N415, G418 and W420 (39, 40). The critical residues for 3/11 binding to E2 are N415, W420 and H421 (39). Since this region is highly conserved, these critical residues are conserved in all patient-derived variants. Interestingly, by expanding the sequence analysis up- and downstream of AA-region 412-423, we discovered additional binding residues for AP33 as well as for 3/11. Using overlapping peptides (15-mer with 1 AA overlap) spanning AA-region 406 to 427 of E2, the overall binding pattern for AP33 as well as 3/11 differed for gt1a- and gt1b-derived sequences (Figure 4A). The presence of Q412 and H421 at both ends of the minimal epitope is important for binding of both mAbs. Furthermore, residues at AA-positions 408 and 410 represent additional anchor residues. Remarkably, the involvement of position 408 is more pronounced in gt1a, whereas position 410 influences binding in gt1b: (i) absence of K408 in gt1a decreases AP33 as well as 3/11-binding (AP33>3/11), (ii) absence of A/T408 in gt1b only slightly reduces AP33-binding and does not influence 3/11-binding, (iii) absence of N410 in gt1a has a minor influence on binding of both mAbs, (iv) but in gt1b it drastically decreases AP33 as well as 3/11-binding. Based on these peptide-binding assays, K-residues at AA-positions 408 and 410 seem important for optimal binding of both mAbs. For 3/11 binding, additional anchor residues were observed: (i) absence of Q409 reduces binding (gt1a>gt1b), (ii) addition of N423 (gt1b) and A426 enhances binding.

To unravel whether peptides with good binding potential in Figure 4A (AA408-422, AA409-423 and AA412-426; marked with an asterisk in Figure 4A) represent the complete mAb-epitope, we compared the relative binding affinity of (i) these peptides, (ii) functional full-length E1E2-cell-lysates (E1E2_FL) and (iii) peptides spanning AA-region 384-447 (Figure 4B). Dose response

experiments demonstrate that AP33-binding to AA408-422 and AA384-447 is similar and largely exceeds AA412-426-binding, suggesting the presence of additional anchor-residues upstream of the minimal epitope (AA412-423). The suboptimal AP33-binding of E1E2_FL suggests potential sterical hindrance, probably caused by N-glycosylation at position 417 (termed E2N1, only present in the native particle). 3/11 binding of different peptides, on the other hand, never equals E1E2_FL binding affinity, suggesting additional, possibly conformation-dependent, binding residues in E2. As an alternative explanation, the suboptimal peptide-binding of mAb 3/11 as compared to the full-length E2 protein could be attributed to the fact that peptides are not glycosylated, since it was previously demonstrated that the glycan at position 423 potentially contributes to the 3/11 binding (49).

To confirm the involvement of K-residues at positions 408 and 410, peptides were generated with K- or A-substitutions at these positions (Figure 5A). Dose response experiments clearly demonstrate a hierarchy in AP33-affinity: K408-K410 > A/T408-K410 > K408-A410 > A/T408-A410. The 3/11 binding was not influenced by AA-changes at these positions. Since the affinity of AP33 for peptides with alanine substitutions at positions 408 and 410 (■ in Figure 5A) still largely exceeds the 3/11 binding, additional binding of these anchor residues can only partially explain the superiority of AP33.

An extensive analysis of the residues present at AA-positions 408 and 410 in E2 envelope glycoproteins of a panel of 72 gt1a and 101 gt1b HCV strains within the NIH GenBank HCV database shows that these residues are genotype specific (Figure 5B). K-residues are observed much more frequently at position 408 and 410 in gt1a- and gt1b-strains, respectively. Based on the presence of K-residues in natural variants and its involvement in AP33-binding, we conclude that AP33, but not 3/11, is a relevant candidate for neutralization of gt1-infected patient-derived virions.

mAb AP33 protects humanized mice against a patient-derived HCV challenge.

The *in vivo* protection capacity of mAbs AP33 and 3/11 was compared using mice with a humanized liver (46, 47). Chimeric mice were injected with 1 mg of either antibody and three

days later, they were challenged with a 100% infectious dose of mouse passaged P05 virus (mLT_P05). Clonal sequence analysis based on the diversity observed at AA384-427 showed that the inoculum was composed of 66% P05_VF, 28% P05_VE and 6% P05_VL variants. Interestingly, in the LT_P05_VL variant, an asparagine to threonine mutation was observed at position 415 (N415T), one of the critical residues of both the AP33 and 3/11 epitope. In contrast to AP33-treated mice, of which only one became infected (K1149RR), HCV-RNA could be readily detected in 3 out of 4 mice loaded with mAb 3/11 (Figure 6A). Compared to the control mice, there was no delay in the kinetics of the infection in the treated, but non-protected mice. This is in contrast to previous *in vivo* challenge studies using polyclonal antibodies (15, 17). Although the overall human albumin levels were comparable between the different treatment groups, it is worth noting that the non-infected 3/11-treated mouse has the lowest huAlb concentration (3.2 mg/mL), whereas the infected AP33-treated mouse has the highest huAlb concentration (10.0 mg/mL).

We sequenced the E1E2 region of the virions that were isolated one week after viral challenge from the plasma of the control mice and the mAb-treated mice that became infected. The virion diversity in control and 3/11-treated mice was comparable to that of the inoculum (Figure 6B), with a dominant presence of variant LT_P05_VF. Mutations in the region spanning the AP33 and 3/11 epitope were identified in a limited number of clones isolated from control (4 out of 62 clones) as well as 3/11-treated mice (2 out of 46 clones). The HCV-RNA^{+} AP33-treated mouse became infected with a monophyletic viral pool that was uniquely composed of variant P05_VF with a conserved antibody-binding region. In 1 out of 14 clones generated from control animal B311 we identified the E655G mutation that was previously described to be associated with decreased sensitivity to AP33 (50). The mutations S501N, V506A and N415Y described previously (50, 51) to affect the potency of the two mAbs were not detected in any of the viral clones analyzed in this study.

Discussion.

HCV infection of the graft in patients with detectable serum HCV RNA at time of LT occurs immediately, is universal and greatly compromises patient and graft survival. While DAA are currently evaluated for prevention (4) and treatment (5, 6) of liver graft infection, a clinically approved strategy for prevention of liver graft infection is still absent. Our previous functional studies have demonstrated that viral entry and escape from antibody-mediated neutralization are key factors defining HCV reinfection in LT suggesting that cross-neutralizing antibodies targeting conserved epitopes within the HCV envelope region may be a viable antiviral strategy to prevent HCV recurrence (43, 45, 52).

In the present study, we compared the ability of two well-characterized neutralizing anti-E2 mAbs targeting the same conserved region, AA412-423, to neutralize patient-derived viral variants and to protect mice against a patient-derived HCV challenge. The therapeutic potential of this region as an antiviral target is strengthened by its conserved nature and its seemingly low natural immunogenicity, evidenced by the very low prevalence of Abs directed to this epitope in chronically infected patients (53). Using a LT-patient-derived viral inoculum we demonstrate for the first time that anti-E2 antibody AP33 can protect humanized mice against HCV genotype 1b challenge *in vivo*. Despite targeting the same region, mAb 3/11 failed to prevent infection of humanized mice challenged with the same isolate.

Several anti-E2 mAbs have been reported (AP33, 3/11, HCV1 and 95-2) that recognize epitopes within AA412-423 (29, 37). This region encompasses the highly conserved E2 residue W420, which plays an essential role in CD81 binding and represents a critical contact residue for the four mAbs (54). Previous studies using prototype HCV strains showed that the four Abs broadly neutralize the most common HCV genotypes *in vitro*, although IC_{50} differ between isolates (29, 39, 40). Here, we show that although both mAbs neutralize infection of the prototype and patient-derived viral variants, the neutralizing potential of AP33 is greater compared to 3/11. Furthermore, using the HCVcc system we demonstrate that AP33 can neutralize VL/JFH1, a LT-patient-derived variant that harbors mutations mediating escape from neutralizing antibodies through multiple mechanisms and that is resistant to autologous and

heterologous serum-derived antibodies as well as to many broadly neutralizing human mAbs (43, 45).

ELISA binding studies show that mAb AP33 had a 3- to 15-fold higher relative affinity to prototype and patient-derived viral variants compared to 3/11. Sequence analysis of the AA-region 412-423 of H77c (1a) and patient-derived viral variants (1b) didn't reveal any AA differences within this region, suggesting that additional AA residues in the E1E2 envelope glycoprotein contribute to optimal binding. Epitope mapping by expanding binding analysis upstream of AA412-423, demonstrates that K-residues at AA-positions 408 and 410 largely contribute to AP33-binding. The 3/11 binding was not influenced by AA-changes at these positions. Since K-residues are abundantly present at AA-positions 408 and 410 in natural gt1 variants, these additional anchor residues may explain the increased binding affinity and neutralization potential of AP33. Furthermore, differences in the length of the heavy chain CDR3 previously described between AP33 and 3/11 may also explain binding differences between the two mAbs (39).

Recently Meola and colleagues reported that the epitope targeted by AP33 and 3/11 may adopt different conformations that are influenced by up- and downstream sequences (41). In addition they showed that HVR1 hampers the binding of 3/11 to soluble E2 while it contributed to the stability of the AP33-glycoprotein complex. The additional contact residues mentioned above could be responsible for this increased binding stability and neutralization capacity of AP33; or they may at least modulate the structure of the epitope towards the orientation that is favored by AP33.

Here, *in vivo* data using a patient-derived viral challenge, demonstrate that AP33 at doses of 100 mg/Kg can protect 3 out of 4 mice against HCV infection. Treatment with 3/11, however, protected only 1 of 4 mice and no delay in the kinetics of infection were observed. Sequence analysis of E1E2 envelope glycoproteins of viral variants detected after viral rebound in the treated mice showed no relevant mutations in the 412-423 region. Furthermore, minor mutations observed outside this region were similar to those detected in viral variants that became

dominant in the control mice, indicating that these were random mutations rather than the consequence of viral escape.

These data clearly demonstrate that despite sharing the same conserved epitope, mAb AP33 shows an increased neutralizing capacity compared to mAb 3/11, which is in line with its higher affinity and neutralizing capacity in both the HCVpp and HCVcc systems. In contrast to most recently published studies that use "prototype" strains for neutralization assays and animal challenge, the present study was performed using viral variants derived from LT-patients and results in comparable *in vitro* and *in vivo* neutralization data. Patient-derived variants probably better reflect "real life" HCV reinfection of the liver graft and may therefore be a superior tool for anti-HCV mAbs evaluation.

Until now, only few anti-HCV mAbs have been tested in an *in vivo* setting. The human mAb MBL-HCV1 was recently evaluated for its neutralizing capacity in chimpanzees and humans (32, 35). In preclinical evaluation, MBL-HCV1 was infused into two chimpanzees at doses of 50 and 250 mg/Kg, respectively, 30 minutes prior to challenge with 32 CID of H77c genotype 1a virus. Only the chimpanzee receiving 250 mg/Kg MBL-HCV1 was protected while the chimpanzee receiving 50 mg/Kg was not protected (32). In a pilot clinical study, multiple infusions of mAb MBL-HCV1 at doses of 50 mg/Kg delayed the median time to viral rebound compared to placebo treatment in HCV-patients undergoing LT, but failed to prevent HCV reinfection of the liver graft (35). Since the primary goal - prevention of infection - was not achieved in this study, more potent mAbs are still welcome. Although AP33 and MBL-HCV1 target approximately the same region in E2, they appear to have different characteristics: i) when complexed with epitope peptides they have a different structural orientation (40, 55-57); ii) the major determinants within the minimal binding epitope are different (29, 39); iii) we here defined additional epitope residues important for AP33 binding; and iv) AP33 is endowed with higher neutralizing capacities (57).

These findings led to our *in vivo* experiments and form the major rationale for performing the present study. Using a similar set-up with humanized uPA-SCID mice Law *et al.* evaluated the ability of 6 human mAbs against an HCV challenge with patient-derived serum (gt 1a). In

this set-up, 2 out of 5 mice receiving mAb AR3A and 3 out of 4 mice receiving mAb AR3B were still protected. However, high concentrations of mAbs (200 mg/Kg) were required for protection precluding their future use in humans (22). In the present study we demonstrate the efficacy of mouse AP33 at 100 mg/Kg against pre-transplant viral variants. The recent humanization of mAb AP33 (55) warrants further exploration in a human setting, not only during LT but also as a safe and efficient immunoprophylactic approach before LT to decrease viral load in circulation.

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Figure legends

Figure 1. Binding characteristics of mAbs AP33 and 3/11. Cell-lysates containing HCV E1E2 envelope glycoproteins derived from prototype isolates (H77c, JFH1, J4) and patient-derived viral isolates (gt1b) selected during liver graft infection (LT_P05_VD, LT_P05_VE, LT_P05_VF) or selected during transmission to humanized mice (CC_P08_VD, CC_P09_VA, CC_P12_VA) were incubated with serially diluted mAbs AP33 and 3/11 for 1 hour at room temperature. Bound antibodies were detected with an HRP-conjugated anti-species secondary antibody. Absorbance values were determined at 450 nm. Dose response binding, expressed as percentage of maximal binding in EIA (means +/- SEM), (A) and EC_{50} (B) are shown. The difference in EC_{50}-values between mAbs AP33 and 3/11 can be considered very significant (*P*=0,0039).

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not significantly different. (D) Alignment of the amino acid sequence of the envelope region spanning AA384-427 of the different CC and LT patient isolates.

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Figure 6. mAb AP33 protects humanized mice against an HCV-gt 1b challenge. (A) Chimeric animals were challenged with 10^4 IU HCV RNA of mLT_P05 without pretreatment (no IgG) or after passive immunization on day -3 with 1 mg of mAb 3/11 or AP33. Plasma HCV RNA levels (IU/mL) were measured until 8 weeks after viral challenge. Mice that remained non-infected after inoculation are shown as HCV-RNA <750 IU/ml (LOQ, dotted line). Animal B338RL died 4 weeks after viral challenge. (B) Clonal sequence analysis of recovered virions in selected week 1 samples (week 2 for B311R) revealed viable E1E2 sequences in all samples. Each color represents a unique sequence in AA-region 384-427 (left panel). Columns represent the percentage of each clone detected in the inoculum or in the plasma of the animals. For each animal, the total number of clones analyzed is shown beneath the mouse ID. Viral outcome after mLT_P05 challenge is summarized as % protection. The sequence of each quasispecies clone is shown in the right panel. AA highlighted in red, represent AP33 anchor residues identified in this and previous papers.

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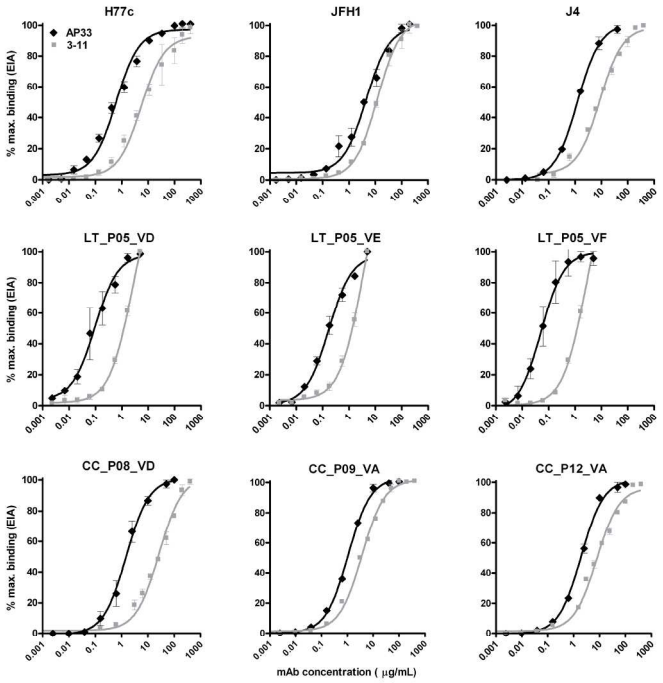
FIGURE 1

A



B

EC50 (µg/ml)

				LT (gt1b)			CC (gt1b)		
	H77c	JFH	J4	P05_VD	P05_VE	P05_VF	P08_VD	P09_VA	P12_VA
3/11	5.52	12.04	8.77	2.43	3.24	2.48	26.39	3.65	7.52
AP33	0.61	4.53	1.25	0.09	0.17	0.05	1.49	1.06	2.04

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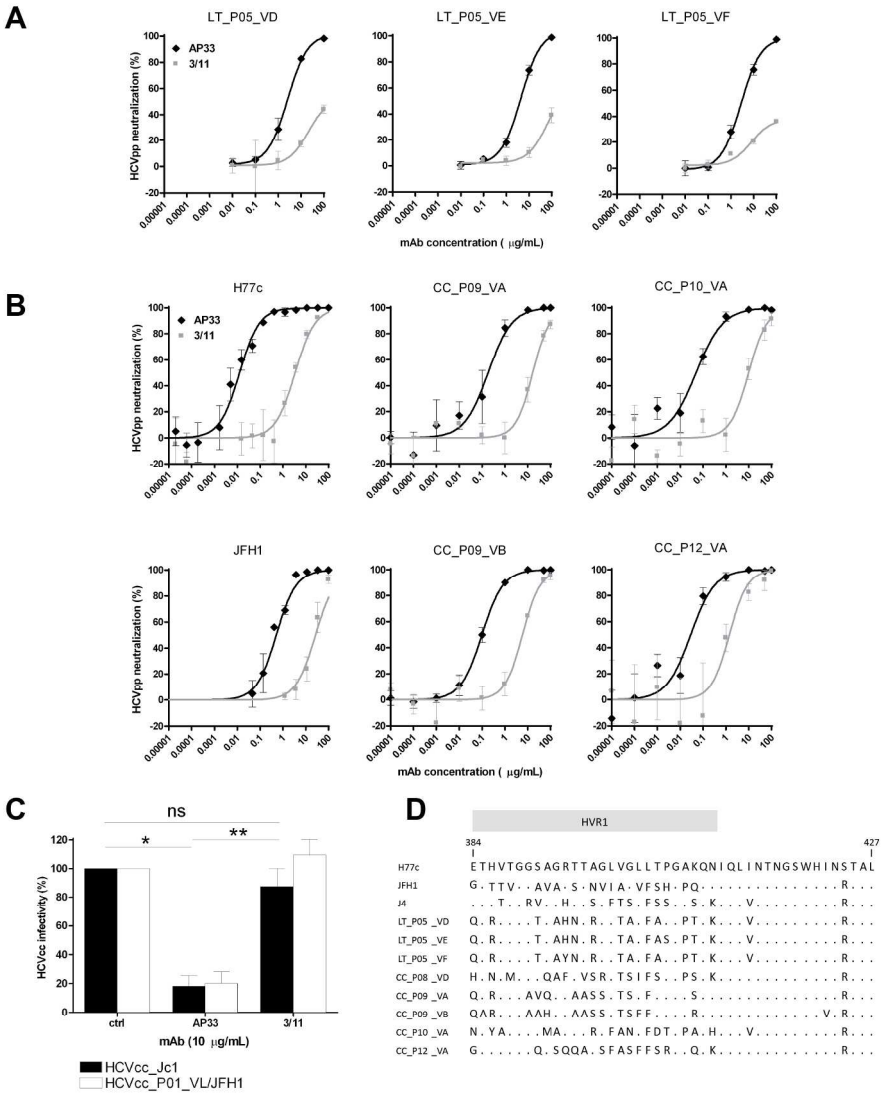
FIGURE 2



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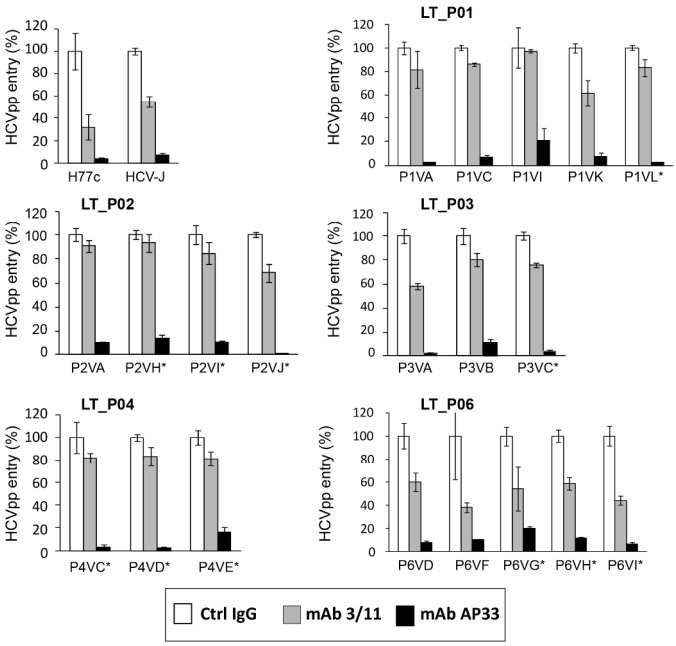

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FIGURE 4

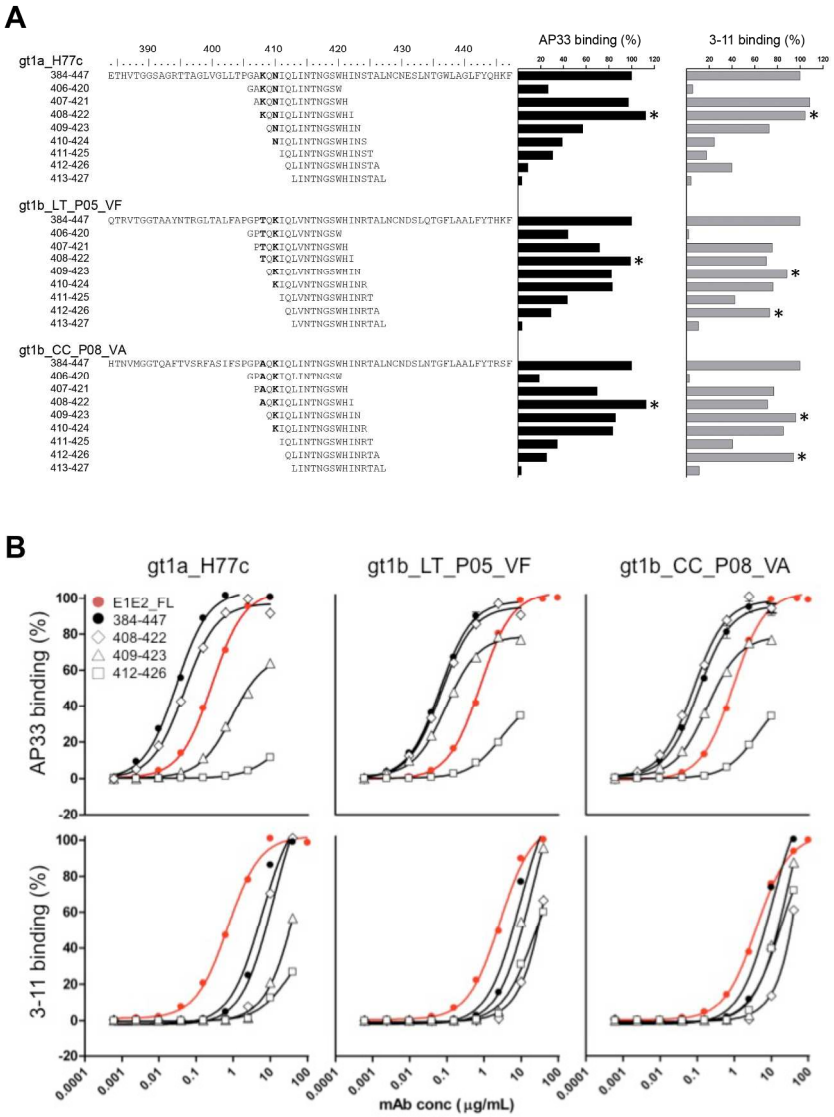

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FIGURE 5

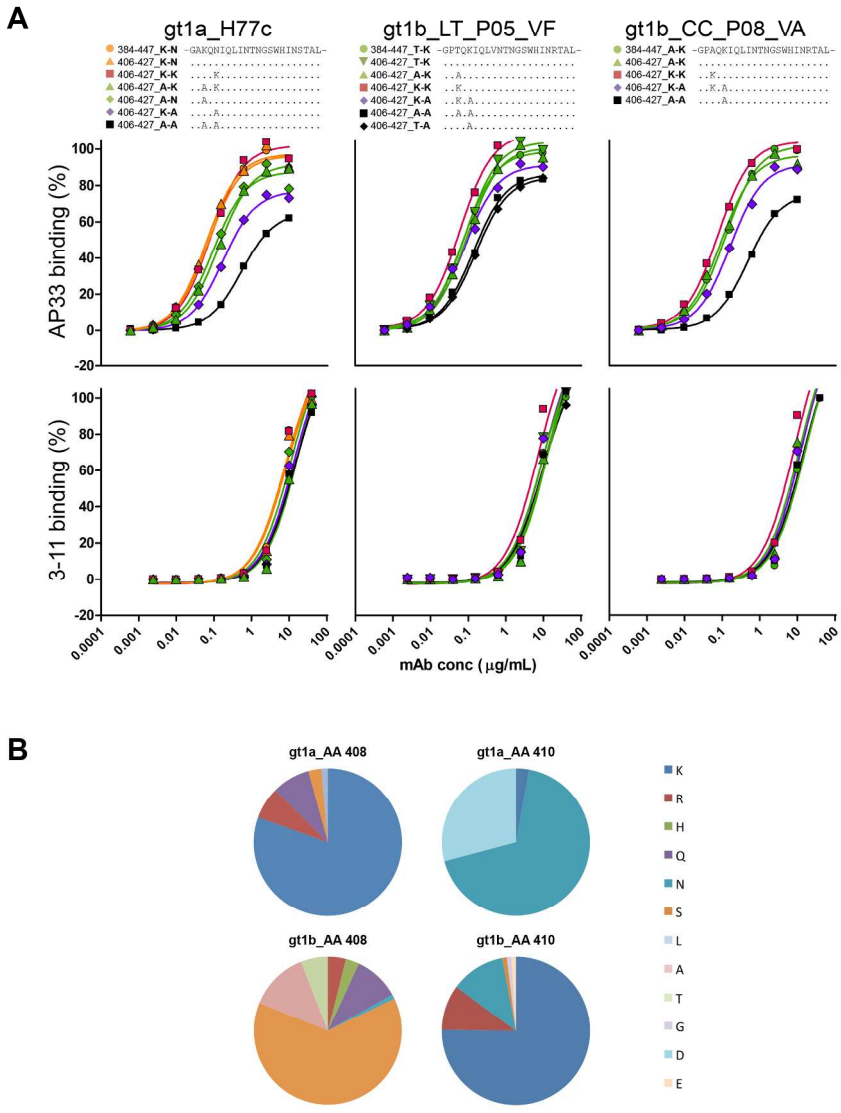

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FIGURE 6

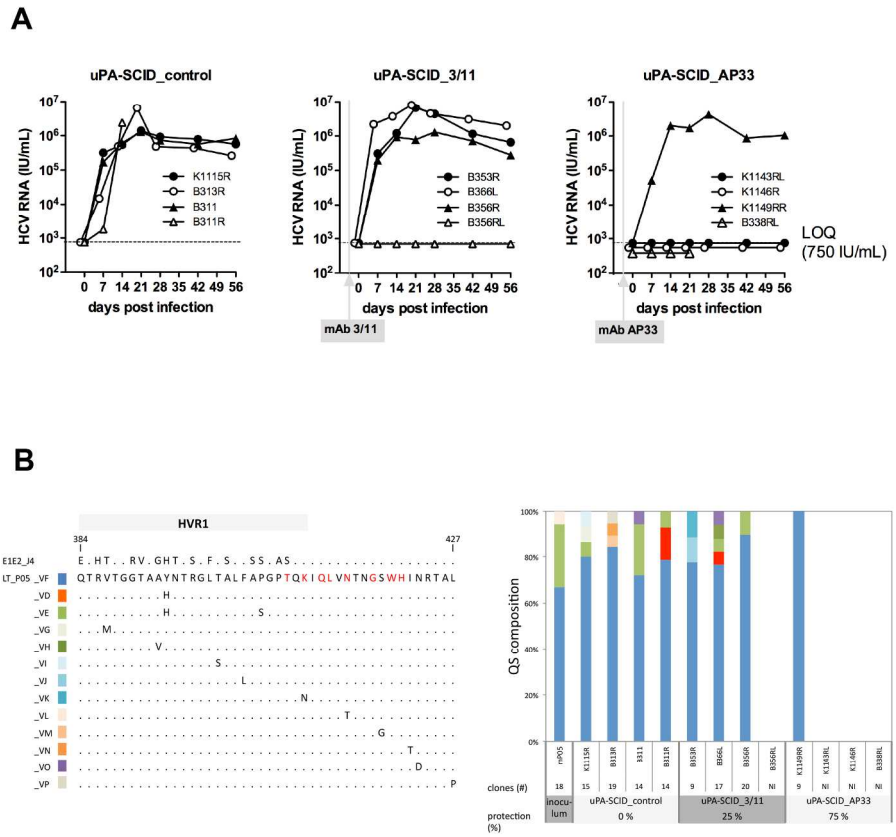
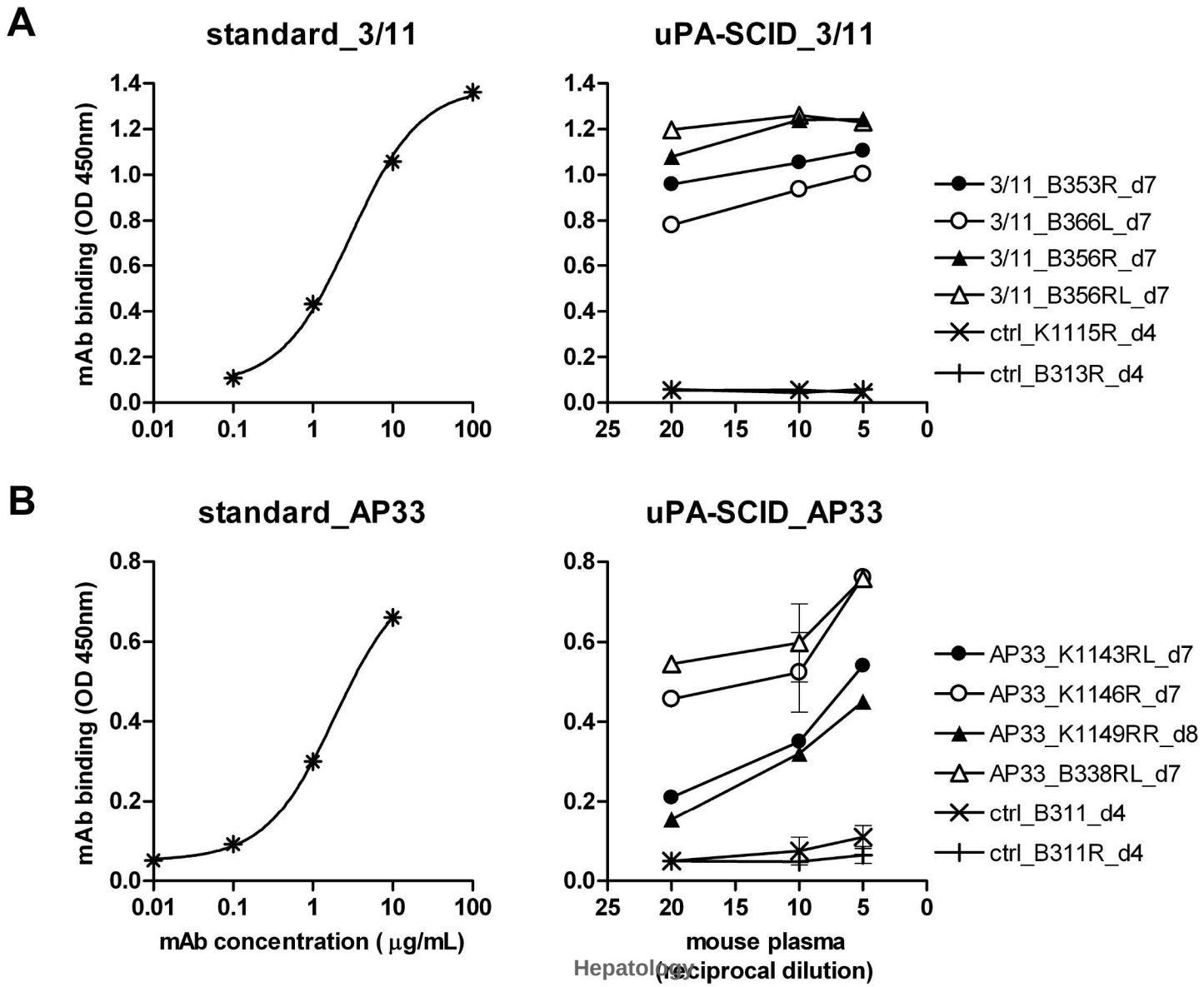


Figure 6. mAb AP33 protects humanized mice against an HCV-gt 1b challenge. (A) Chimeric animals were challenged with 104 IU HCV RNA of mLT_P05 without pretreatment (no IgG) or after passive immunization on day -3 with 1 mg of mAb 3/11 or AP33. Plasma HCV RNA levels (IU/mL) were measured until 8 weeks after viral challenge. Mice that remained non-infected after inoculation are shown as HCV-RNA <750 IU/ml (LOQ, dotted line). Animal B338RL died 4 weeks after viral challenge. (B) Clonal sequence analysis of recovered virions in selected week 1 samples (week 2 for B311R) revealed viable E1E2 sequences in all samples. Each color represents a unique sequence in AA-region 384-427 (left panel). Columns represent the percentage of each clone detected in the inoculum or in the plasma of the animals. For each animal, the total number of clones analyzed is shown beneath the mouse ID. Viral outcome after mLT_P05 challenge is summarized as % protection. The sequence of each quasispecies clone is shown in the right panel. AA highlighted in red, represent AP33 anchor residues identified in this and previous papers.
190x254mm (300 x 300 DPI)

Supplementary Figure 1: Bioavailability of circulating mAb AP33 and 3/11 in immunized mice. After viral challenge, the presence of mAb 3/11 in 3/11-treated (A) and of AP33 in AP33-treated (B) uPA-SCID mice was measured using EIA. Cell-lysates containing HCV E1E2 envelope proteins derived from prototype isolate H77c were incubated with 3 dilutions of mouse plasma (1/5, 1/10 and 1/20) for 1 hour at room temperature. Bound antibodies were detected with an HRP-conjugated anti-species secondary antibody. Absorbance values were determined at 450 nm. For a semi-quantitative estimation of the circulating mAb-level, dilutions of both mAbs were added in parallel (termed 'standard'). In each set-up, the plasma of two control mice was added. The suffix added to the mouse ID number refers to the day of plasma collection.

Supplementary Figure 1 for online publication



Supplementary Table 1 for online publication

Supplementary Table 1. Neutralizing properties of mAbs AP33 and 3/11*

			LT (gt1b)			CC (gt1b)			
	H77c	JFH	P05_VD	P05_VE	P05_VF	P09_VA	P09_VB	P10_VA	P12_VA
3/11	3,42	26,62	21,08	65,43	7,86	19,16	5,85	8,92	1,09
AP33	0,01	0,51	2,56	4,42	2,78	0,20	0,10	0,06	0,03

*IC_{50}-results (µg/mL) of the HCVpp-experiments shown in Figure 2 panel A and B.

Detailed Materials and Methods for online publication.

Primary human hepatocytes, cell lines and monoclonal antibodies. Primary human hepatocytes (PHH) were obtained from patients undergoing partial liver resection. Huh7.5.1, HEK293FT and HEK293T cell lines have been described previously (1, 2). The isolation and production of the anti-HCV E2 mouse mAb AP33 and the rat anti-E2 mAb 3/11 have been described previously (3-6)**.**

Patient-derived HCV-variants. A first group of viral variants used in the *in vitro* experiments (Figure 1 and 2) and termed *LT_P0#_variant*, originates from post-LT serum isolated from HCV-infected patients that underwent LT and became re-infected. These variants, selected during liver graft infection, are considered as LT escape variants. Human pre-transplant serum from LT_P05 was injected in uPA$^{+/+}$-SCID mice, resulting in mouse plasma with a restricted diversity mimicking the post-LT human serum (7). This mouse-passaged P05 virus (termed *mLT_P05* in Figure 6) lacks the patient's neutralizing antibodies and was used as the challenge virus in the *in vivo* experiments. A second group of viral variants used in the *in vitro* experiments and termed *CC_P0#_variant*, originates from purified B-lymphocytes isolated from HCV-gt1b infected chronic carriers that were injected in chimeric uPA$^{+/+}$-SCID mice. After passage, the infectious mouse plasma was mostly composed of a single viral variant (used to generate HCVpp), reflecting variant-selection during transmission to humanized mice and escape from the patients' humoral immune response (Desombere *et al.*, *HCV-genotype 1b escapes antibody-mediated neutralization by viral transmission through peripheral blood B-lymphocytes,* meeting abstract P5.06 at the 21st International Symposium on Hepatitis C Virus and Related Viruses, September 7-11, 2014, Banff, Canada).

GNA-capture and peptide binding ELISA. To measure the relative binding affinity of mAbs AP33 and 3/11 to HCV E1E2 glycoproteins, a cell-lysate based ELISA was performed as previously described (8). Briefly, HEK-293T cells were transfected with plasmids expressing the E1E2 glycoproteins of (i) prototype isolates H77c (gt1a), JFH1 (gt2a) and J (gt1b) (9), (ii) LT-escape variants P05_VD, P05_VE and P05_VF (gt1b), and (iii) chronic carrier (CC)-variants P08_VD, P09_VA and P12_VA (gt1b). Optimal dilution of E1E2 lysates derived from transfected HEK-293T cells was determined using GNA (*Galanthus nivalis*) lectin-coated microtiter plates (Sigma). To measure the relative affinity of mAbs AP33 and 3/11 to different peptides, a standard ELISA was performed wherein peptides (25 µg/mL) were surface-attached using a 50mM carbonate-bicarbonate buffer (pH9.6). Synthetic peptides were obtained from JPT Peptide Technologies GmbH (Berlin, Germany) (>95% purity) and dissolved in water plus 10% dimethyl sulfoxide (DMSO) at 10 mg/ml. AP33 and 3/11 were serially diluted and incubated for 1 hour at room temperature. Bound antibodies were detected with an HRP-conjugated anti-species secondary antibody. Absorbance values were determined at 405 nm. Results were expressed as % of maximal binding.

HCVpp and HCVcc production, infection and neutralization. Lentiviral HCVpp expressing patient-derived envelope glycoproteins were produced as previously described (7, 10-11). HCVpp entry was determined by measuring the luciferase reporter gene expression. The detection limit for positive luciferase reporter protein expression was $3x10^3$ RLU/assay corresponding to the mean ± 3 SD of background levels, i.e. luciferase activity of naïve non-infected cells or cells infected with pseudotypes without HCV envelopes. Patient-derived HCVpp infectivity in PHH and Huh7.5.1 ranged from $7x10^3$ to $9x10^5$ RLU and $3x10^5$ to $2x10^6$ RLU, respectively. Chimeric HCVcc expressing patient-derived structural proteins have been produced previously and their infectivity was determined by the $TCID_{50}$ assay in Huh7.5.1 cells (12). HCVpp and HCVcc neutralization were performed as described (2, 7, 13). Briefly, HCVpp or HCVcc were

mixed with mAbs AP33, 3/11 or control isotype IgG, pre-incubated for 1 hour at 37°C and added to PHH (for HCVpp) or Huh7.5.1 cells (for HCVpp and HCVcc) for 72 hours at 37°C. For dose-dependent response, mAbs were serially diluted to determine the IC_{50}, the concentration of antibody that gave a 50% inhibition of HCVpp entry. The specific neutralization was determined as described (7). Background levels of HCVpp and HCVcc infection were taken into account by subtracting infectivity in the presence of irrelevant isotype control IgG used at the same dilution as for anti-HCV mAbs.

Sequencing analysis. Clonal sequence analysis was performed on all HCV-positive mouse samples and the inoculum. Briefly, total RNA was purified from plasma using the High Pure Viral Nucleic Acid kit (Roche) and converted into cDNA using Superscript™III Reverse Transcriptase (Invitrogen) and random primers (125 ng/µL). The region spanning the C-terminal part of core and the full-length E1E2 region was amplified using LongAmp DNA polymerase (NEB) using the following primers: 5'-CGTAGGTC-GCGTAACTTGGGTAA-3' and 5'-GTGCGCCTCGGCTCTGGT-GATAAA-3'. A second, nested PCR was performed using Pfu DNA polymerase (Fermentas) and the following primers: 5'-TATAGATATCATGGGGTACATCCGCTCGTC-3' and 5'-ATATGATATCTTACTCAGCCTGAGCTATCAG-3' (2). The generated amplicons were then run on a 1% agarose gel, purified and cloned into the Zero Blunt cloning vector (Invitrogen). After transformation, 9 to 20 clones were sequenced on an ABI3730XL DNA analyzer (Applied Biosystems), using plasmid (M13) and HCV-specific (5'-TGGGATATGATGATGAACTGGTC-3', 5'-AGGGCAGTCCTGTTGATGTGCCAG-CTGCC-3', 5'-TGCCCCACGGACTGCTTCCGGA-3', 5'-CCTTCAGACCGGGTTCCTTG-3' and 5'-CATG-CRGCATTGAGCCTGTG-3') primers.

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