Mutations that alter use of hepatitis C virus cell entry factors mediate escape from neutralizing antibodies.

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Abstract:	Background & Aims: The development of vaccines and other strategies to prevent hepatitis C virus (HCV) infection is limited by rapid viral evasion. HCV entry is the first step of infection; this process involves several viral and host factors and is targeted by host neutralizing responses. Although the roles of host factors in HCV entry have been well characterized, their involvement in evasion of immune responses is poorly understood. We used acute infection of liver graft as a model to investigate the molecular mechanisms of viral evasion. Methods: We studied factors that contribute to evasion of host immune responses using patient-derived antibodies, HCV pseudoparticles, and cell culture-derived HCV that express viral envelopes from patients who have undergone liver transplantation. These viruses were used to infect hepatoma cell lines that express different levels of HCV entry factors. Results: Using reverse genetic analyses, we identified altered use of host-cell entry factors as a mechanism by which HCV evades host immune responses. Mutations that alter use of the CD81 receptor also allowed the virus to escape neutralizing antibodies. Kinetic studies demonstrated that these mutations affect virus-antibody interactions during post-binding steps of the HCV entry process. Functional studies with a large panel of patient-derived antibodies showed that this mechanism mediates viral escape, leading to persistent infection in general. Conclusion: We identified a mechanism by which HCV evades host immune responses, in which use of cell entry factors evolves with escape from neutralizing antibodies. These findings advance our understanding of the pathogenesis of HCV infection and might be used to develop antiviral strategies and vaccines.

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Mutations that Alter Use of Hepatitis C Virus Cell Entry Factors Mediate Escape from Neutralizing Antibodies

Molecular mechanisms of viral evasion in HCV infection

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Abbreviations: CLDN - claudin; CTRL - control; HCV - hepatitis C virus; HCVpp - HCV pseudoparticles; HMAb - human monoclonal antibody; HVR - hypervariable region; IgG - immune globulin G; mAb - monoclonal antibody; RLU - relative light units; SR-BI - scavenger receptor class B type I; LT - liver transplantation; OCLN – occludin ; V- viral variant.

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ABSTRACT

Background & Aims: The development of vaccines and other strategies to prevent hepatitis C virus (HCV) infection is limited by rapid viral evasion. HCV entry is the first step of infection; this process involves several viral and host factors and is targeted by host neutralizing responses. Although the roles of host factors in HCV entry have been well characterized, their involvement in evasion of immune responses is poorly understood. We used acute infection of liver graft as a model to investigate the molecular mechanisms of viral evasion.

Methods: We studied factors that contribute to evasion of host immune responses using patient-derived antibodies, HCV pseudoparticles, and cell culture-derived HCV that express viral envelopes from patients who have undergone liver transplantation. These viruses were used to infect hepatoma cell lines that express different levels of HCV entry factors.

Results: Using reverse genetic analyses, we identified altered use of host-cell entry factors as a mechanism by which HCV evades host immune responses. Mutations that alter use of the CD81 receptor also allowed the virus to escape neutralizing antibodies. Kinetic studies demonstrated that these mutations affect virus–antibody interactions during post-binding steps of the HCV entry process. Functional studies with a large panel of patient-derived antibodies showed that this mechanism mediates viral escape, leading to persistent infection in general.

Conclusion: We identified a mechanism by which HCV evades host immune responses, in which use of cell entry factors evolves with escape from neutralizing antibodies. These findings advance our understanding of the pathogenesis of HCV infection and might be used to develop antiviral strategies and vaccines.

Keywords: virology; liver disease; tissue culture model; immunity

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of liver disease.[1] A vaccine is not available and antiviral treatment is limited by resistance and adverse effects.[2] HCV-induced liver disease is a leading indication for liver transplantation (LT).[3] A major limitation of LT is the universal re-infection of the liver graft with accelerated recurrence of liver disease. A strategy to prevent re-infection is lacking.[3] Thus, there is an urgent unmet medical need for the development of efficient and safe antivirals and vaccines.

HCV entry is required for initiation, maintenance and dissemination of infection. Viral entry is a key target for adaptive host responses and antiviral strategies.[4, 5] Functional studies in clinical cohorts highlight that viral entry and escape from antibody-mediated neutralization play an important role in viral persistence and liver disease.[6-12] HCV entry is a highly orchestrated process mediated by viral envelope glycoproteins E1 and E2 and several host factors including heparan sulfate, CD81, scavenger receptor BI (SR-BI), claudin-1 (CLDN1), occludin (OCLN) (reviewed in [5]) and kinases.[13] While the role of E1E2 in antibody-mediated neutralization has intensively been studied,[4, 5, 14] the role of host factors for viral evasion *in vivo* is only poorly understood.

Acute graft infection is an established *in vivo* model to study viral evasion since viral infection and host neutralizing responses can be precisely monitored.[8] Viral entry and escape from host neutralizing responses are important determinants allowing the virus to rapidly infect the liver during transplantation.[8] However, the molecular mechanisms by which the virus evades host immunity to persistently re-infect the liver graft are unknown.

To uncover viral and host factors mediating enhanced viral entry and escape, we functionally analyzed genetically closely related prototype variants derived from a

well-characterized patient undergoing LT.[8] One variant P01VL re-infecting the liver graft was characterized by high infectivity and escape from neutralizing antibodies present in autologous pre-transplant serum.[8] The other closely related variants, P01VA and VC, were not selected during LT and characterized by lower infectivity and high sensitivity to neutralization by autologous pre-transplant serum.[8] Previous studies had indicated that an E2 region comprising amino acids 425-483 most likely contained mutations responsible for the phenotype of enhanced entry and viral evasion of variants re-infecting the liver graft.[8]

MATERIALS AND METHODS

Patients. Evolution and functional analysis of viral variants of patient P01 have been described.[8] Anti-HCV-positive serum samples from patients undergoing transplantation and chronic HCV infection were obtained with approval from the Strasbourg University Hospital IRB (ClinicalTrial.gov Identifiers NCT00638144 and NCT00213707).

Plasmids. Plasmids for HCVpp production of variants VL, VA and VC have been described.[8] E1E2-encoding sequences were used as templates for individual and combinations of mutations using the QuikChange II XL site-directed mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequence analysis (GATC Biotech) for the desired mutation and for exclusion of unexpected residue changes in the full-length E1E2 encoding sequences. Mutated constructs were designated X#Y, where # is the residue location in H77c,[15] X is the mutated and Y the original amino acid.

Antibodies. Monoclonal anti-E1 (11B7) and anti-E2 (AP33, IGH461, 16A6), human anti-HCV IgG,[10, 16] HMAbs CBH-2, CBH-5, CBH-23 and HC-1 have been described.[9, 17] Anti-CD81 (JS-81) was from BD Biosciences, AP33 from Genentech, 11B7, IGH461 and 16A6 from Innogenetics.

Cell lines. HEK 293T and Huh7.5.1 cells were cultured as described.[10, 13, 16] Huh7.5.1 cells overexpressing HCV entry factors were created by stable lentiviral gene transfer of CLDN1, OCLN, SR-BI or CD81.[18] Huh7.5 stably transduced with retroviral vectors encoding for CD81 and CD13-specific shRNAs have been described.[19] Receptor expression was assessed by flow cytometry .[13]

HCVpp and HCVcc production, infection and neutralization. Lentiviral HCVpp bearing patient-derived envelope glycoproteins were produced as described.[8, 10, 20] The amount of HCVpp was normalized following quantification of HIV p24 antigen expression (Innotest HIV Antigen mAb Kit, Innogenetics) and HCVpp entry was performed as described. [8, 10, 11, 16] Chimeric HCVcc expressing patient-derived structural proteins were constructed and produced as described in Supplementary Materials and Methods. HCVcc infectivity was determined by determining the $TCID_{50}$[21] or intracellular HCV RNA levels as described.[13, 21, 22] HCVpp and HCVcc neutralization were performed as described.[8, 10, 11, 16]

Kinetic assays. HCVpp kinetic assays were performed in Huh7.5.1 cells using anti-CD81 (JS-81) and anti-E2 (CBH-23) mAbs as described.[16, 23]

Statistical analysis. Statistical analysis (Repeated Measures ANOVA) was performed using the SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL).

RESULTS

HCV E2 residues at positions 447, 458 and 478 confer enhanced viral entry of a high-infectivity variant re-infecting the liver graft. To investigate the molecular mechanism of enhanced entry of the variant VL re-infecting the liver graft, we first introduced individual mutations of region $E2_{425-483}$[8] of the low-entry and neutralization-sensitive mutant VC into HCV pseudoparticles (HCVpp) expressing envelope glycoproteins of the highly infectious escape variant VL (Fig. 1A). Previous studies had indicated that this region most likely contains the mutations responsible for the high-infectivity phenotype of VL.[8] Following normalization of HCVpp levels by p24 antigen expression, viral entry was quantified relative to the escape variant VL. The entry level of the nonselected variant VC was 5% compared to the escape variant VL (Fig. 1B). By introducing the mutations S458G and R478C into VC, chimeric HCVpp showed similar viral entry level as the paternal variant VL whereas introduction of individual or combination of other mutations only had a partial effect (Fig. 1B, Fig. S1). To explore the impact of other positions on viral entry we introduced mutations from another nonselected variant termed VA into VL (Fig. 1A) and identified position F447 as an additional residue relevant for enhanced entry of the escape variant VL (Fig. 1C). These results demonstrate that residues F447L, S458G and R478C are largely responsible for the high-infectivity of the escape variant VL.

Enhanced viral entry by mutations F447L, S458G and R478C of the escape variant is the result of altered use of CD81. To address whether the mutations affect viral entry by different usage of cell entry factors SR-BI, CD81, CLDN1 and OCLN, we studied viral entry of HCVpp derived from parental and chimeric variants in Huh7.5.1 cells stably overexpressing individually the four main entry factors (Fig.

2A). Overexpression of either SR-BI, CD81, CLDN1 or OCLN, did not affect the stability or proportion of other cell surface HCV receptors (Fig. 2B and data not shown).

Overexpression of CD81 significantly enhanced viral entry of VL (3.2fold) and VC (2fold) compared to parental cells ($P < .001$) (Fig. 2C). The fold change in HCVpp entry was significantly higher for VL than for VC ($P < .001$). Exchanging the two residues at position 458 and 478 similarly increased viral entry. This suggests that combination of the two individual mutations modulates viral entry by altering CD81-dependency. Overexpression of SR-BI also increased viral entry of VL and VC, but no specific increase was observed for the chimeric strains containing substitutions at positions 458 and 478 (Fig. 2C). These data confirm an important role for SR-BI as an entry factor for patient-derived variants, but also demonstrate that positions 458 and 478 do not significantly alter SR-BI-dependency. Thus, increased entry efficiency of VL in SR-BI-overexpressing cells is most likely due to other mutations, e. g. in HVR1. Viral entry enhancement was less pronounced in cells overexpressing CLDN1 or OCLN than CD81 and SR-BI (Fig. 2C) and no specific modulation of viral entry was associated with the two variants or chimeric strains.

The CD81 usage of viral variants VL, VC and VA was further investigated using Huh7.5 cells with silenced CD81 expression (Fig. 3A).[19] The escape variant VL showed the highest decrease (5.4fold) of viral entry in shCD81-Huh7.5 cells compared to the decrease of variants VC (4.3fold, $P < .001$) and VA (2.9fold, $P < .001$) (Fig. 3B-C). Exchange of the mapped residues into chimeric expression plasmids conferred the phenotype of decreased entry of VL (Fig. 3B-C) confirming that identified residues modulate viral entry by different CD81 usage. Moreover, using a relevant model system for HCV-CD81 interactions occurring *in vivo* consisting of cell surface-expressed CD81, we demonstrate that E1E2 complexes of

the escape variant VL bound less efficiently to shCD81-Huh7.5 cells than glycoproteins of variants VC and VA (Fig. S2A). Exchange of the mapped residues conferred similar phenotypes as the parental glycoproteins (Fig. S2B) suggesting that the residues at positions 447, 458 and 478 alter E1E2 interactions with cell surface CD81.

Taken together, these data demonstrate that (i) the escape variant is characterized by markedly altered CD81 usage and (ii) altered CD81 usage of the variant is mediated by residues at positions 447, 458 and 478.

Since the levels of E1E2 incorporation into HCVpp and lentiviral p24 antigen expression were similar for all strains (Fig. S3A-D), it is unlikely that the differences in viral entry are the result of impaired HCVpp assembly or release.

Next, to assess whether enhanced entry is due to more rapid internalization of viral particles we investigated internalization kinetics of the parental and chimeric variants in the presence of anti-CD81 antibody.[16, 21, 23, 24] Since entry kinetics of parental and chimeric variants were similar (Fig. 3D), it is unlikely that the mutant-induced modulation of CD81-dependency alters the velocity of viral entry.

Positions 447, 458 and 478 mediate escape from autologous transplant serum during graft re-infection. To assess whether the residues in region $E2_{425-483}$ influencing viral entry (Fig. 1) were also responsible for escape from antibody-mediated neutralization, we studied the impact of each single and combined substitutions of the nonselected variant VC on neutralization by autologous pre-transplant serum. Autologous pre-transplant serum only poorly neutralized the selected variant VL as well as the variants substituted at position 434, 444, and 445 while individual substitution at positions 458 and 478 significantly ($P < .001$, $P \leq .05$) increased the sensitivity of $VLVC_{458}$ and $VLVC_{478}$ to autologous neutralizing

antibodies (1:400 and 1:200) (Fig. 4A). Noteworthy, only the variant $VLVC_{458+478}$ showed a similar neutralization titer as the nonselected variant VC (1:6,400, $P < .001$). To confirm that these mutations were indeed responsible for the phenotype of the parental variant VL, we investigated neutralization of $VCVL_{458+478}$ by autologous serum. The variant $VCVL_{458+478}$ escaped autologous neutralization similarly to the escape variant VL (Fig. 4A). A similar phenotype was observed when mutation 447 of VA was introduced into the VL cDNA (Fig. 4B). In contrast, the introduction of other residues into VL only had a minor effect on neutralization (Fig. 4B). Taken together, these findings suggest that the residues at positions 447, 458 and 478 are simultaneously responsible for both enhanced viral entry and evasion from antibody-mediated neutralization.

Positions 447, 458 and 478 define a conformational epitope involved in evasion from host neutralizing responses. To further elucidate the mechanism of viral evasion of the escape variant VL from patient-derived neutralizing antibodies, we investigated whether the identified mutations F447L, S458G and R478C confer resistance or sensitivity to a panel of mAbs directed against conformational[9, 17] and linear E2 epitopes.[16] The conformational HMAbs (CBH-2, CBH-5, CBH-23, HC-1) have been shown to exhibit a broad cross-neutralizing activity by interfering with E2-CD81 interaction[9, 17] and their epitopes are only partially defined (Table S1). AP33 is directed against a conserved epitope comprising aa 412-423.[25] While the escape variant VL was poorly neutralized by several HMAbs directed against conformational epitopes, VC and VA were efficiently neutralized by all HMAbs (Fig. 5A-B). Moreover, by substituting the residues at positions 458 and 478 or 447, the well neutralized nonselected variants VC ($VCVL_{458+478}$) and VA ($VAVL_{447}$) became neutralization-resistant as the escape variant VL. Introducing the residues of VC or VA into VL

($VLVC_{458+478}$ and $VLVA_{447}$) restored neutralization by HMAbs, suggesting that these residues are part of the HMAbs epitopes. In contrast, anti-E2 antibodies (AP33, 16A6, IGH461) targeting linear epitopes similarly neutralized parental and chimeric variants (Fig. 5A-B and Table S1).

Antibody-mediated neutralization occurs at binding and post-binding steps during viral entry.[16] To map the entry step involved in viral evasion from neutralizing antibodies by VL, we investigated the neutralization kinetics of parental and chimeric variants.[16, 21, 23] The anti-E2 HMAb CBH-23 inhibited viral entry of VC and $VLVC_{458+478}$ at post-binding steps during time points closely related to HCV-CD81 interaction (Fig. 5C). Partial inhibition at post-binding steps by CBH-23 was also observed for VA and $VLVA_{447}$ (Fig. 5D). The VL variant escaped antibody-mediated neutralization at the same steps.

Interestingly, purified HCVpp expressing envelope glycoproteins of the escape variant bound similarly to neutralizing anti-E2 antibody CBH-23 as the envelope glycoproteins of non selected variants or variants containing mutations of the identified escape residue (Fig. S4). Thus, it is likely that viral evasion is not due to decreased antibody-binding to circulating virions but rather occurs during post-binding steps of viral entry where E2-host entry factor interactions result in conformational changes of the envelope and failure of antibodies to inhibit entry. Taken together, these data indicate that positions 447, 458 and 478 mediate viral evasion from neutralizing antibodies at post-binding steps and time points closely related to HCV-CD81 interaction.

Positions 447, 458 and 478 mediate escape from antiviral antibodies in non-related patients with chronic HCV infection. To investigate whether these mutations not only result in escape from antibodies from the same patient but also

confer resistance to antiviral antibodies of non-related HCV infected patients, we studied the neutralization of the parental variants by a large panel of sera randomly selected from chronically infected patients (n = 102). While VL was not neutralized by 53 out of 102 patient sera (mean neutralizing titer, 1:144) VC was significantly neutralized by 90 out of 102 patient sera (mean neutralizing titer, 1:1,088, $P < .001$) (Fig. 6 and Table S2). Similar results were obtained for VA (neutralization by 80 out of 102 patient sera, mean neutralizing titer of 1:322, $P = .01$). Functional analysis of HCVpp expressing chimeric envelope glycoproteins demonstrated that neutralization of VC and VA was predominantly mediated by the identified mutations in residues 447, 458 and 478 (Fig. 6).

Confirmation of differential cell entry factor usage and viral evasion using chimeric HCVcc. Finally, we confirmed the functional impact of the three residues on virus-host interactions using the HCVcc system. To address this issue we constructed chimeric JFH-1 based HCVcc expressing the VL wild-type envelope or VL containing VC and VA-specific functional residues. Viruses containing patient-derived envelopes showed similar levels of replication and envelope production (data not shown). Phenotypic analyses of infection and neutralization of chimeric HCVcc confirmed the relevance of the identified residues for enhanced entry, differential CD81 usage and viral evasion (Fig. 7A-D). While the escape variant VL was poorly neutralized, the identified mutations at positions 447, 458 and 478 restored its sensitivity to conformational HMAb CBH-23 (Fig. 7C) as well as to heterologous sera from chronically infected patients (Fig. 7D). These data confirm the functional relevance of the obtained results in the HCVcc system expressing authentic patient-derived envelopes.

DISCUSSION

Using acute infection of the liver graft as an *in vivo* model, we identified a novel, clinically and therapeutically important mechanism of viral evasion, where co-evolution simultaneously occurs between cellular entry factor usage and escape from neutralization.

Several host selection forces operate concomitantly during HCV infection. These include pro-viral host factors resulting in selection of most infectious viruses best adapted to host factors and anti-viral host immune responses leading to escape from immune responses. Antibody-mediated selective pressure is thought to be an important driver of viral evolution.[8, 11] The immune response may fail to resolve HCV infection because neutralizing antibody-mediated response lags behind the rapidly and continuously evolving HCV glycoprotein sequences.[11] However, continuous generation of escape mutations during chronic HCV infection may also compromise virus infectivity: indeed, it has been reported that structural changes in E2 leading to complete escape from neutralizing antibodies simultaneously compromised viral fitness by reducing CD81-binding.[9] Moreover, escape from T cell responses has been associated with impaired viral replication.[26, 27] We show for the first time that clinically occurring mutations simultaneously lead to enhanced viral infectivity by optimizing host factor usage and escape from host immune responses. Since this mechanism was uncovered in patient strains isolated during acute liver graft infection it is likely that the novel and unique mechanism of co-evolution between host factor usage and viral evasion ensures optimal initiation, dissemination and maintenance of viral infection in the early phase of liver graft infection. In addition, since the VL strain escapes autologous antibodies from the transplant patient (Fig. 4) and resists to monoclonal and polyclonal antibodies of heterologous patients (Figs. 5, 6, 7 and Tables S1, S2), and given the high prevalence of the identified mutations in a large

genomic database of viral isolates (Fig. S5 and Supplementary Results), the co-evolution of receptor usage and escape from neutralizing antibodies may also play an important role for viral evasion in chronic HCV infection in general.

Our mechanistic studies demonstrate that the identified viral evasion factors are part of a conformational neutralizing epitope modulating E2-CD81 interactions at post-binding entry steps.[28, 29] Noteworthy, the same mutations were also responsible for immune escape of VL. Neutralization studies using HMAbs directed against discontinuous envelope glycoprotein regions termed domain B and C[30, 31] demonstrate that the three positions are part of an epitope which plays a key role for neutralization and viral evasion. Since the mutations are outside the known contact residues within the epitopes of the HMAbs CBH-2, CBH-5, CBH-23 and HC-1[9, 17] (Table S1) and complementary to previously identified regions associated with escape from neutralizing monoclonal antibodies,[25] positions 447, 458 and 478 either modulate the interaction of the majority of antibodies directed against domain B and C epitopes or are part of a novel E2 epitope mediating evasion from host neutralizing antibodies.

Based on previous functional observations and structural predictions, Krey and colleagues proposed a model for a potential tertiary organisation of E2. In this model, E2 comprises three subdomains with the CD81 binding regions located within domain I (W420, A440LFY, Y527, W529, G530 and D535) and potential CD81 binding sites overlapping with domain III (Y613RLWHY).[28, 29, 32, 33] In this model, positions 447, 458 and 478 are located outside but in close proximity of the previously suggested CD81 binding domains. Moreover, position 447 is located immediately downstream a conserved motif between HVR1 and HVR2 which has been shown to play an important role in CD81 recognition as well as pre- or post-

CD81 dependent stages of viral entry.[32] Position 478 is located within HVR2 which modulates, by a complex interplay with HVR1, binding of E2 glycoprotein to CD81.[34]

Since mutations F447L, S458G and R478C (i) modulate CD81-dependency of HCV entry (Fig. 2 and 3), (ii) alter the interaction with cell surface CD81 (Fig. S2), (iii) mediate viral evasion from antibodies at post-binding steps closely related to HCV-CD81 interactions (Fig. 5) and (iv) are located within E2 loops of the predicted E2 secondary structure and tertiary organization[29] positions 447, 458 and 478 may be part of two loops belonging to a larger cluster of closely related surface-exposed E2 loops. These loops are most likely involved in E2-CD81 binding either directly or indirectly as a key point for structural rearrangement during viral entry. [34, 35]

The polar S and R residues present in the escape variant can form non-bonded interactions with other residues by hydrogen bonds and salt bridge, respectively. These interactions could increase the stability of the interacting E2-CD81 interface allowing efficient entry of the VL escape variant through E2-CD81-CLDN1 co-receptor complexes which are key determinants for viral entry.[13, 23, 36] Furthermore, the E2 cluster of loops containing the mutations bears linear epitopes but also defines at least one conformational epitope that is a target of neutralizing antibodies. According to residue physical-chemical properties, the VL variant S458 and R478 residues enhance the hydrophilicity of the loops they belong to and may promote the surface exposure of the loops. This change could further modulate E2-CD81 interactions and impair the binding of neutralizing antibodies by blocking access to their target epitopes. The F to L substitution present in the VA strain most likely does not profoundly alter the tertiary or quaternary structure of E2. This is suggested by the fact that this position is located in a loop as predicted by the proposed E2 model.[29] Thus, it is conceivable that this mutation which increases E2 hydrophobicity may reduce accessibility of the loop and its interactions with CD81 or

CD81-CLDN1 co-receptor complexes. Alternatively, allosteric mechanisms may play a role in the observed virus-antibody-host interactions.

Taken together, our data identified key determinants of immune evasion *in vivo.* Mutations conferring neutralization escape altered CD81 receptor usage and enhanced cell entry. Moreover, our data suggest that mutations in HVR1 which may modulate entry and neutralization by altering SR-BI-dependency (Fig. 1, 2, 4 and data not shown) may contribute to the high-entry and escape phenotype of the escape variant. Furthermore, interfering non-neutralizing antibodies may constitute another mechanism of escape (data not shown).

Although proof-of-concept studies in animal models have demonstrated a potential role for HMAbs in prevention of HCV infection,[37, 38] the partial or complete escape of the VL variant from autologous and heterologous serum-derived antibodies as well as many broadly cross-neutralizing HMAbs (Fig. 5; Table S1) demonstrates the ability of the virus to evade cross-neutralizing anti-envelope mAbs. By identifying viral and host factors mediating immune evasion in the HCV-infected patient, our results may open new perspectives for the development of broadly cross-neutralizing anti-envelope or anti-receptor antibodies overcoming viral escape.

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FIGURE LEGENDS

Figure 1. Positions 447, 458 and 478 confer enhanced viral entry of a high-infectivity variant re-infecting the liver graft. (A) Genomic organisation and mutations of envelope glycoproteins of escape variant VL and nonselected variants VC and VA. HVR1 and HVR2 are depicted in green; E2 domains in red (DI), yellow (DII) and blue (DIII); and CD81 binding domains in dark blue. [29, 33, 39] Positions 447, 458 and 478 are highlighted in black vertical lines. Differences between VL, VC and VA in region $E1E2_{384-483}$ are displayed. (B-C) Viral entry in Huh7.5.1 cells of the escape variant VL, the nonselected variants VC and VA as well as chimeric variants containing defined mutations of VC and VA in VL or vice-versa (see Fig. S1). HCVpp infection was analyzed by luciferase reporter gene expression. Results are expressed as percentage of viral entry compared to VL. Means±SD from at least four independent experiments performed in triplicate are shown. Significant differences in HCVpp entry between variants are indicated (*, $P \leq .05$; **, $P < .001$). Abbreviations: aa - amino acid; BD - binding domain; n.s. - not significant

Figure 2. Altered usage of CD81 is responsible for enhanced viral entry of the escape variant. (A) Entry factor expression in clones of SR-BI-, CD81-, CLDN1- or OCLN-transduced Huh7.5.1 cells. The relative overexpression of each entry factors was determined by flow cytometry and is indicated as fold expression compared to parental Huh7.5.1 cells. (B) Entry factor expression in pools of CD81-overexpressing Huh7.5.1 cells (grey bars). The relative entry factor expression was determined as described in (A). (C) Receptor-dependency of patient-derived HCVpp entry. Parental and transduced Huh7.5.1 cells were incubated with parental or chimeric HCVpp and viral entry was determined as described in Fig. 1. Viral entry is expressed as fold change of viral entry compared to parental cells. Means±SD from three independent

experiments performed in triplicate are shown. Significant differences in HCVpp entry between variants are indicated (**, $P < .001$).

Figure 3. Different CD81 usage of viral variants in Huh7.5 cells with silenced CD81 expression. (A) Entry factor expression in Huh7.5 cells with silenced CD81 (grey bars) or CD13 (black bars) expression. CD81 expression was determined by flow cytometry and is indicated as fold expression compared to control shCD13-Huh7.5 cells. (B-C) Entry of patient-derived HCVpp VL, VC (B) and VA (C). Huh7.5 cells with silenced CD81 or CD13 expression were incubated with parental or chimeric HCVpp and viral entry was determined as described in Fig. 1. Viral entry is expressed as fold change of viral entry compared to shCD13-Huh7.5 control cells. Means±SD from three independent experiments performed in triplicate are shown. Significant differences in HCVpp entry between wildtype and chimeric variants are indicated (**, $P < .001$). (D) Entry kinetics of patient-derived variants. Kinetics of HCVpp entry was performed using anti-CD81 or isotype control antibody (5 µg/ml). HCV entry was determined as described in Fig.1. A representative experiment out of four is shown.

Figure 4. Positions 447, 458 and 478 mediate viral escape from neutralization by autologous transplant serum. Neutralization of the escape variant VL, variants VC and VA and the chimeric strains. HCVpp were incubated with autologous anti-HCV positive or control serum in serial dilutions for 1 h at 37°C before incubation with Huh7.5.1 cells. Neutralization titers obtained by endpoint dilution are indicated. Dotted line indicates the threshold for a positive neutralization titer (1/40). Means±SD from at least four experiments performed in triplicate are shown. (A) Neutralization of variants VL, VL containing individual or combined mutations of VC and VC with

double substitutions of VL by autologous anti-HCV positive pre-transplant serum. (B) Neutralization of variants VL, VL containing individual mutations of VA and VA with single substitution of VL by autologous anti-HCV positive pre-transplant serum. Significant differences in neutralization between variants are indicated (*, $P \leq .05$; **, $P < .001$).

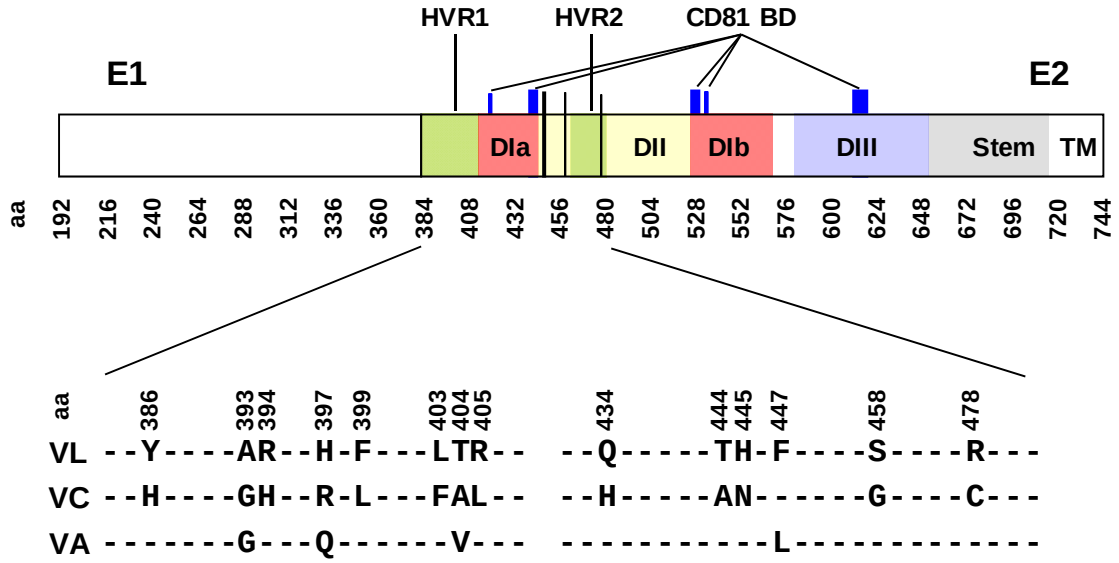
Figure 5. Mechanisms of viral evasion from neutralizing antibodies. (A-B) Escape from neutralization by HMAbs directed against conformational and linear epitopes. HCVpp produced from isolates shown in Fig. 1 were incubated with HMAbs (Table S1) or control Ab (10 µg/ml) for 1 h at 37°C prior to incubation with Huh7.5.1 cells. Results are expressed as percentage of viral entry relative to HCVpp incubated with control mAb. Means±SD from at least four experiments performed in triplicate are shown. Significant differences in HCVpp entry between variants are indicated (**, $P < .001$). (C-D) Escape from neutralization of anti-E2 antibody CBH-23 in kinetic assays. Kinetics were performed as described in Fig. 3 (HMAb 10 µg/ml; JS-81: 5 µg/ml). A representative experiment out of four is shown.

Figure 6. HCV VL strain is poorly neutralized by antibodies present in sera from a large panel of non-related patients with chronic HCV infection. Parental HCVpp (VL, VC and VA) and chimeric HCVpp ($VLVC_{458+478}$ and $VLVA_{447}$) strains, adjusted for p24 antigen expression, were preincubated for 1 h with serial dilutions of anti-HCV positive sera from randomly selected patients with chronic hepatitis C prior to incubation with Huh7.5.1 target cells. Patient number, gender, HCV genotype and viral load are indicated in Table S2. Neutralization was determined as in Fig. 4. Mean neutralization titers are marked by lines. Means from at least three independent

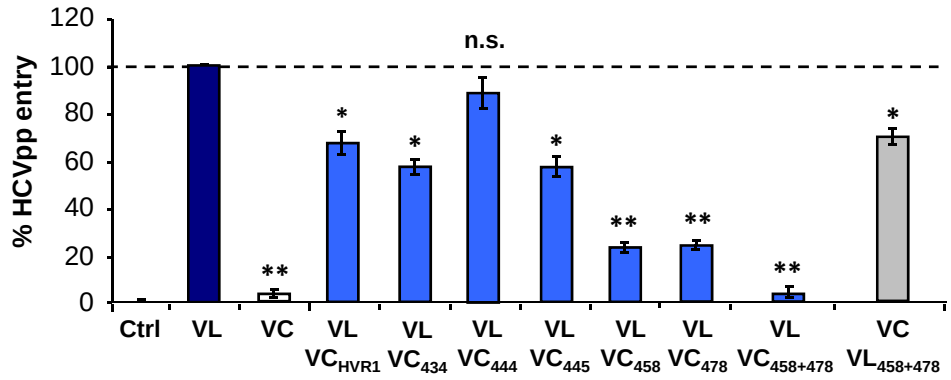
experiments performed in triplicate are shown. Significant differences in neutralization are indicated.

Figure 7. Entry viral and escape from neutralization of chimeric HCVcc expressing patient-derived viral envelopes. (A) Infectivity of HCVcc expressing envelopes of variant VL and functional residues of VA and VC is indicated by $TCID_{50}$. Means±SD from one representative experiment are shown. (B) Relative infectivity of chimeric HCVcc expressing patient-derived viral envelopes in Huh7.5 cells with silenced CD81 or CD13 expression. Means±SD from three independent experiments performed in triplicate are shown. (C) Escape from neutralization by HMAb CBH-23. Neutralization was performed as described in Fig. 5. Results are expressed as percentage of viral infectivity relative to HCVcc incubated with control mAb. Means±SD from at least three experiments performed in triplicate are shown. (D) Inhibition of HCVcc infection by anti-HCV positive sera described in Table S2B. Neutralization was performed as described in Fig. 6. Means from one representative experiment performed in triplicate are shown. Significant differences in HCVcc infection between wildtype and chimeric variants are indicated (*, $P \leq .05$; **, $P < .001$)

A.


HVR1
HVR2
CD81 BD
E1
E2
DIa
DII
DIb
DIII
Stem
TM
aa
192 216 240 264 288 312 336 360 384 408 432 456 480 504 528 552 576 600 624 648 672 696 720 744
aa
386 393 394 397 399 403 404 405 434 444 445 447 458 478
VL --Y----AR--H-F---LTR-- --Q-----TH-F----S----R---
VC --H----GH--R-L---FAL-- --H-----AN------G----C---
VA -------G---Q------V--- ----------L-------------

B.


% HCVpp entry
120
100
80
60
40
20
0
n.s.
*
**
Ctrl
VL
VC
VL VC$_{HVR1}$
VL VC$_{434}$
VL VC$_{444}$
VL VC$_{445}$
VL VC$_{458}$
VL VC$_{478}$
VL VC$_{458+478}$
VC VL$_{458+478}$

C.

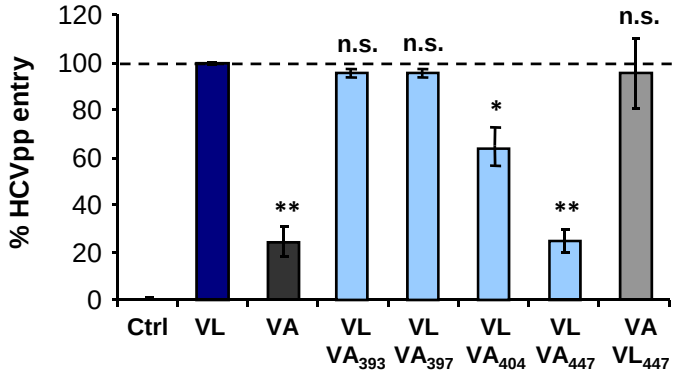

% HCVpp entry
120
100
80
60
40
20
0
n.s.
*
**
Ctrl
VL
VA
VL VA$_{393}$
VL VA$_{397}$
VL VA$_{404}$
VL VA$_{447}$
VA VL$_{447}$

Figure 1

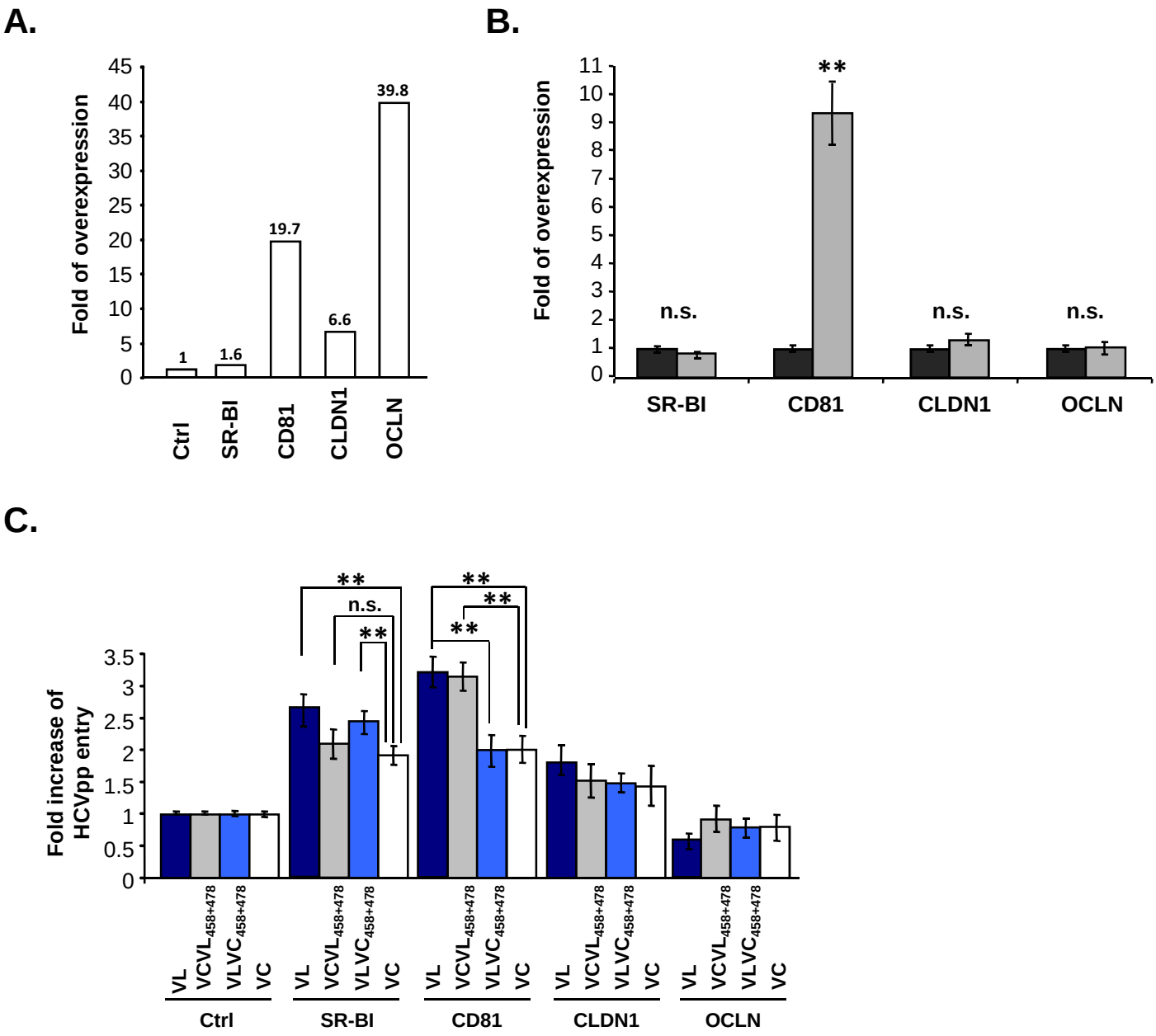

A.
Fold of overexpression
45
40
35
30
25
20
15
10
5
0
1
1.6
19.7
6.6
39.8
Ctrl
SR-BI
CD81
CLDN1
OCLN
B.
Fold of overexpression
11
10
9
8
7
6
5
4
3
2
1
0
**
n.s.
n.s.
n.s.
SR-BI
CD81
CLDN1
OCLN
C.
Fold increase of HCVpp entry
3.5
3
2.5
2
1.5
1
0.5
0
**
n.s.
**
**
**
**
VL
VCVL458+478
VLVC458+478
VC
Ctrl
SR-BI
CD81
CLDN1
OCLN

Figure 2

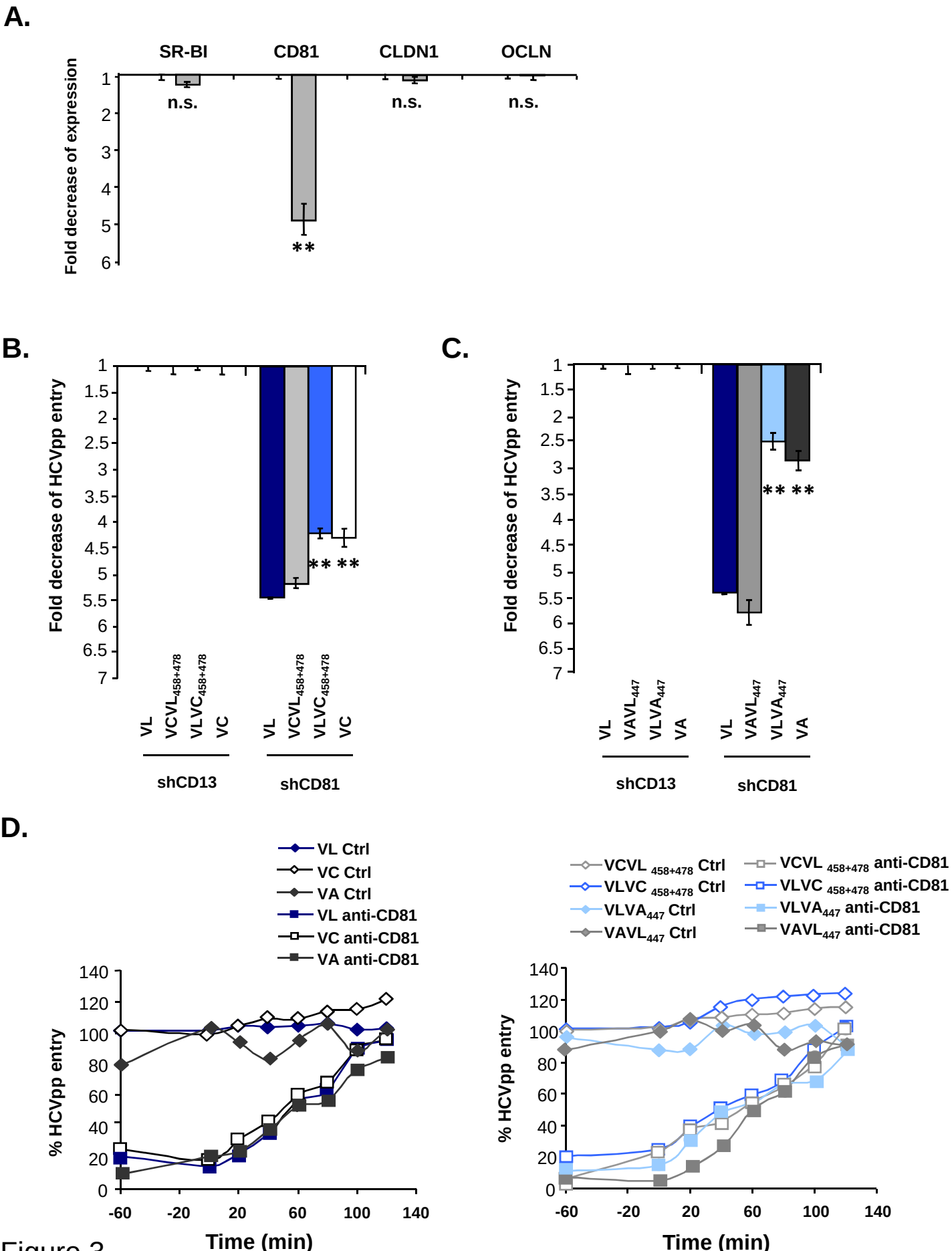
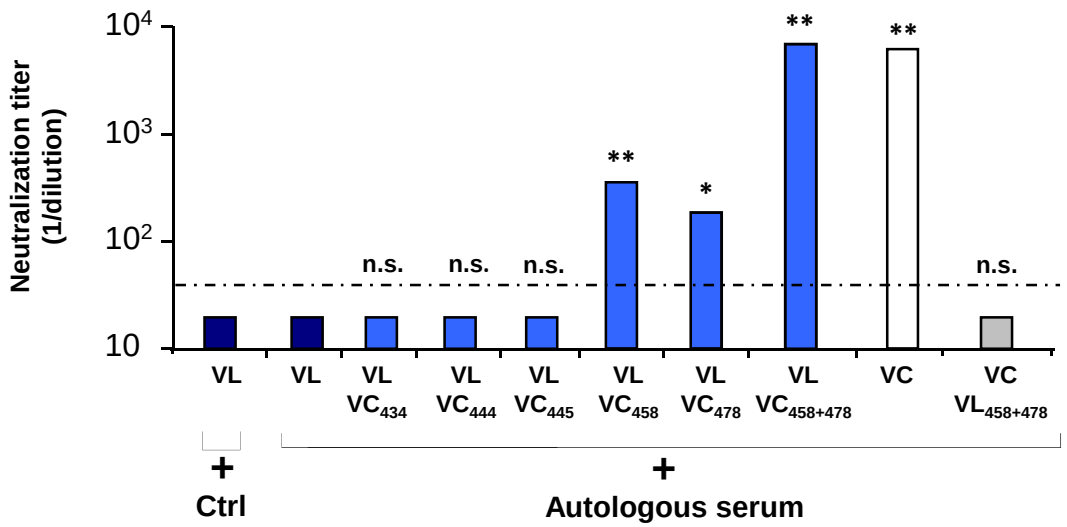

A.
SR-BI
CD81
CLDN1
OCLN
Fold decrease of expression
n.s.
**
n.s.
n.s.
B.
Fold decrease of HCVpp entry
** **
VL
VCVL458+478
VLVC458+478
VC
shCD13
shCD81
C.
Fold decrease of HCVpp entry
** **
VL
VAVL447
VLVA447
VA
shCD13
shCD81
D.
VL Ctrl
VC Ctrl
VA Ctrl
VL anti-CD81
VC anti-CD81
VA anti-CD81
% HCVpp entry
Time (min)
VCVL 458+478 Ctrl
VLVC 458+478 Ctrl
VLVA447 Ctrl
VAVL447 Ctrl
VCVL 458+478 anti-CD81
VLVC 458+478 anti-CD81
VLVA447 anti-CD81
VAVL447 anti-CD81
% HCVpp entry
Time (min)

Figure 3

A.


Neutralization titer (1/dilution)
10⁴
10³
10²
10
n.s.
n.s.
n.s.
**
*
**
**
n.s.
VL
VL
VL VC434
VL VC444
VL VC445
VL VC458
VL VC478
VL VC458+478
VC
VC VL458+478
+
Ctrl
+
Autologous serum

B.

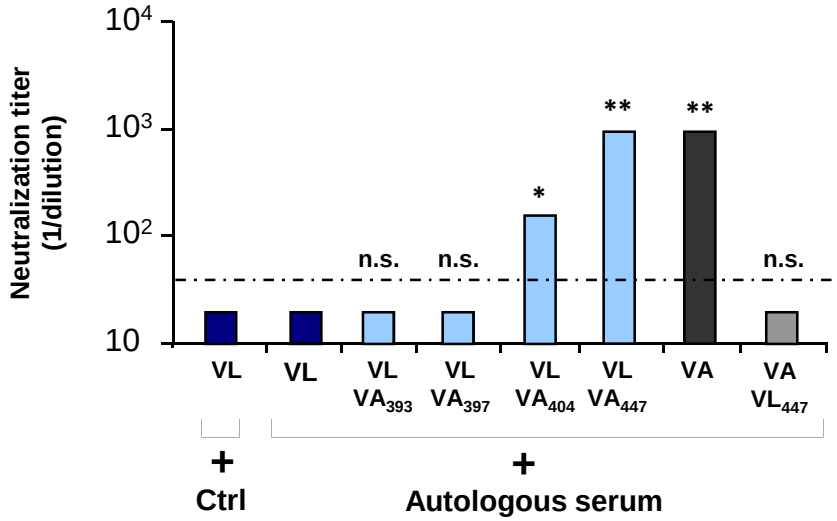
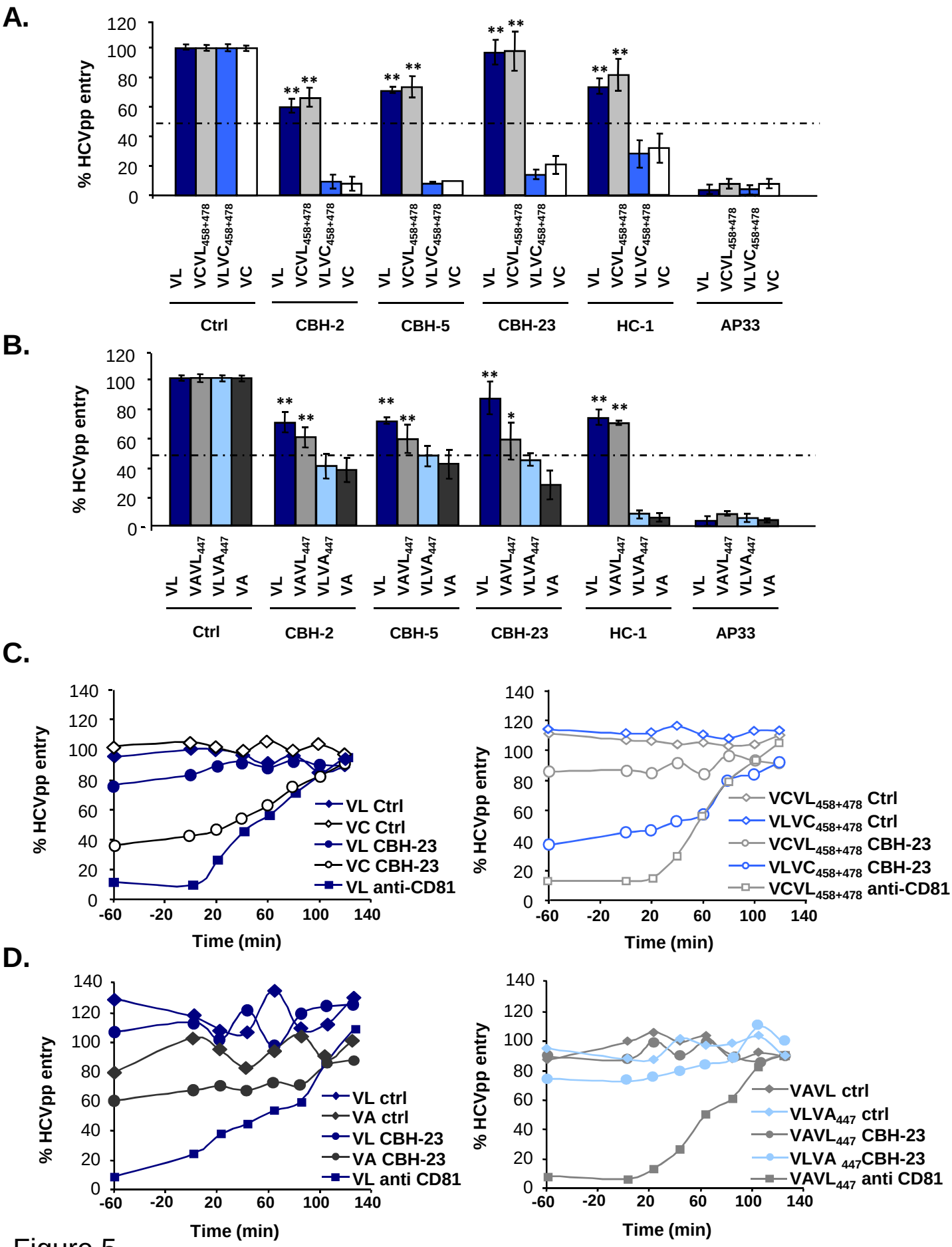

Neutralization titer (1/dilution)
10⁴
10³
10²
10
n.s.
n.s.
*
**
**
n.s.
VL
VL
VL VA393
VL VA397
VL VA404
VL VA447
VA
VA VL447
+
Ctrl
+
Autologous serum

Figure 4


A.
% HCVpp entry
120
100
80
60
40
20
0
**
VL
VCVL458+478
VLVC458+478
VC
Ctrl
CBH-2
CBH-5
CBH-23
HC-1
AP33
B.
% HCVpp entry
VAVL447
VLVA447
VA
*
C.
% HCVpp entry
Time (min)
VL Ctrl
VC Ctrl
VL CBH-23
VC CBH-23
VL anti-CD81
VCVL458+478 Ctrl
VLVC458+478 Ctrl
VCVL458+478 CBH-23
VLVC458+478 CBH-23
VCVL458+478 anti-CD81
D.
VL ctrl
VA ctrl
VL CBH-23
VA CBH-23
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VLVA447 ctrl
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VLVA 447CBH-23
VAVL447 anti CD81

Figure 5

Figure 6

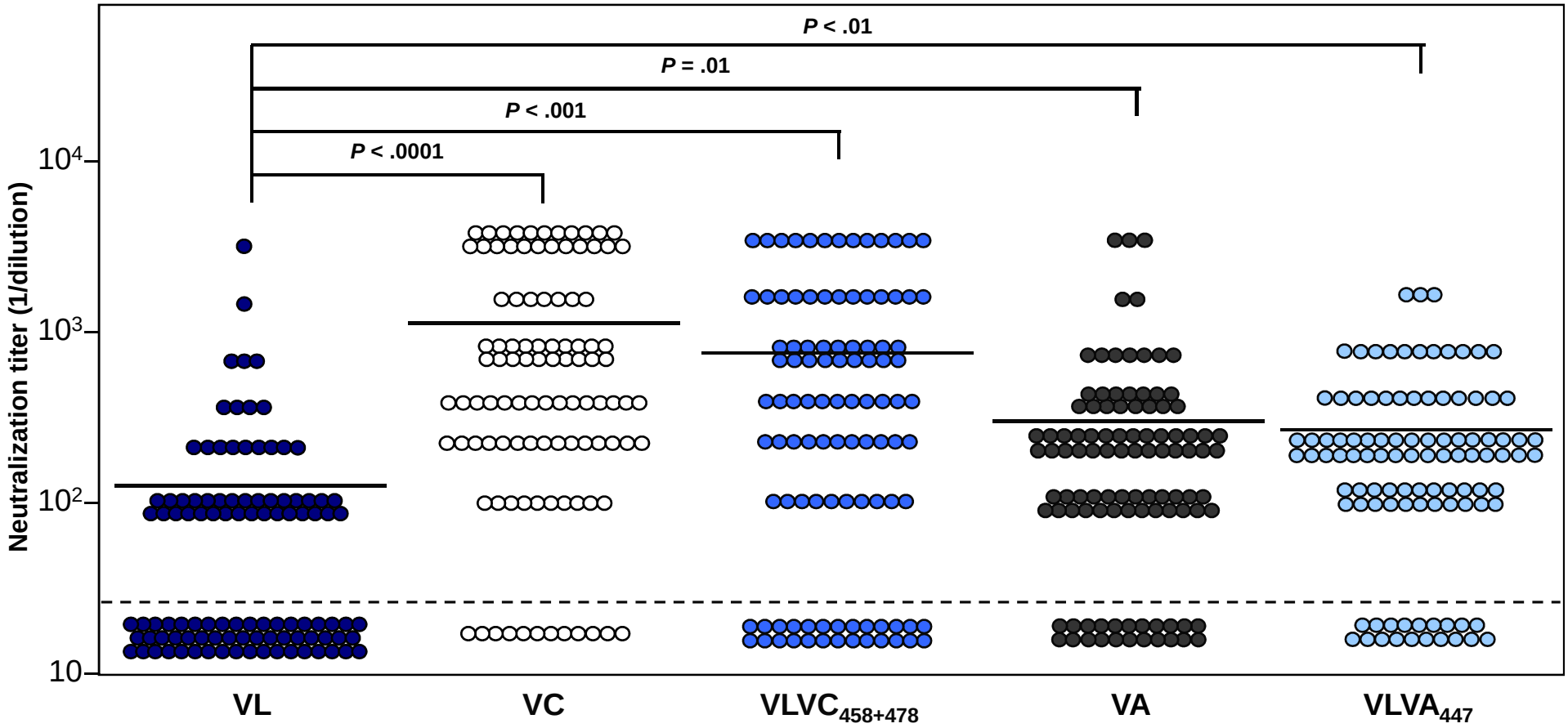

Figure 6

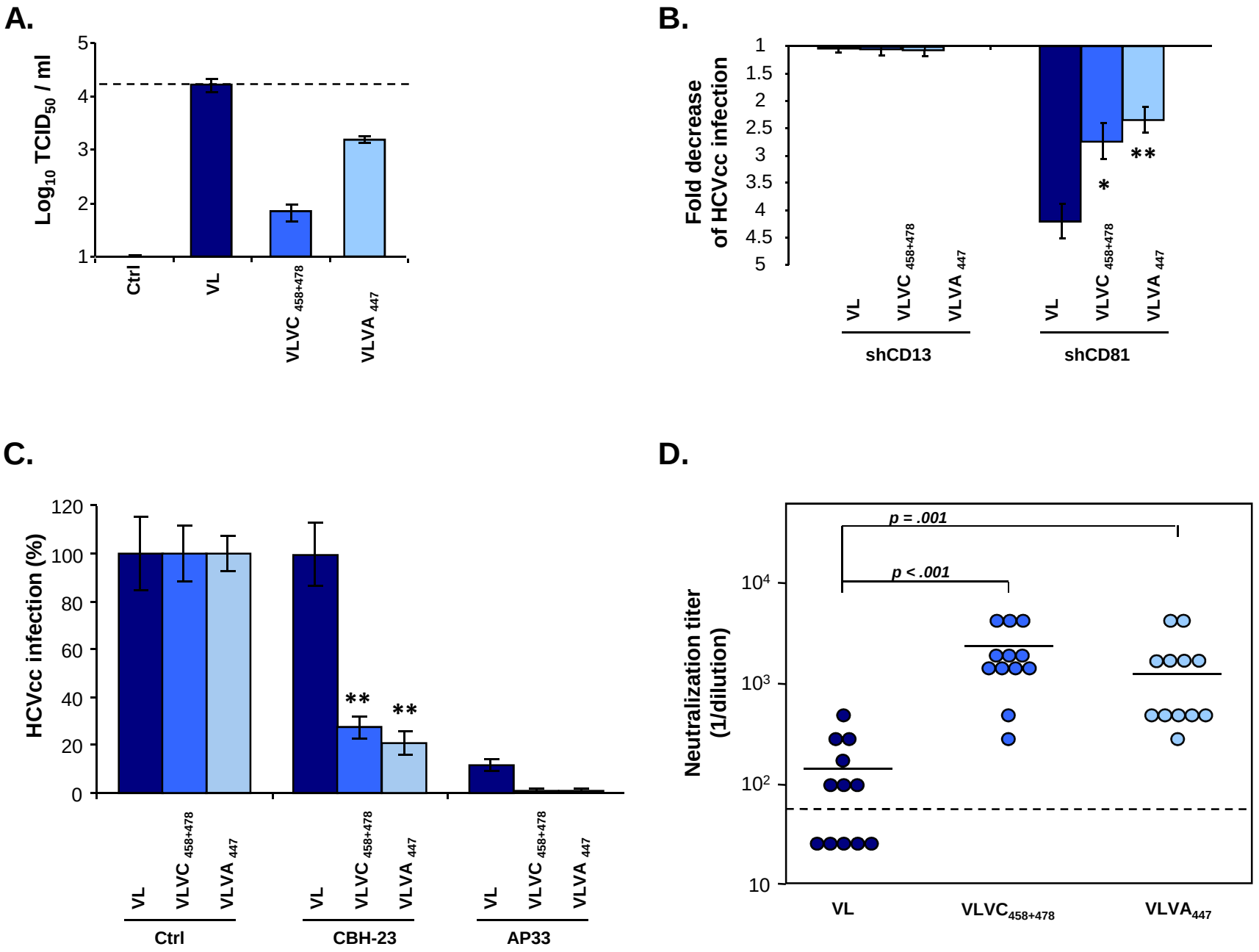
A.
Log10 TCID50 / ml
5
4
3
2
1
Ctrl
VL
VLVC 458+478
VLVA 447
B.
Fold decrease of HCVcc infection
1
1.5
2
2.5
3
3.5
4
4.5
5
*
**
VL
VLVC 458+478
VLVA 447
shCD13
shCD81
C.
HCVcc infection (%)
120
100
80
60
40
20
0
**
**
Ctrl
CBH-23
AP33
D.
Neutralization titer (1/dilution)
p = .001
p < .001
10⁴
10³
10²
10
VL
VLVC458+478
VLVA447

Figure 7

SUPPLEMENTARY DATA

SUPPLEMENTARY MATERIALS AND METHODS

Analysis of HCVpp envelope glycoprotein expression. Expression of HCV glycoproteins was characterized in HEK 293T producer cells and HCVpp purified through a 20% sucrose cushion ultracentrifugation as described.[1] Immunoblots of HCV glycoproteins were performed using anti-E1 11B7 and anti-E2 AP33 mAbs as described.[2]

Cellular binding of envelope glycoproteins. Envelope glycoprotein-expressing HEK 293T cells were lysed in PBS by four freezing and thawing cycles. Cell debris and nuclei were removed by low-speed centrifugation and supernatants containing native intracellular E1E2 complexes were used for binding studies. shCD81- or shCD13-Huh7.5 cells (2 x 10^5 cells per well) were seeded in 96 well plates. Following incubation with lysates containing patient-derived E1E2 proteins, Huh7.5.1 target cells were first incubated with mAb AP33 (10 µg/ml) and then with phycoerythrin-conjugated anti-mouse Ab (5 µg/ml, BD). Bound E2 was analyzed by flow cytometry as described.[3]

Construction of plasmids for production of chimeric HCVcc expressing patient-derived envelopes. Genotype 1 JFH-based HCVcc chimeras expressing the structural proteins of patient-derived viruses were produced as previously described for Con1/C3-JFH1-V2440L.[4, 5] Briefly, the cDNA region encoding for the HCV core to first transmembrane domain of NS2 (C3 junction site) from variant VL was inserted into pFK-Con1/C3-JFH1-V2440L using fusion polymerase chain reaction (PCR) with Pfu DNA polymerase (Stratagene) and standard cloning procedures using

appropriate restriction sites including BsmI and AvrII. The obtained construct was designated VL/JFH1. VL/JFH1 encoding sequence was used as template to insert individual and combined mutations using the QuikChange II XL site-directed as described previously. [1]

GNA Capture ELISA. Binding of HMAb CBH-23 to viral envelopes was analyzed using an ELISA with HCVpp as a capture antigen as described.[6] HCVpp expressing the E1E2 glycoproteins of HCV variants or control (Ctrl) pseudoparticles with absent HCV envelope glycoprotein expression were partially purified and enriched through ultracentrifugation as described.[1] Purified particles were quantified as described before.[1] Partially purified HCVpp or control pseudoparticles were captured onto GNA-coated microtiter plates as described.[6] Soluble E2 (sE2, derived from strain HCV-H77 and expressed in 293T cells as described previously[3]) was used as a positive control for antibody binding. Neutralizing human anti-E2 antibody CBH-23 (25 µg/ml diluted in PBS) was then added to captured HCVpp or sE2 (1 h at RT). Following washing and removal of nonbound antibody, mAb binding to HCV envelopes was detected using horseradish peroxidase anti-human IgG (GEhealthcare) at a concentration of 1/3000 for 1 h at RT, followed by incubation with 1-step™ Turbo TMB-ELISA (Thermo Scientific) for color development. Absorbance was measured at 450 nm using a microplate reader (Molecular Devices) and the Softmax program.

Bioinformatics. Multiple sequence alignment of complete E2 proteins was performed using the European HCV databases (http://euhcvdb.ibcp.fr).[7] Two amino-acid repertoires were computed with all E2 sequences of provisional/confirmed genotype 1b using the *ComputeRepertoire* tool as part of the euHCVdb *Extract* tool.

SUPPLEMENTARY RESULTS

Prevalence of the identified mutations in a large genomic database of viral isolates. Bioinformatic sequence analysis of a large panel of 2,074 HCV strains within the European HCV database further supports the potential relevance of the identified positions for pathogenesis of HCV infection in general.[7] Residues F, S and R are much more frequently observed at positions 447, 458 and 478 than L, G and C. F and S are the most predominant residues at positions 447 and 458 in the large majority of 1b strains, respectively (F447 all: 98.4%, 1b: 96.2%; S458 all: 94% for 1b: 90.3%; Fig. S5). The position 478 is variable but R (all: 2.4% for 1b: 10.8%) is more frequent than C (all: 0.2%, 1b: 0.9%) (Fig. S5). The high prevalence of identified residues supports their functional relevance for virus survival and selection as more structurally and functionally relevant residues will be more frequently observed. These data suggest that the epitope containing the identified residues at positions 447, 458 and 478 is not only responsible for viral evasion from autologous antiviral antibodies during LT but may also contribute to viral evasion in chronic HCV infection in general.

SUPPLEMENTARY TABLE LEGENDS AND TABLES

Table S1. Neutralization of patient-derived and chimeric HCVpp by monoclonal anti-envelope antibodies. HCVpp produced from isolates shown in Figure 1 were incubated with mAbs (10 µg/ml) for 1 h at 37°C. HCVpp-antibody complexes were then added to Huh7.5.1 cells. Viral epitopes targeted by the respective antibody, percentage of HCV entry in the presence of antibody (strains VL, VC, $VCVL_{458+478}$, $VLVC_{458+478}$, VA, $VAVL_{447}$ and $VLVA_{447}$) and source or reference of antibody are shown. Means±SD from at least three experiments each performed in triplicate are shown. Abbreviations: V - viral variant; aa - amino acid.

Antibody	Source or reference	Epitope (aa)	HCVpp entry (%)						
			VL	VC	$VCVL_{458+478}$	$VLVC_{458+478}$	VA	$VAVL_{447}$	$VLVA_{447}$
AP33	8	412-423	6 ± 3	12 ± 1	3 ± 1	11 ± 5	2 ± 1	5 ± 1	3 ± 1
IGH461	9	436-448	58 ± 4	56 ± 8	51 ± 7	53 ± 3	55 ± 2	56 ± 6	52 ± 7
16A6	9	523-530	76 ± 10	74 ± 8	83 ± 9	82 ± 2	73 ± 9	74 ± 4	81 ± 9
CBH-2	10	Domain B, conformational 431, 523-540	60 ± 5	8 ± 5	65 ± 6	9 ± 5	39 ±8	61 ± 4	39 ± 10
CBH-5	10	Domain B, conformational 523-540	71 ± 2	10 ± 4	73 ± 7	8 ± 1	36 ± 5	59 ± 7	47 ± 8
CBH-23	Keck and Foung, unpublished	Domain C, conformational	97 ± 9	21 ± 6	98 ± 13	14 ± 3	32 ± 7	53 ± 12	44 ± 3
HC-1	11	Domain B, conformational 523-540	73 ± 5	31 ± 9	81 ± 10	27 ± 9	2 ± 1	2 ± 1	77 ± 1

Table S2. Characteristics of patients and viruses used for neutralization studies. (A) HCVpp were incubated with anti-HCV positive sera from 102 patients with chronic HCV infection (ClinicalTrial.gov Identifier NCT00638144). Patient number, age, gender, viral genotype and load in serum are indicated. HCVpp-antibody complexes were added to Huh7.5.1 cells and infection was analyzed as described in Fig. 4. Calculation of neutralization and determination of background and thresholds for neutralization were performed as described in Fig. 6. Neutralization titers obtained by endpoint dilution are indicated for each variant. (B) Results were confirmed using chimeric HCVcc expressing the HCV envelope glycoproteins depicted in Fig. 7 and using 12 representative sera from patients. Neutralization assays were performed using a similar protocol as described in (A). Means from at least three independent experiments each performed in triplicate are shown. Abbreviations: V - viral variant ; M - male ; F - female.

A.

Patient number	Age	Gender	Genotype	Viral Load (IU/mL)	HCVpp neutralization titer (1/dilution)		
					VL	VC	VA
1	65	M	1b	2.29×10^5	100	100	100
2	27	F	1b	9.7×10^4	100	3200	200
3	31	F	1b	1.53×10^5	400	3200	400
4	47	M	3a	1.02×10^6	20	20	100
5	58	M	1b	1.15×10^6	100	3200	200
6	72	M	1b	1.50×10^6	20	200	100
7	51	M	4	4.38×10^6	20	20	20
8	69	F	1b	9.7×10^5	20	400	100
9	36	F	1	1.29×10^5	800	1600	100
10	46	M	1a	1.05×10^6	100	800	100
11	55	M	1a	1.54×10^6	400	3200	200
12	56	M	4c/4d	2.41×10^4	20	800	200
13	56	F	4a	1.09×10^6	100	400	400
14	59	F	1b	3.54×10^5	200	800	200
15	62	M	1a	3.37×10^6	20	20	20
16	50	M	4a	1.48×10^6	20	200	20

17	46	M	4a	4×10^5	20	200	100
18	70	F	1b	1.3×10^6	100	800	20
19	77	F	1b	6.2×10^4	20	100	100
20	61	F	1b	2.58×10^4	200	800	200
21	46	F	1b	2.11×10^5	100	400	800
22	36	M	1a	2.04×10^6	20	200	400
23	52	F	4a	9.12×10^5	20	3200	400
24	54	M	1a	9.77×10^5	100	800	200
25	54	M	1b	1.12×10^6	20	100	200
26	54	F	1a	3.38×10^6	20	400	20
27	47	M	3a	6.16×10^5	100	3200	3200
28	43	M	1a	5.75×10^6	20	800	200
29	51	M	4a	1.44×10^6	100	400	400
30	54	M	2c	4.67×10^5	100	100	3200
31	51	M	1a	6.16×10^6	100	400	100
32	39	M	4a	1.12×10^6	20	200	800
33	62	F	4f	2.88×10^6	20	800	20
34	46	M	4k	3.54×10^5	20	20	100
35	42	M	1a	9.54×10^5	400	800	400
36	54	M	2c	4.67×10^5	200	3200	100
37	34	M	3a	3.23×10^6	20	20	100
38	47	M	3a	7.94×10^4	20	400	20
39	30	F	1b	1.00×10^6	20	200	400
40	47	F	1b	2.29×10^6	100	400	200
41	52	M	1a	1.73×10^6	200	3200	400
42	34	M	1b	1.45×10^6	3200	3200	200
43	46	M	1a	4.34×10^6	200	800	400
44	66	F	1b	3.89×10^5	200	1600	200
45	29	F	1a	1.08×10^5	400	400	200
46	45	M	3a	2.78×10^5	20	200	200
47	65	F	4f	1.46×10^6	20	3200	20
48	55	M	1a	8.81×10^6	20	800	100
49	53	M	1a	1.15×10^6	100	100	100
50	40	M	3a	2.46×10^6	100	3200	200
51	48	F	1a	1.00×10^5	20	800	20
52	37	M	1a	5.08×10^6	20	400	200
53	47	M	3a	6.8×10^6	100	1600	400
54	37	M	1a	1.84×10^6	800	800	200
55	65	F	1b	2.18×10^6	100	100	800
56	45	F	1a	3.93×10^6	1600	1600	400
57	49	M	4a	2.06×10^6	800	3200	200

58	30	M	1b	7.21×10^5	100	800	200
59	31	M	3a	6.66×10^6	100	200	200
60	37	M	1a	6.70×10^6	20	100	100
61	49	M	1a	3.16×10^5	20	800	20
62	43	M	1	6.83×10^5	20	20	20
63	69	M	1b	4.7×10^5	20	20	200
64	48	M	1a	3.28×10^6	20	3200	100
65	46	M	3a	8.55×10^5	20	800	100
66	51	M	1b	1.07×10^6	20	200	1600
67	43	M	1b	4.27×10^5	20	100	800
68	36	M	3a	1.14×10^6	20	800	20
69	53	F	1b	3.06×10^5	20	400	20
70	24	F	3a	1.29×10^6	20	20	20
71	63	M	1b	3.01×10^6	100	200	100
72	44	M	1	1.10×10^5	20	3200	200
73	28	M	3a	1.85×10^6	20	3200	20
74	54	M	1b	1.29×10^5	20	3200	20
75	17	F	1b	2.41×10^5	20	20	200
76	40	M	3a	1.26×10^6	20	20	100
77	35	M	1b	8.89×10^5	20	20	800
78	36	F	6a	1.4×10^7	20	100	400
79	70	F	1b	1.13×10^5	100	100	400
80	62	M	1a	2.68×10^6	100	200	20
81	70	M	1b	2.85×10^5	20	200	3200
82	63	M	1b	1.95×10^5	200	400	400
83	33	M	1a	1.76×10^6	100	200	800
84	35	M	1a	2.78×10^6	20	20	200
85	60	F	1	6.39×10^5	20	200	100
86	57	M	3a	1.22×10^6	200	3200	400
87	60	M	1	3.6×10^6	100	3200	20
88	49	M	4	2.24×10^6	20	1600	20
89	37	M	4	9.35×10^5	100	800	100
90	55	M	1a	3.77×10^6	20	3200	100
91	47	M	1a	2.36×10^6	20	1600	20
92	72	M	3a	3.83×10^5	20	400	20
93	79	M	1b	2.81×10^5	100	1600	100
94	58	F	1b	6.58×10^5	100	3200	200
95	50	M	3a	6.07×10^5	20	3200	100
96	67	F	1b	4.13×10^5	100	800	20
97	49	M	3a	5.22×10^5	200	400	200
98	53	F	1b	2.31×10^6	20	400	1600

99	37	M	1a	1.87×10^5	100	3200	200
100	54	F	4a	9.23×10^5	20	200	100
101	39	M	1a	1.76×10^5	100	800	200
102	51	F	2b	1.10×10^6	100	3200	800

B.

Patient number	HCVcc neutralization titer (1/dilution)		
	VL	$VLVC_{458+478}$	$VLVA_{447}$
11	400	1600	800
28	20	1600	800
33	20	400	400
35	400	1600	1600
36	200	1600	3200
45	800	1600	800
65	20	1600	1600
66	20	3200	800
68	20	1600	1600
94	100	3200	800
98	100	800	3200
99	100	3200	1600

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Actual viral infectivity of HCVpp derived from variants VL, VC and VA shown as relative light units of luciferase reporter gene expression. (A-B) Comparative analysis of viral entry of HCVpp shown in Fig. 1. Results are expressed in relative light units (RLU) plotted in a logarithmic scale. The threshold for a detectable infection in this system is indicated by dashed lines. The detection limit for positive luciferase reporter protein expression was 3×10^3 RLU/assay, corresponding to the mean±3 SD of background levels, i.e., luciferase activity of naive noninfected cells or cells infected with pseudotypes without HCV envelopes.[1, 12, 13] Background levels of the assay were determined in each experiment. Means±SD from at least four independent experiments performed in triplicate are shown. Significant differences in HCVpp entry VC, VA and VL wildtype and mutant variants are indicated by stars (*, $P \leq .05$; **, $P < .001$). Abbreviations: Ctrl - control; HVR - hypervariable region; n.s. - not significant; V - viral variant.

Figure S2. Positions 447, 458 and 478 modulate binding of envelope glycoproteins to CD81 expressed at the cell surface. Binding of native E1E2 complexes expressed from patient-derived cDNAs to Huh7.5 cells with silenced CD81 expression (described in Fig. 3) was detected by flow cytometry. Results are expressed as percentage of E1E2 binding compared to shCD13-Huh7.5 control cells. Means±SD from three independent experiments performed in triplicate are shown. Significant differences in binding between variants are indicated by stars (**, $P < .001$).

Figure S3. Differences in viral entry are not due to impaired HCVpp production. (A) Analysis of envelope glycoprotein expression. Protein expression was analyzed by immunoblotting as described in Materials and Methods. Molecular markers (kDa) are indicated on the right. (B) Transfection efficiency during HCVpp production. Transfection effciency was analyzed for each variant and quantified by determining luciferase expression in HEK 293T producer cells expressed as normalized percentage compared to control transfected cells. (C) Envelope glycoprotein expression in HCVpp. HCVpp were purified as described previously[1, 2] and subjected to immunoblot as described in panel (A). (D) Lentiviral p24 antigen expression was analyzed by ELISA and is indicated as optical density (O.D.) values at 450 nm. Abbreviations: Da - Dalton; MW - molecular weight; n.s. - not significant.

Figure S4. Binding of neutralizing anti-E2 HMAb CBH-23 to patient derived-envelope glycoproteins expressed on HCVpp as capture antigens in an ELISA. HCVpp expressing envelope glycoproteins of variants VL, VA, VC, $VLVA_{447}$ and $VLVC_{458+478}$ were used as capture antigens on GNA-coated ELISA plates. Control (Ctrl) pseudoparticles with absent HCV envelope glycoprotein expression and recombinant soluble E2 (sE2 derived from strain H77)[14] served as negative and positive controls, respectively. Anti-E2 CBH-23 reactivity was detected as described in supplementary Materials and Methods and is indicated as optical density (O.D.) values at 450 nm. Means±SD from one representative experiment are shown.

Figure S5. Distribution of residues at positions 447, 458 and 478 of HCV E2 sequences in the European HCV databases. Distribution of residues at positions 447, 458 and 478 for HCV complete E2 sequences from all subtypes (black) and from subtype 1b only (white) within the European Hepatitis C Virus databases [7],

(http://euhcvdb.ibcp.fr). F and S are the predominant residue at positions 447 and 458 (F447: 98.4%, 1b: 96.2%; S458 all: 94%, 1b: 90.3%). The position 478 is variable (it belongs to HVR2) but R (all: 2.4% for, 1b: 10.8%) is more frequent than C (all: 0.2%, 1b: 0.9%).

Supplementary Figure S1
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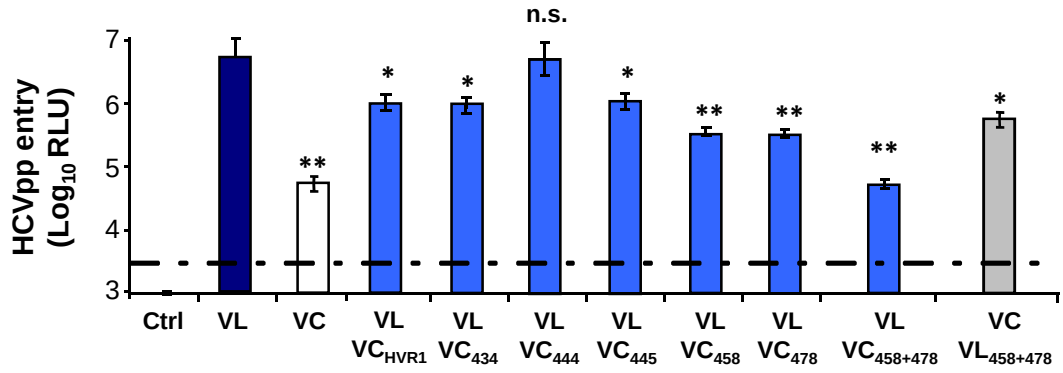
A.



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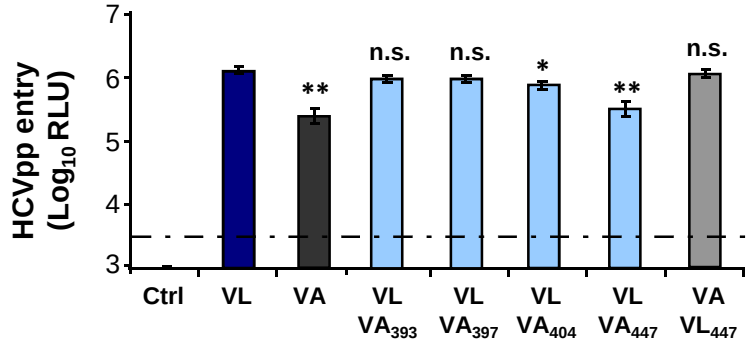

Figure S1

Supplementary Figure S2
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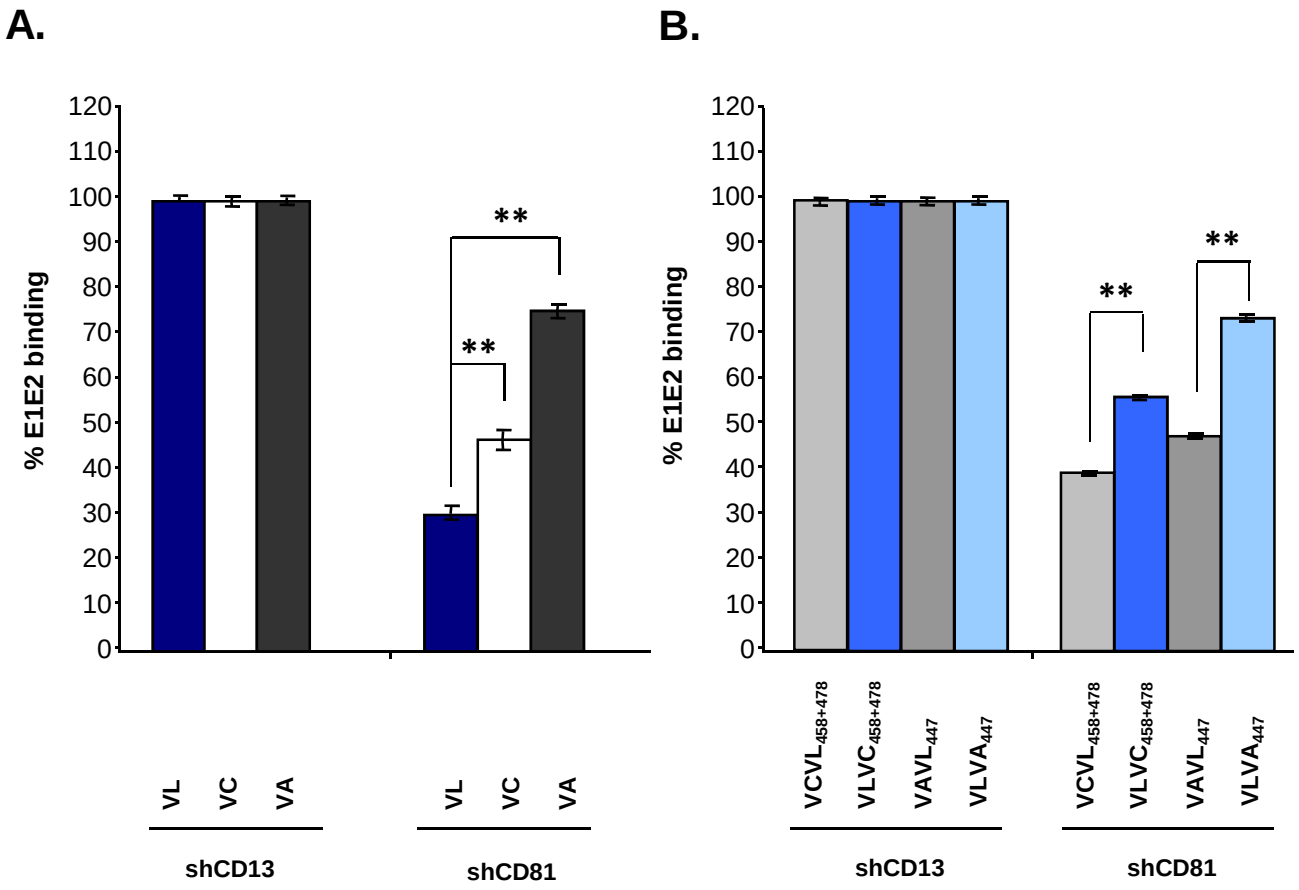

Figure S2

Supplementary Figure S3

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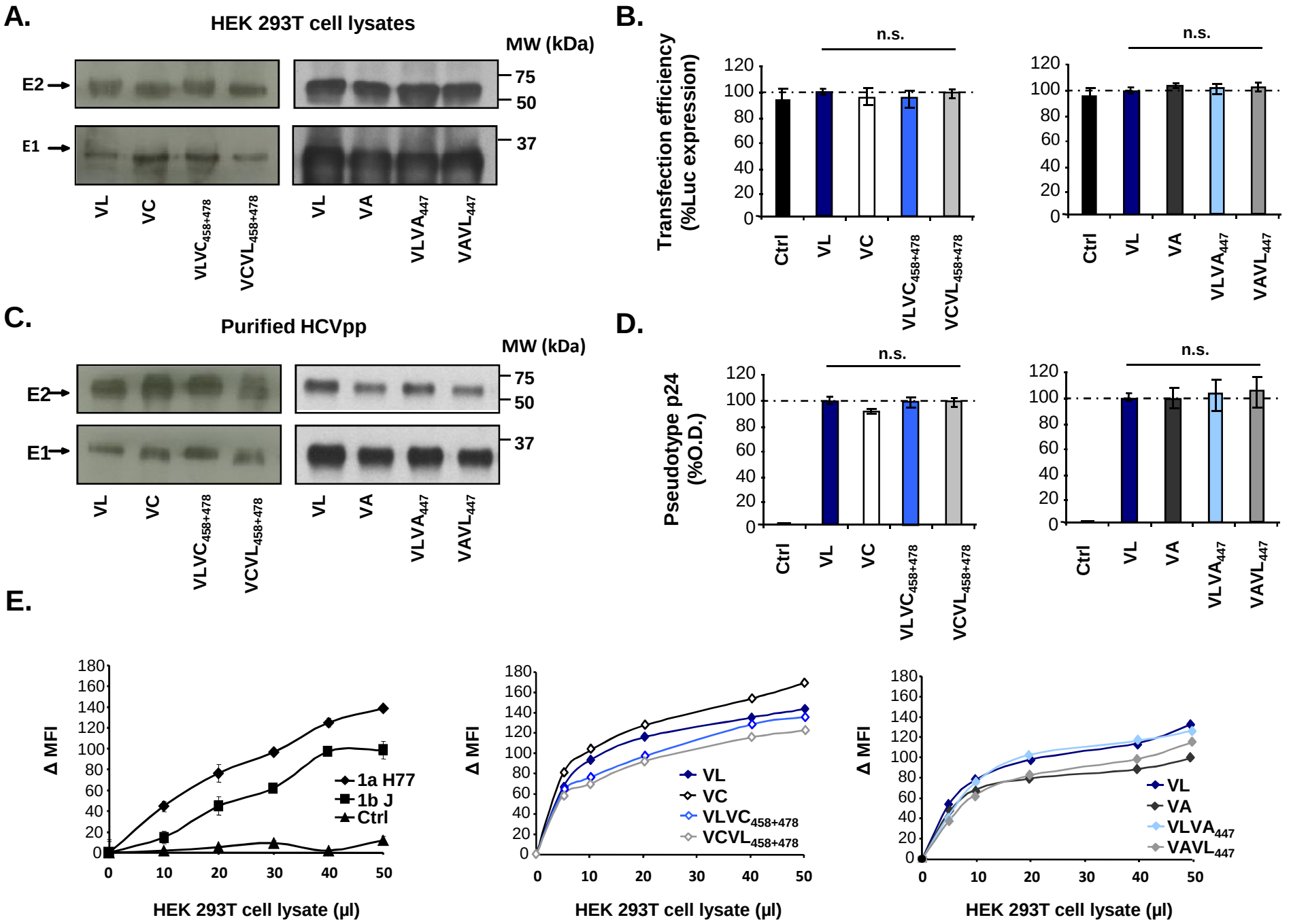

Figure S3

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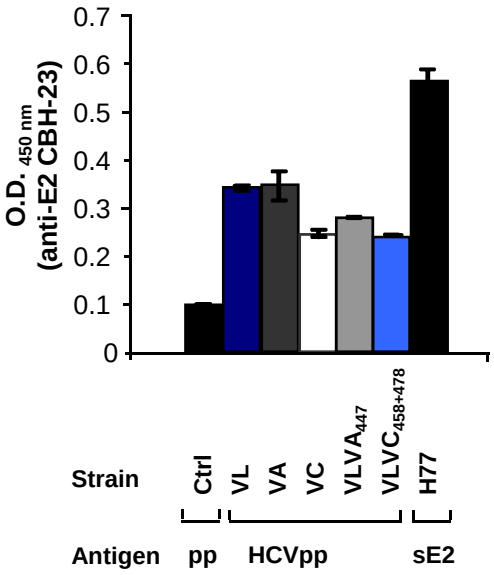

Figure S4

Supplementary Figure S5
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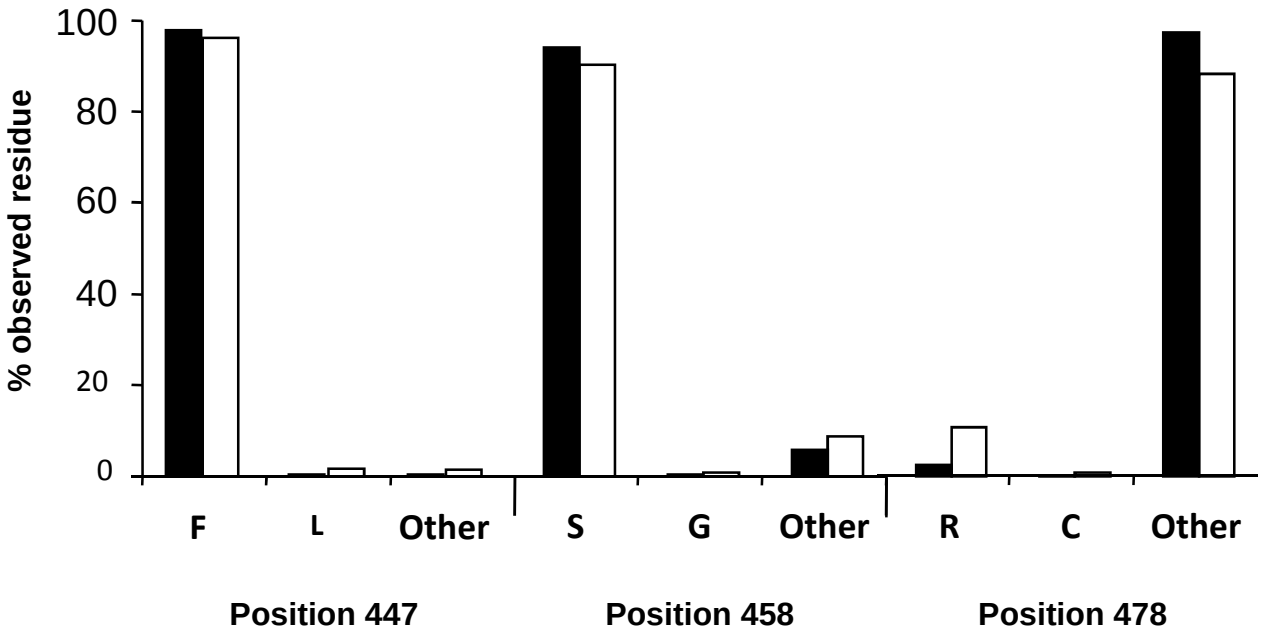

Figure S5

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