

## **Monoclonal anti-claudin 1 antibodies prevent hepatitis C virus infection of primary human hepatocytes.**

Isabel Fofana, Sophie Krieger, Fritz Grunert, Sandra Glauben, Fei Xiao, Samira Fafi-Kremer, Eric Soulier, Cathy Royer, Christine Thumann, Christopher Mee, et al.

► **To cite this version:**

Isabel Fofana, Sophie Krieger, Fritz Grunert, Sandra Glauben, Fei Xiao, et al.. Monoclonal anti-claudin 1 antibodies prevent hepatitis C virus infection of primary human hepatocytes.. Gastroenterology, WB Saunders, 2010, 139 (3), pp.953-64, 964.e1-4. <10.1053/j.gastro.2010.05.073>. <inserm-00741442>

**HAL Id: inserm-00741442**

**<http://www.hal.inserm.fr/inserm-00741442>**

Submitted on 12 Oct 2012

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Monoclonal anti-claudin 1 antibodies prevent hepatitis C virus infection of primary human hepatocytes

Isabel Fofana<sup>1,2</sup>, Sophie E. Krieger<sup>1,2</sup>, Fritz Grunert<sup>3</sup>, Sandra Glauben<sup>3</sup>, Fei Xiao<sup>1,2</sup>,  
5 Samira Fafi-Kremer<sup>1,2</sup>, Eric Soulier<sup>1,2</sup>, Cathy Royer<sup>1,2</sup>, Christine Thumann<sup>1,2</sup>,  
Christopher J. Mee<sup>4</sup>, Jane A. McKeating<sup>4</sup>, Tatjana Dragic<sup>5</sup>, Patrick Pessaux<sup>6</sup>,  
Francoise Stoll-Keller<sup>1,2</sup>, Catherine Schuster<sup>1,2</sup>, John Thompson<sup>3</sup>  
and Thomas F. Baumert<sup>1,2,7</sup>

10

<sup>1</sup>Inserm, U748, 67000 Strasbourg, France, <sup>2</sup>University of Strasbourg, 67000  
Strasbourg, France, <sup>3</sup>Genovac GmbH, 79111 Freiburg, Germany, <sup>4</sup>Hepatitis C  
Research Group, Institute for Biomedical Research, University of Birmingham,  
Birmingham, B15 2TT, United Kingdom, <sup>5</sup>Department of Microbiology and  
15 Immunology, Albert Einstein College of Medicine, Bronx, 10461 New York, USA,  
<sup>6</sup>Pôle des Pathologies Digestives, Hépatiques et Transplantation and <sup>7</sup>Pôle Hépatodigestif,  
Hôpitaux Universitaires de Strasbourg, 67000 Strasbourg, France

Word count: title – 89 characters, abstract – 205 words, text – 5,481 words

20

## Correspondence

Address requests for reprints to: Thomas F. Baumert, M. D., Inserm Unit 748,  
Université de Strasbourg, 3 rue Koeberlé, F-67000 Strasbourg, France; Phone:  
(++33) 3 68 85 37 03, Fax: (++)33) 3 68 85 37 24, e-mail:

25 [Thomas.Baumert@unistra.fr](mailto:Thomas.Baumert@unistra.fr)

**Abbreviations:** CLDN - claudin; CMFDA - 5-chloromethylfluorescein di-acetate; CTRL - control; EL1 - extracellular loop 1; HBV - hepatitis B virus; HCV - hepatitis C virus; HCVcc - cell culture-derived HCV; HCVpp - HCV pseudoparticles; mAb - monoclonal antibody; IgG - immune globulin G; MFI - mean fluorescence intensity; 5 MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P - patient; PHH - primary human hepatocytes; RLU - relative light units; TJ - tight junction.

### **Author contribution**

I. F. and S. E. K. contributed equally to this work, F. G., J. T. and T. F. B. designed 10 research. I. F., S. E. K., E. S., F. X., C. J. M., C. T., S. F.-K., F. G., C. S., S. G. and T. F. B. performed research. I. F., S. E. K., E. S., C. J. M., J. A. McK., S. F.-K. C. S., F. X., J. T., F. G., and T. F. B. analyzed data. S. F.-K., F. S.-K., P. P., T. D. and C. R. provided reagents. T. F. B. wrote the paper. The authors declare no conflict of interest.

15

### **Grant support**

This work was supported by Inserm, University of Strasbourg, the chair of excellence program of the Agence Nationale de la Recherche France (ANR-05-CEXC-008), GENOVAC, the Bundesministerium für Wirtschaft und Technologie (BMW: Pro-INNO 20 II). I. F. and S. E. K. were supported by the French Ministry for Research and Education within program ANR-05-CEXC-008.

25

## ABSTRACT

**BACKGROUND & AIMS:** Hepatitis C virus (HCV) infection is a challenge to prevent and treat because of the rapid development of drug resistance and escape. Viral entry is required for initiation, spread, and maintenance of infection, making it an attractive target for antiviral strategies. The tight junction protein claudin-1 (CLDN1) has been shown to be required for entry of HCV into the cell.

**METHODS:** Using genetic immunization, we produced six monoclonal antibodies against the host entry factor CLDN1. The effects of antibodies on HCV infection were analyzed in human cell lines and primary human hepatocytes.

**RESULTS:** Competition and binding studies demonstrated that antibodies interacted with conformational epitopes of the first extracellular loop of CLDN1; binding of these antibodies required the motif W(30)-GLW(51)-C(54)-C(64) and residues in the N-terminal third of CLDN1. The monoclonal antibodies against CLDN1 efficiently inhibited infection by HCV of all major genotypes as well as highly variable HCV quasispecies isolated from individual patients. Furthermore, antibodies efficiently blocked cell entry of highly infectious escape variants of HCV that were resistant to neutralizing antibodies.

**CONCLUSION:** Monoclonal antibodies against the HCV entry factor CLDN1 might be used to prevent HCV infection, such as after liver transplantation, and might also restrain virus spread in chronically infected patients.

Keywords: antiviral, genetic barrier, host factor, receptor, treatment

## INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver cirrhosis and hepatocellular carcinoma world-wide.<sup>1</sup> Current antiviral treatment consisting of pegylated interferon-alpha (IFN- $\alpha$ ) and ribavirin is limited by resistance, adverse effects and high costs.<sup>2</sup> Although the clinical development of novel antivirals targeting HCV protein processing has been shown to improve sustained virological response, toxicity of the individual compounds and development of viral resistance remain major challenges.<sup>3</sup>

The absence of strategies for prevention of HCV infection is a major problem for patients undergoing liver transplantation for HCV-related end-stage liver disease.<sup>1</sup> Due to viral evasion from host immune responses and immunosuppressive therapy, re-infection of the graft is universal and characterized by accelerated progression of liver disease.<sup>1, 4</sup> Recurrent HCV liver disease in the graft with poor outcome has become an increasing problem facing hepatologists and transplant surgeons. Thus, novel antiviral preventive and therapeutic strategies are urgently needed.<sup>5</sup>

HCV entry into target cells is a promising target for antiviral preventive and therapeutic strategies<sup>6, 7</sup> since it is essential for initiation, spread and maintenance of infection.<sup>6, 7</sup> Furthermore, cross-neutralizing antibodies inhibiting HCV entry have been shown to be associated with control of HCV infection and prevention of HCV re-infection in cohorts with self-limited acute infection.<sup>8, 9</sup>

HCV entry is a multistep process involving several host factors including heparan sulfate,<sup>10</sup> CD81,<sup>11</sup> scavenger receptor B1,<sup>12</sup> claudin-1 (CLDN1)<sup>13</sup> and occludin.<sup>14</sup> Among the host cell entry factors, tight junction (TJ) protein CLDN1 is a promising antiviral target since it is essential for HCV entry and to date there is no evidence for CLDN1-independent HCV entry.<sup>13, 15</sup> Furthermore, CLDN1 has been suggested to play an important role in cell-cell transmission.<sup>16</sup> In contrast to other

HCV entry factors such as CD81 or scavenger receptor BI, CLDN1 is predominantly expressed in the liver.<sup>17</sup> CLDN1 is also expressed in the kidney.<sup>17</sup>

To date, the exploration of CLDN1 as an antiviral target has been hampered by the lack of antibodies targeting surface expressed epitopes.<sup>13</sup> In this study, we demonstrate for the first time successful production of anti-CLDN1 monoclonal antibodies (mAbs) which inhibit HCV infection. These results suggest that targeting CLDN1 with specific mAbs may constitute a novel antiviral approach to prevent primary HCV infection, such as after liver transplantation and might also restrain virus spread in chronically infected patients.

10

## MATERIALS AND METHODS

**Primary hepatocytes and cell lines.** Culture of primary human hepatocytes (PHH),<sup>18</sup> Huh7.5.1,<sup>19</sup> HEK293T,<sup>9</sup> HepG2,<sup>20</sup> BOSC23 and CHO<sup>18</sup> cells have been described.

5

**Production and screening of anti-CLDN1 mAbs.** Anti-CLDN1 mAbs were raised by genetic immunization of Wistar rats using an eukaryotic expression vector encoding the full-length human CLDN1 cDNA according to proprietary GENOVAC technology. Following completion of immunization, antibodies were selected by flow  
10 cytometry for their ability to bind to human CLDN1 expressed on the cell surface of non-permeabilized HEK293T-BOSC23 cells and CHO cells which had been transfected with pCMV-SPORT6/CLDN1. For imaging studies Huh7.5.1 cells were stained with rat isotype control or anti-CLDN1 mAb OM-4A4-D4 (10 µg/ml) and analyzed as described.<sup>18</sup>

15

**Epitope mapping.** Competition between anti-CLDN1 mAbs for cellular binding was measured by a cell-based ELISA and labeled antibodies: Huh7.5.1 cells were incubated for 60 min with 0.1 µg/ml biotinylated anti-CLDN1 mAb (Sulfo-NHS-LC-Biotin; Thermo Scientific) together with increasing concentrations of unlabeled anti-  
20 CLDN1 mAbs as competitors. Following washing with PBS, binding of biotinylated antibody was detected by incubation with streptavidin labeled with horseradish peroxidase. Curves determined by measurement of binding in the presence of an isotype-matched control were compared to those determined in the presence of the competing antibody. Epitope mapping was performed using plasmids encoding for  
25 CLDN1 containing defined mutations and a cytoplasmic N-terminal hemagglutinin-tag

(HA-tag).<sup>21</sup> To study binding of anti-CLDN1 mAbs to mutant CLDN1, binding of mAb OM-7D3-B3 to BOSC23 transfected with CLDN1 expression constructs was determined by flow cytometry. Flow cytometric quantitation of HA-tag expression using an anti-HA antibody (Covance) served as internal control. The transfected cells were either permeabilized with Cytoperm/Cytofix (BD) for analysis of cytoplasmic HA-tag expression or untreated for analysis of anti-CLDN1-mutant CLDN1 interactions. For FACS analysis, transfected cells were incubated with 20 µg/ml anti-CLDN1 mAb or anti-HA for 30 min followed by incubation with 10 µg/ml anti-rat IgG mAb (for anti-CLDN1) or 10 µg/ml anti-mouse IgG mAb labeled with phycoerythrin (for anti-HA). The half-saturating concentrations (apparent  $K_d$ ) were determined as described.<sup>22</sup>

**HCVcc and HCVpp production and infection.** HCVcc (Luc-Jc1, Luc-Con1),<sup>23</sup> HCVpp (strains H77, HCV-J, JFH1, UKN3A1.28, UKN4.21.16, UKN5.14.4, UKN6.5.340, P01VL, P02VH, P02VI, P02VJ, P03VC, P04VC, P04VD, P04VE, P05VD, P05VE, P05VF, P06VG, P06VH, P06VI)<sup>24</sup> and VSVpp were produced as described.<sup>24</sup> Patient-derived HCVpp were produced from 6 patients (P01-P06) undergoing liver transplantation using full-length E1E2 expression constructs generated from circulating HCV as described.<sup>9, 24</sup> Huh7.5.1, Huh7 cells or PHH were pre-incubated with antibodies for 1 h and incubated for 4 h at 37°C with HCVcc or HCVpp. Viral infection was analyzed as described.<sup>18, 23</sup> For antibody-mediated neutralization, HCVpp were pre-incubated with autologous anti-HCV serum,<sup>24</sup> anti-E2 mAb (IGH461; Innogenetics)<sup>25</sup> and anti-HCV IgG purified from a chronically infected patient as described.<sup>25, 26</sup>



**Toxicity assays.** Cytotoxic effects on cells were assessed by analyzing the ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously.<sup>27</sup> Huh7.5.1 cells and PHH from three different donors were pre-incubated with isotype control, anti-CLDN1 OM-7D3-B3 mAbs, anti-Fas (BD),  
5 flavopiridol (Sigma) or compound C (Sigma). TJ integrity was analyzed as described.<sup>20</sup>

**Statistical analysis.** Results are expressed as means  $\pm$  standard deviation (SD). Statistical analyses were performed using Student's *t* test with a *P* value of  $<0.05$   
10 being considered statistically significant.

## RESULTS

**Production of anti-CLDN1 mAbs with high affinity to CLDN1 expressed on human hepatocytes.** To explore CLDN1 as a target for antiviral strategies, we generated anti-CLDN1 mAbs by genetic immunization using a full-length CLDN1 cDNA expression vector. Genetic immunization generated six mAbs that reacted with native human CLDN1 expressed on BOSC23 and CHO cells, showing negligible staining of parental cells (Fig. 1A, B). Analyses of the antibody isotypes revealed IgG2a and IgG2b with a kappa light chain. All of the mAbs bound to the surface of Huh7.5.1 cells and PHH (Fig. 1C, D).

Next, we characterized the binding properties of anti-CLDN1 mAbs to PHH (Fig. 1E). The half-saturating concentrations for binding (corresponding to the apparent  $K_d$ ) were in the low nanomolar range demonstrating that anti-CLDN1 mAbs produced by genetic immunization bind with high affinity to CLDN1 expressed on human hepatocytes ( $K_d$  OM-7C8-A8, 7 nM; OM-8A9-A3, 2 nM; OM-6D9-A6, 8 nM; OM-4A4-D4, 9 nM; OM-6E1-B5, 3 nM; OM-7D3-B3, 4 nM).

Since anti-CLDN1 mAbs failed to stain primary mouse hepatocytes but bound to primary hepatocytes of *Macaca fascicularis* (data not shown), it is likely that the epitopes targeted by the antibodies are conserved among primates but different in rodents. Negligible staining of CLDN1 deficient cells transfected to express human CLDN2, 3, 4, 6, 7, 9, 11, 12, 15, 17 and 19 suggests specific interaction of the mAbs with CLDN1 but not with other members of the CLDN family (Fig. 2A and Fig. S1).

**Anti-CLDN1 mAbs bind to conformational epitopes in CLDN1 extracellular loop 1 (EL1) comprising motif W(30)-GLW(51)-C(54)-C(64).** To investigate whether the six different anti-CLDN1 mAbs recognize similar or unrelated epitopes, we performed

cross-competition experiments with the mAbs. Labeled anti-CLDN1 mAb OM-7D3-B3 was incubated with Huh7.5.1 cells in the presence of increasing concentrations of the five remaining unlabeled anti-CLDN1 mAbs. All of the unlabeled mAbs reduced the binding of OM-7D3-B3. In contrast, the isotype control mAb had no effect (Fig. 2B).

5 Similar results were obtained for cross-competition experiments with labeled versions of the other mAbs (Fig. 2C). The mutual cross-competition between all six mAbs suggests that they recognize similar epitopes on CLDN1.

To further define the motif(s) targeted by the mAbs, we studied the interaction of mAb OM-7D3-B3 with a panel of CLDN1 mutants.<sup>21</sup> Using an alanine scanning  
10 mutagenesis approach, Cukierman and colleagues identified residues in the highly conserved claudin motif W(30)-GLW(51)-C(54)-C(64) within the first extracellular loop that are critical for HCV entry.<sup>21</sup> To investigate whether this motif is recognized by anti-CLDN1 mAbs, CLDN1-deficient BOSC23 cells were transduced to express CLDN1 mutants and binding of anti-CLDN1 OM-7D3-B3 was analyzed by flow  
15 cytometry. CLDN1 mutants contained alanine substitutions of critical residues in the highly conserved claudin motif (Fig. 2D, left panel) as well as single alanine substitutions at positions occupied by bulky, charged or polar residues in the CLDN1 N-terminal region (Fig. 2D, right panel). To monitor correct protein expression, mutant CLDN1 proteins contained an HA-tag (Fig. 2D). Mutation of amino acid  
20 residues at positions 30, 33, 35, 49, 50, 51, 54 and 64 of CLDN1 reduced OM-7D3-B3 binding to CLDN1 mutants, whereas the other mutations had a minimal effect (Fig. 2D). These results suggest that the antibody OM-7D3-B3 recognizes conformation-dependent epitopes in CLDN1 EL1 which are dependent on the W(30)-GLW(51)-C(54)-C(64) motif as well as residues in the N-terminal third of EL1.

25

**Cross-inhibition of infection of HCV isolates from all major genotypes.** To investigate whether the antibodies produced by genetic immunization inhibit HCV infection, Huh7.5.1 cells were infected with chimeric luciferase reporter viruses Luc-Con1 (HCV structural proteins of genotype 1b) and Luc-Jc1 (genotype 2a).<sup>23</sup> Fig. 3A shows that anti-CLDN1 mAbs inhibit Luc-Jc1 infection of Huh7.5.1 cells in a dose-dependent manner ( $IC_{50}$  1-6  $\mu$ g/ml). In contrast, an isotype control mAb had no effect. Comparable results were obtained for Luc-Con1 (Fig. 3B), suggesting that inhibition of HCV infection was not dependent on the viral genotype. To investigate whether anti-CLDN1 mAbs were effective against different HCV envelope glycoproteins, we analyzed their inhibition of HCVpp bearing envelope glycoproteins from HCV genotypes 1-6. All of the anti-CLDN1 mAbs inhibited the infectivity of HCVpp from genotypes 1-6 in a dose dependent manner ( $IC_{50}$  0.1-5  $\mu$ g/ml; Fig. 3C). A similar inhibition of HCVpp infection was observed in PHH (Fig. S2). Inhibition of HCV infection was confirmed using whole HCV derived from patient serum (Fig. S3). Taken together, these data demonstrate that anti-CLDN1 mAbs efficiently inhibit HCV infection of all major genotypes.

**Inhibition of infection of HCV variants escaping autologous neutralizing antibodies and re-infecting the liver graft.** End-stage liver disease due to chronic HCV infection is a leading cause for liver transplantation. Using PHH and HCVpp bearing viral envelope glycoproteins derived from HCV-infected patients undergoing liver transplantation, we previously demonstrated that enhanced viral entry and escape from autologous antibody-mediated neutralization are key determinants for the selection of viral variants during HCV re-infection of the liver graft.<sup>24</sup>

To assess whether anti-CLDN1 mAbs inhibit infection by HCV escape variants, we investigated the effect of anti-CLDN1 mAbs on entry of HCVpp bearing envelope glycoproteins from highly infectious HCV strains selected during transplantation and re-infecting the liver graft.<sup>24</sup> As shown in Fig. 4, pre-incubation of PHH with anti-CLDN1 mAbs inhibited entry of patient-derived HCVpp in PHH (Fig. 4C), which were only poorly neutralized by antibodies present in autologous pre-transplant serum (Fig. 4B). Similar results were obtained using PHH from different donors (data not shown). These data demonstrate that anti-CLDN1 mAbs specifically inhibit entry of HCV escape variants that are resistant to autologous host responses and re-infect the liver graft.

Recent studies have shown that cross-neutralizing anti-E2 antibodies or purified heterologous anti-HCV IgG obtained from HCV-infected patients are capable of neutralizing genetically diverse HCV isolates and could protect against HCV quasispecies challenge.<sup>28, 29</sup> To investigate whether the combination of anti-envelope and anti-receptor antibodies results in an additive effect on the inhibition of HCV infection, we pre-incubated patient-derived HCVpp with anti-E2 mAb IGH461 (Fig. 5A) or purified heterologous anti-HCV IgG (Fig. 5B) and studied their ability to inhibit HCVpp infection in cells pre-incubated with anti-CLDN1 mAbs. Combination of antiviral and anti-receptor antibodies resulted in a marked additive effect, decreasing the IC<sub>50</sub> of anti-CLDN1 up to 100 fold (Fig. 5).

**Anti-CLDN1 mAbs inhibit entry of highly variable HCV quasispecies from patients with chronic hepatitis C.** A major challenge for the development of antiviral strategies is the high variability of the virus. HCV has a high replication rate and the highly error prone viral polymerase allows for rapid production of minor viral

variants called “quasispecies” that may outpace humoral and cellular immune responses.<sup>26, 30</sup> These variants are under constant immune pressure in the infected host, and selection processes lead to domination of the viral quasispecies by the fittest virus that can evade immune recognition or confer resistance to antiviral therapies or antiviral antibodies. To investigate whether anti-CLDN1 mAbs can inhibit all variants within a quasispecies population within chronically infected patients, we cloned, sequenced and expressed the envelope glycoproteins of an individual patient chronically infected with HCV (Fig. 6). Anti-CLDN1 mAbs broadly inhibited HCV infection of HCVpp bearing highly variable envelope glycoproteins from patient-derived quasispecies (Fig. 6). A similar inhibition of infection of HCV quasispecies was obtained for a second patient chronically infected with HCV (data not shown).

**Absent toxic effects of anti-CLDN1 mAbs in PHH.** To address potential toxic effects of anti-CLDN1 mAbs we performed cell viability analysis in PHH and Huh7.5.1 cells based on MTT testing. Following incubation of hepatoma cells and PHH with anti-CLDN1 no toxic effects were observed (Fig. 7A-D). In contrast, pre-incubation of cells with flavopiridol, compound C and anti-Fas antibody resulted in easily detectable toxicity (Fig. 7A-D). CLDN1 is an important structural component of TJ.<sup>17</sup> To investigate whether anti-CLDN1 mAbs alter TJ function, we studied their effect on TJ integrity and permeability in a well characterized TJ functional assay based on polarized hepatoma cells.<sup>20</sup> As shown in Fig. 7E, the capacity of bile canaliculi lumens to retain 5-chloromethylfluorescein di-acetate (CMFDA) was similar in polarized HepG2 cells treated with anti-CLDN1 mAbs OM-7D3-B3, OM-8A9-A3, control mAb, or PBS, whereas CMTDA retention was reduced in PMA, TNF $\alpha$  and

interferon- $\gamma$ -treated HepG2 cells (Fig. 7E). These data indicate that anti-CLDN1 mAbs do not affect TJ integrity.

## DISCUSSION

Here we demonstrate, for the first time, the successful production of anti-CLDN1 mAbs which broadly inhibit HCV infection including patient-derived escape variants that are resistant to autologous host neutralizing responses.

5           Due to the absence of preventive strategies for re-infection and limited efficacy and tolerability of interferon-based antiviral therapies in liver transplant recipients, there is a major medical need for the development of novel approaches preventing HCV infection of the liver graft.<sup>4, 31</sup> In contrast to chronic hepatitis B, where antiviral antibodies efficiently prevent re-infection of the liver graft in combination with  
10 nucleoside analogues, the development of prophylactic strategies for HCV re-infection remains a challenge.<sup>31</sup> A key limitation for the development of preventive strategies is the high variability of the virus resulting in rapid and constant escape from neutralizing anti-envelope antibodies.<sup>26, 32</sup> In a detailed longitudinal analysis of six patients undergoing liver transplantation and HCV re-infection, we have  
15 previously shown that HCV evades host neutralizing responses due to rapidly emerging adaptive mutations in the HCV envelope glycoproteins.<sup>24</sup> The liver graft is infected by highly infectious HCV escape variants that are resistant to autologous host responses.<sup>24</sup> Escape from neutralizing antibodies has been also described as an important mechanism for viral persistence in chronic HCV infection.<sup>26</sup> Thus, blocking  
20 essential cellular entry factors may be a promising alternative antiviral strategy by increasing the genetic barrier for viral resistance.

In this study we demonstrate for the first time that anti-CLDN1 mAbs efficiently inhibit infection of PHH with HCV isolates having escaped host neutralizing antibodies during liver transplantation. The broad neutralization of a large panel of  
25 HCV escape variants from six patients undergoing liver transplantation (Fig. 4)



suggests that cross-inhibiting anti-CLDN1 mAbs, with or without concomitant antiviral therapy, may offer a promising strategy to prevent HCV re-infection of the liver graft. As anti-CLDN1 mAbs inhibit viral infection already by more than 99% (Fig. 3), the combination of anti-CLDN1 mAbs with direct antiviral agents such as anti-envelope antibodies (Fig. 5) or viral protease inhibitors<sup>3</sup> most likely will completely block HCV infection.

Interestingly, the doses of anti-CLDN1 mAbs required for efficient inhibition (>90%) of escape variants or quasispecies present in chronically infected patients (10-50 µg/ml) were markedly lower than anti-HBs plasma concentrations required for prevention of HBV infection (500 µg/ml anti-HBs IgG during the first week post-transplantation and 100 µg/ml after week 12).<sup>33</sup> Inhibitory anti-CLDN1 concentrations were in a similar range as trough levels of plasma concentrations of approved therapeutic or preventive mAbs used in cancer or antiviral treatment.<sup>34, 35</sup> Thus, anti-CLDN1 concentrations required for efficient inhibition of HCV infection are in a range of plasma antibody concentrations that can be achieved following intravenous administration of antibodies *in vivo*.

Moreover, anti-CLDN1 mAbs may also be of interest to target antiviral resistance in chronic hepatitis C. Since anti-CLDN1 mAbs broadly inhibit infection of highly diverse viral quasispecies and escape variants in a genotype-independent manner, it is conceivable that anti-CLDN1 mAbs may complement ongoing efforts to block intracellular replication events with inhibitors of the HCV proteases and polymerase. This concept is supported by the successful clinical use of entry inhibitors for viral resistance in combination with other antivirals in HIV infection.<sup>36</sup>

Using a panel of well characterized CLDN1 mutants we demonstrate that mAb OM-7D3-B3 recognizes a cluster of amino acid residues comprising W(30)-GLW(51)-

C(54)-C(64) as well as residues of the CLDN1 EL1 N-terminal third. Interestingly, these CLDN1 regions have been shown to be important for HCV entry in mutagenesis studies.<sup>13,21</sup> Since the identified epitopes are structurally not grouped together, it is likely that recognized epitopes are conformation-dependent. This hypothesis is further supported by the finding that (i) anti-CLDN1 mAbs did not show an easily detectable interaction with linear CLDN1 peptides as antigens in an ELISA and that (ii) pre-incubation of antibodies with linear peptides encoding for amino acids of the CLDN1 EL1 were not able to revert antibody-mediated inhibition of infection (data not shown). Thus, our results indicate that the targeted epitopes are conformational in nature, rather than linear, confirming that genetic immunization focuses on recognition of native proteins and offers an explanation why this technology has proven successful. Taken together, our results define epitopes in CLDN1 EL1 which are crucial for HCV entry and are accessible to antibodies blocking HCV infection.

A potential limitation of the clinical use of anti-receptor antibodies could be adverse effects. Host cell factors have important functions which may be linked to mechanism of viral entry. Antibodies binding to cellular entry factors may alter the function or expression of receptors resulting in side effects. Interestingly, no toxic effects were detected in PHH based on MTT testing. Furthermore, anti-CLDN1 mAbs had no adverse effect on TJ integrity in polarized hepatoma cells (Fig. 7). This finding suggests that TJ CLDN1 is not accessible for anti-CLDN1 mAbs or that CLDN1 domains targeted by anti-CLDN1 mAbs are different from functional domains required for TJ integrity. Although further studies are needed to address toxicity in hepatic and extrahepatic tissues (such as the kidney) *in vivo*, our studies in PHH and

hepatoma cells did not reveal any findings precluding the further development of the antibodies.

In conclusion, our findings demonstrate that targeting CLDN1 EL1 by anti-CLDN1 mAbs broadly cross-neutralizes HCV infection and therefore constitutes a novel antiviral approach to prevent primary HCV infection, such as after liver transplantation and might also restrain virus spread in chronically infected patients. Following humanization of the anti-CLDN1 mAbs, proof-of-concept studies in animal models and humans are the next step for the clinical development.

## 10 **Acknowledgements**

The authors would like to thank F. V. Chisari (The Scripps Research Institute, La Jolla, CA) for the gift of Huh7.5.1 cells, R. Bartenschlager (University of Heidelberg, Germany), F.-L. Cosset (Inserm U758, ENS Lyon, France) and J. Ball (University of Nottingham, UK) for providing plasmids for production of HCVcc and HCVpp, KaLy-Cell (Illkirch, France) for providing primary human hepatocytes, M. Parnot and M. Bastien-Valle (all Inserm U748, Strasbourg, France) for excellent technical assistance, J. Dubuisson (Institut Pasteur Lille, France), M. B. Zeisel (Inserm U748, Strasbourg) and C. M. Rice (Rockefeller University, New York) for helpful discussions.

20

25

## REFERENCES

1. Lauer GM, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001;345:41-52.
2. Tai AW, Chung RT. Treatment failure in hepatitis C: mechanisms of non-response. *J Hepatol* 2009;50:412-20.
3. Hezode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, Bronowicki JP, Bourliere M, Gharakhanian S, Bengtsson L, McNair L, George S, Kieffer T, Kwong A, Kauffman RS, Alam J, Pawlotsky JM, Zeuzem S. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 2009;360:1839-50.
4. Brown RS. Hepatitis C and liver transplantation. *Nature* 2005;436:973-8.
5. Pereira AA, Jacobson IM. New and experimental therapies for HCV. *Nat Rev Gastroenterol Hepatol* 2009;6:403-11.
6. Timpe JM, McKeating JA. Hepatitis C virus entry: possible targets for therapy. *Gut* 2008;57:1728-37.
7. Zeisel MB, Cosset FL, Baumert TF. Host neutralizing responses and pathogenesis of hepatitis C virus infection. *Hepatology* 2008;48:299-307.
8. Osburn WO, Fisher BE, Dowd KA, Urban G, Liu L, Ray SC, Thomas DL, Cox AL. Spontaneous control of primary hepatitis C virus infection and immunity against persistent reinfection. *Gastroenterology* 2009;138:315-324.
9. Pestka JM, Zeisel MB, Blaser E, Schurmann P, Bartosch B, Cosset FL, Patel AH, Meisel H, Baumert J, Viazov S, Rispeter K, Blum HE, Roggendorf M, Baumert TF. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc Natl Acad Sci U S A* 2007;104:6025-30.

10. Barth H, Schafer C, Adah MI, Zhang F, Linhardt RJ, Toyoda H, Kinoshita-Toyoda A, Toida T, Van Kuppevelt TH, Depla E, Von Weizsacker F, Blum HE, Baumert TF. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J Biol Chem* 2003;278:41003-12.
- 5 11. Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. Binding of hepatitis C virus to CD81. *Science* 1998;282:938-41.
12. Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A. The human scavenger receptor  
10 class B type I is a novel candidate receptor for the hepatitis C virus. *Embo J* 2002;21:5017-25.
13. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wolk B, Hatzioannou T, McKeating JA, Bieniasz PD, Rice CM. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 2007;446:801-5.
- 15 14. Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, Rice CM. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 2009;457:882-6.
15. Haid S, Windisch MP, Bartenschlager R, Pietschmann T. Mouse-specific residues of claudin-1 limit hepatitis C virus genotype 2a infection in a human  
20 hepatocyte cell line. *J Virol* 2010;84:964-75.
16. Timpe JM, Stamatakis Z, Jennings A, Hu K, Farquhar MJ, Harris HJ, Schwarz A, Desombere I, Roels GL, Balfe P, McKeating JA. Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. *Hepatology* 2008;47:17-24.

17. Furuse M, Fujita K, Hiiiragi T, Fujimoto K, Tsukita S. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol* 1998;141:1539-50.
18. Krieger SE, Zeisel MB, Davis C, Thumann C, Harris HJ, Schnober EK, Mee C, 5 Soulier E, Royer C, Lambotin M, Grunert F, Dao Thi VL, Dreux M, Cosset FL, McKeating JA, Schuster C, Baumert TF. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-Claudin-1 associations. *Hepatology* 2010;51:1144-57.
19. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland 10 SF, Uprichard SL, Wakita T, Chisari FV. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A* 2005;102:9294-9.
20. Mee CJ, Harris HJ, Farquhar MJ, Wilson G, Reynolds G, Davis C, van ISC, Balfe P, McKeating JA. Polarization restricts hepatitis C virus entry into HepG2 hepatoma cells. *J Virol* 2009;83:6211-21.
- 15 21. Cukierman L, Meertens L, Bertaux C, Kajumo F, Dragic T. Residues in a highly conserved claudin-1 motif are required for hepatitis C virus entry and mediate the formation of cell-cell contacts. *J Virol* 2009;83:5477-84.
22. Catanese MT, Graziani R, von Hahn T, Moreau M, Huby T, Paonessa G, Santini C, Luzzago A, Rice CM, Cortese R, Vitelli A, Nicosia A. High-avidity 20 monoclonal antibodies against the human scavenger class B type I receptor efficiently block hepatitis C virus infection in the presence of high-density lipoprotein. *J Virol* 2007;81:8063-71.
23. Koutsoudakis G, Kaul A, Steinmann E, Kallis S, Lohmann V, Pietschmann T, Bartenschlager R. Characterization of the early steps of hepatitis C virus 25 infection by using luciferase reporter viruses. *J Virol* 2006;80:5308-20.

24. Fafi-Kremer S, Fofana I, Carolla P, Soulier E, Meuleman P, Leroux-Roels G, Patel AH, Cosset FL, Pessaux P, Doffoel M, Wolf P, Stoll-Keller F, Baumert TF. Escape from antibody-mediated neutralization and viral entry are key determinants for hepatitis C virus re-infection in liver transplantation. in revision 2010.
- 5
25. Haberstroh A, Schnober EK, Zeisel MB, Carolla P, Barth H, Blum HE, Cosset FL, Koutsoudakis G, Bartenschlager R, Union A, Depla E, Owsianka A, Patel AH, Schuster C, Stoll-Keller F, Doffoel M, Dreux M, Baumert TF. Neutralizing host responses in hepatitis C virus infection target viral entry at postbinding steps and membrane fusion. *Gastroenterology* 2008;135:1719-1728.
- 10
26. von Hahn T, Yoon JC, Alter H, Rice CM, Rehermann B, Balfe P, McKeating JA. Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* 2007;132:667-78.
- 15
27. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
28. Law M, Maruyama T, Lewis J, Giang E, Tarr AW, Stamataki Z, Gastaminza P, Chisari FV, Jones IM, Fox RI, Ball JK, McKeating JA, Kneteman NM, Burton DR. Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge. *Nat Med* 2008;14:25-7.
- 20
29. Vanwolleghem T, Bukh J, Meuleman P, Desombere I, Meunier JC, Alter H, Purcell RH, Leroux-Roels G. Polyclonal immunoglobulins from a chronic hepatitis C virus patient protect human liver-chimeric mice from infection with a homologous hepatitis C virus strain. *Hepatology* 2008;47:1846-55.
- 25

30. Ray SC, Fanning L, Wang XH, Netski DM, Kenny-Walsh E, Thomas DL. Divergent and convergent evolution after a common-source outbreak of hepatitis C virus. *J Exp Med* 2005;201:1753-9.
31. Roche B, Samuel D. Liver transplantation in viral hepatitis: prevention of recurrence. *Best Pract Res Clin Gastroenterol* 2008;22:1153-69.
32. Keck ZY, Li SH, Xia J, von Hahn T, Balfe P, McKeating JA, Witteveldt J, Patel AH, Alter H, Rice CM, Fong SK. Mutations in hepatitis C virus E2 located outside the CD81 binding sites lead to escape from broadly neutralizing antibodies but compromise virus infectivity. *J Virol* 2009;83:6149-60.
33. Samuel D, Bismuth A, Mathieu D, Arulnaden JL, Reynes M, Benhamou JP, Brechot C, Bismuth H. Passive immunoprophylaxis after liver transplantation in HBsAg-positive patients. *Lancet* 1991;337:813-5.
34. Boeckh M, Berrey MM, Bowden RA, Crawford SW, Balsley J, Corey L. Phase 1 evaluation of the respiratory syncytial virus-specific monoclonal antibody palivizumab in recipients of hematopoietic stem cell transplants. *J Infect Dis* 2001;184:350-4.
35. Leyland-Jones B, Gelmon K, Ayoub JP, Arnold A, Verma S, Dias R, Ghahramani P. Pharmacokinetics, safety, and efficacy of trastuzumab administered every three weeks in combination with paclitaxel. *J Clin Oncol* 2003;21:3965-71.
36. Este JA, Telenti A. HIV entry inhibitors. *Lancet* 2007;370:81-8.
37. Broering TJ, Garrity KA, Boatright NK, Sloan SE, Sandor F, Thomas WD, Jr., Szabo G, Finberg RW, Ambrosino DM, Babcock GJ. Identification and characterization of broadly neutralizing human monoclonal antibodies directed



against the E2 envelope glycoprotein of hepatitis C virus. *J Virol*  
2009;83:12473-82.

5

## FIGURE LEGENDS

**Fig. 1. Production of CLDN1-specific mAbs with high affinity to HCV permissive cells lines and human hepatocytes.** (A) Specific binding of rat anti-human CLDN1 mAb OM-7D3-B3 (20  $\mu\text{g/ml}$ ) to CLDN1 expressed on the cell surface of BOSC23 cells transfected with pCMV-SPORT6-CLDN1 (black histogram) but not to cells transfected with control vector (pCMV-SPORT6; red histogram). MAb binding was revealed by flow cytometry using PE-conjugated anti-rat IgG mAb. The x and y axes show mean fluorescence intensities and relative numbers of stained cells, respectively. (B) Specific binding of anti-CLDN1 mAbs (20  $\mu\text{g/ml}$ ) to human CLDN1 expressed on the cell surface of transfected CHO cells. CHO cells were transfected with pCMV-SPORT6-CLDN1 (grey bars) or control vector (pCMV-SPORT6; black bars). The mean fluorescence intensities (MFIs) for each experiment performed in triplicate are shown. (C) Binding of mAbs to Huh7.5.1 cells and primary human hepatocytes (PHH) was determined by flow cytometry as described above. (D) Staining of cell surface CLDN1 on Huh7.5.1 cells by anti-CLDN1 mAb OM-4A4-D4 was performed as described in Materials and Methods. Cell nuclei were stained with DAPI. (E) Binding properties of anti-CLDN1 mAbs to PHH. Cells were incubated with increasing concentrations of anti-CLDN1 mAbs. MAb binding was revealed by flow cytometry using PE-conjugated anti-rat IgG mAb. As a control, isotype-matched rat IgG2 was used. Mean fluorescence intensities compared to experiments with isotype control antibody are shown. Apparent  $K_d$  are shown in the inset.

**Fig. 2. Mapping of CLDN1 epitopes targeted by mAbs.** (A) Negligible binding of anti-CLDN1 mAb to other members of the CLDN family. BOSC23 cells were transfected with validated CLDN expression constructs and binding of anti-CLDN1

mAbs to cell surface CLDN was analyzed by flow cytometry. Specific binding is expressed in mean fluorescence intensity (MFI) relative to negative control. (B, C) Competition of anti-CLDN1 mAbs for cellular binding. Huh7.5.1 cells were incubated with 0.1 µg/ml biotinylated anti-CLDN1 mAb OM-7D3-B3 together with increasing (B) or saturating concentrations (C) of unlabeled anti-CLDN1 mAbs as competitors. Following washing of cells in PBS, binding of labeled antibody was detected as described in Methods and is shown as relative fluorescence units (RFU). (D) Epitope mapping. Mapping studies were performed using plasmids expressing CLDN1 containing an hemagglutinin-tag (HA-tag) and defined mutations indicated on the x-axis: the left panel shows mutants containing alanine substitutions of critical residues in the highly conserved claudin motif W(30)-GLW(51)-C(54)-C(64);<sup>21</sup> the right panel shows single alanine substitutions at positions occupied by bulky, charged or polar residues.<sup>21</sup> CLDN1-negative BOSC23 cells were transfected with mutant CLDN1 expression constructs and binding of mAb OM-7D3-B3 was analyzed by flow cytometry. MAb binding to mutant CLDN1 relative to binding to wild-type CLDN1 is shown (black bars). Proper expression of CLDN1 proteins was confirmed by flow cytometric analysis of HA-tag expression using an anti-HA antibody (grey bars) except for mutant I32A where the HA-tag was absent and expression of CLDN1 was confirmed by FACS analysis using an unrelated anti-CLDN1 antibody (data not shown). Binding of anti-HA antibody to HA of mutant CLDN1 relative to HA of wild-type CLDN1 is shown as internal control for expression of mutant CLDN1 (grey bars).

**Fig. 3. Genotype-independent inhibition of HCVcc and HCVpp infection by anti-CLDN1 mAbs.** (A, B) Inhibition of HCVcc infection by anti-CLDN1 mAbs. Huh7.5.1 cells were pre-incubated with increasing (A) or a fixed (10 µg/ml) (B) concentration of

anti-CLDN1 or control mAbs for 1 h at 37°C before infection with Luc-Jc1 or Luc-Con1 HCVcc. HCV infection was assessed as described in Methods. (C) Inhibition of infection of HCVpp bearing envelope glycoproteins from genotypes 1-6. Huh7 cells were pre-incubated with antibodies as described in (A) before infection with HCVpp. 5 MLV-based HCVpp bearing envelope glycoproteins of strains H77 (genotype 1a), HCV-J (1b), JFH1 (2a), UKN3A1.28 (3a), UKN4.21.16 (4), UKN5.14.4 (5), UKN6.5.340 (6) and VSVpp were produced as described in Methods. Means  $\pm$  SD from three experiments are shown, respectively.

10 **Fig. 4. Efficient inhibition of infection of HCV variants escaping autologous neutralizing antibodies and re-infecting the liver graft by anti-CLDN1 mAbs.**

HIV-based HCVpp bearing envelope glycoproteins from HCV escape variants derived from 6 patients undergoing liver transplantation (P01VL, P02VH, P02VI, P02VJ, P03VC, P04VC, P04VD, P04VE, P05VD, P05VE, P05VF, P06VG, P06VH, 15 P06VI)<sup>24</sup> were produced from patient-derived full-length E1E2 expression constructs as described.<sup>9</sup> (A) Infectivity of HCV escape variants. HCVpp were added to PHH and infection was analyzed as in Fig. 3. Results are expressed in relative light units (RLU) plotted in a logarithmic scale. (B) Escape of HCVpp infection from neutralization by autologous pre-transplant serum. HCVpp were incubated with 20 autologous pre-transplant anti-HCV positive serum or control anti-HCV negative serum (Ctrl) and HCVpp infection in PHH was analyzed as described in Fig. S1. As a positive control, HCVpp bearing envelope glycoproteins from a heterologous strain (HCV-J) were incubated with pre-transplant serum at the same dilution. (C) Inhibition of infection of viral escape variants and HCV-J by anti-CLDN1 mAb OM-7D3-B3. 25 PHH were pre-incubated with anti-CLDN1 or isotype control mAbs (25  $\mu$ g/mL) and

infection was analyzed as described above. Means  $\pm$  SD from a representative experiment performed in triplicate are shown.

**Fig. 5. Additive effect of antiviral and anti-CLDN1 antibodies on inhibition of HCV infection.** HCVpp of strains P02VJ and P04VJ (see Fig. 4) were pre-incubated with anti-E2 mAb IGH461 (A) or purified heterologous anti-HCV IgG (1 or 10  $\mu$ g/ml) obtained from an unrelated chronically infected subject (B) or isotype control IgG for 1 h at 37°C and added to Huh7 cells pre-incubated with serial dilutions of anti-CLDN1 OM-7D3-B3 or rat isotype control mAbs. HCVpp infection was analyzed as described in Fig. 3.

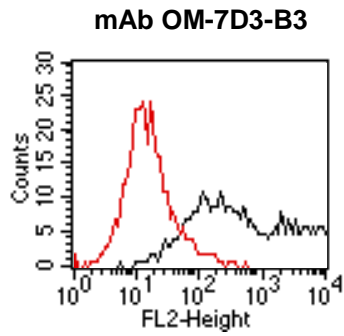
**Fig. 6. Anti-CLDN1 mAbs broadly inhibit infection of viral quasispecies in chronic HCV infection.** Full-length HCV envelope glycoproteins were cloned and sequenced from plasma of an individual patient chronically infected with HCV (genotype 1b) using E1E2-specific primers as described.<sup>9</sup> A total of 30 clones was analyzed by alignment of E1E2 sequences. Lentiviral HCVpp displaying patient-derived envelope were produced as described.<sup>9, 18</sup> (A) Relative distribution of the variants in the HCV-infected patient based on alignment of E1E2 sequences. Variants containing stop codons, insertions or deletions altering the HCV open reading frame are depicted with a star (\*) and were not further analyzed in HCVpp assays. (B) Comparative analysis of viral entry of HCVpp bearing envelope glycoproteins of viral quasispecies in PHH. HIV-HCVpp expressing patient-derived envelope glycoproteins were incubated with PHH and infection was analyzed as described in Fig. S1. Results are expressed in relative light units (RLU) plotted in a logarithmic scale. The threshold for a detectable infection in this system was  $3 \times 10^3$

RLU and is indicated by a dotted line. (C) Inhibition of HCV entry by anti-CLDN1 mAbs. PHH were pre-incubated with rat anti-CLDN1 OM-7D3-B3 or rat isotype control mAbs (25 µg/ml) and infected with HCVpp bearing HCV envelope glycoproteins from quasispecies shown in (A). Means ± SD from a representative experiment performed in triplicate are shown. Abbreviations: NI - non infectious; Ctrl - negative control.

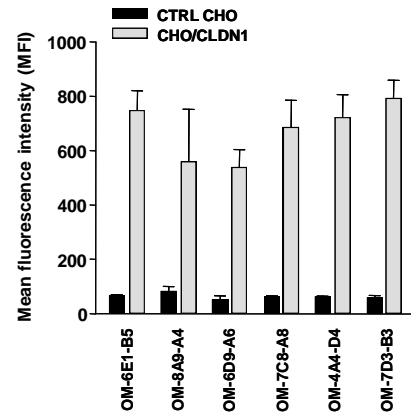
**Fig. 7. Absent toxicity and absent adverse effects on tight junction integrity in PHH and hepatoma cells.** (A-D) Cytotoxic effects on cells were assessed by analyzing the ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).<sup>27</sup> Huh7.5.1 cells (A) and PHH from three different donors (B-D) were incubated either with isotype control or anti-CLDN1 OM-7D3-B3 mAbs (10 µg/ml), anti-Fas (10 µg/ml), flavopiridol (10 µM) or compound C (20 µM) (B-C) or anti-CLDN1 OM-7D3-B3 mAb (0-1000 µg/ml), anti-Fas (0-100 µg/ml) and compound C (0-1000 µM) (D) Relative cell viability was assessed in comparison to mock incubated PHH (=100%). (E) Anti-CLDN1 mAbs do not alter tight junction (TJ) integrity in polarized HepG2 cells. Cells were treated with serum-free medium for 4 hours before being exposed to either control (PBS), irrelevant control, anti-CLDN1 OM-7D3-B3 and OM-8A9-A3 mAbs (10 µg/ml), TNF-α (10 ng/ml), interferon-γ (10 ng/ml), PMA (50 ng/ml) for 1 hour. TJ integrity was analyzed by analyzing the capacity of bile canaliculi<sup>37</sup> to retain 5-chloromethylfluorescein diacetate (CMFDA) compared to the total BC as described.<sup>20</sup> Means ± SD from representative experiments performed in triplicate are shown (A-E).

Figure 1

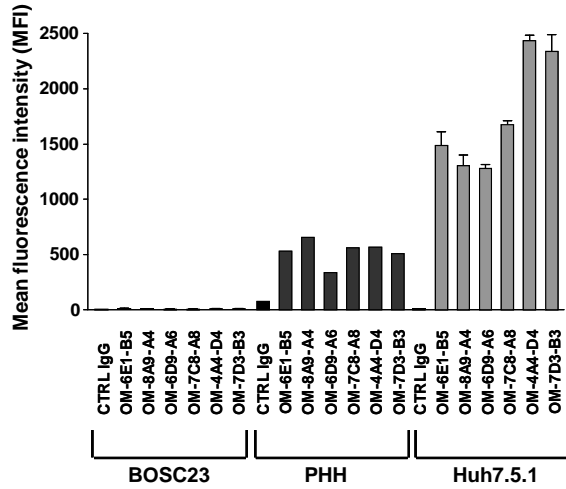
A



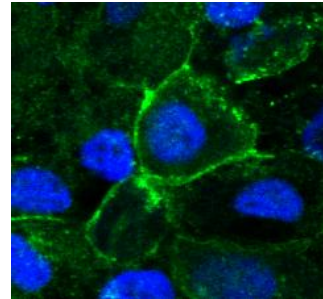
B



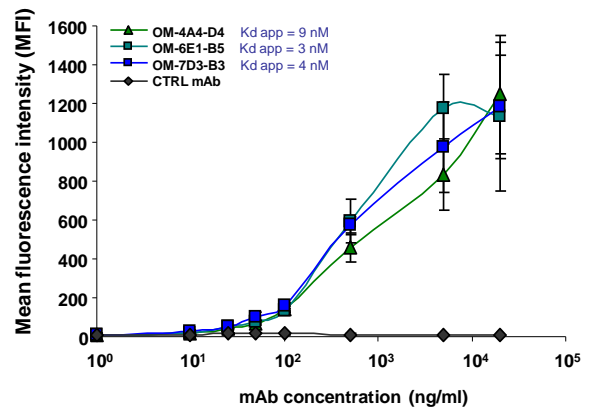
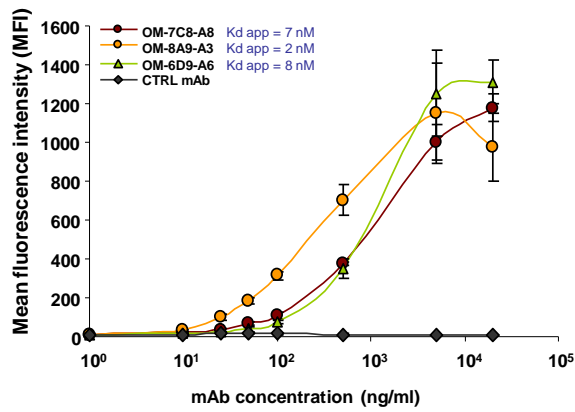
C



D

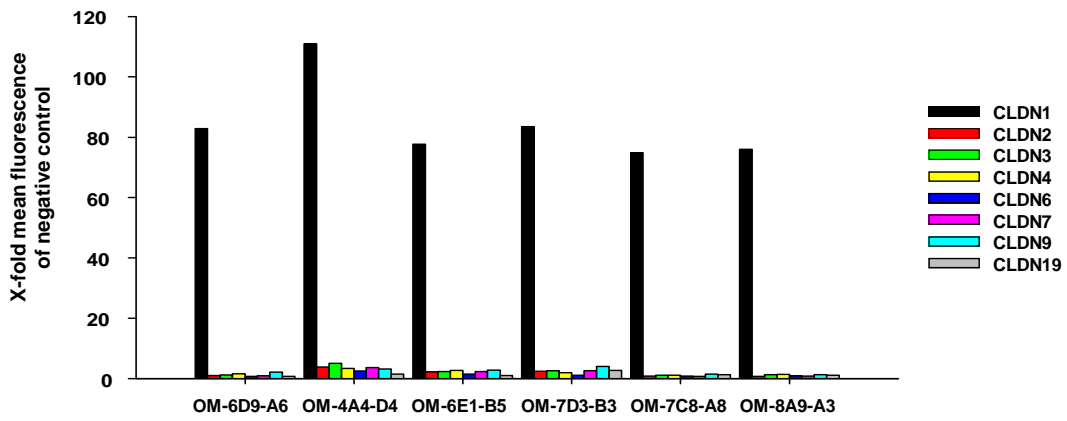


E

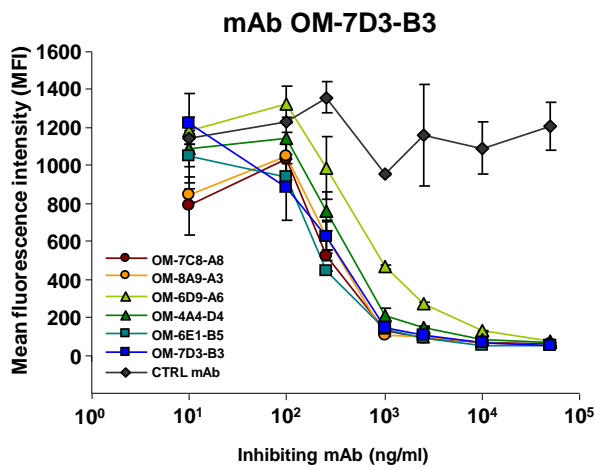


**Figure 2**

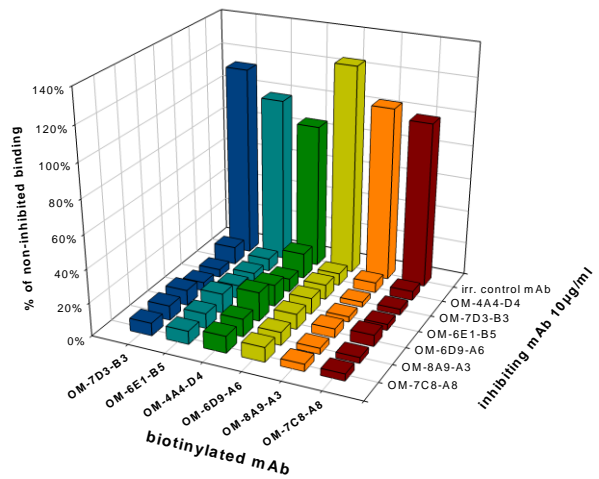
**A**



**B**



**C**



**D**

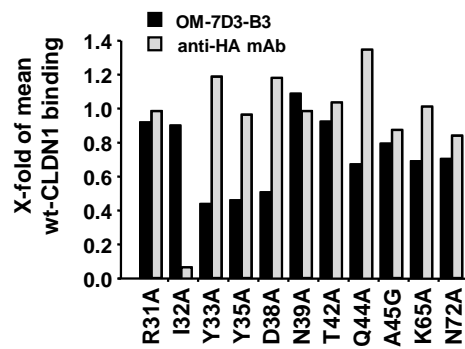
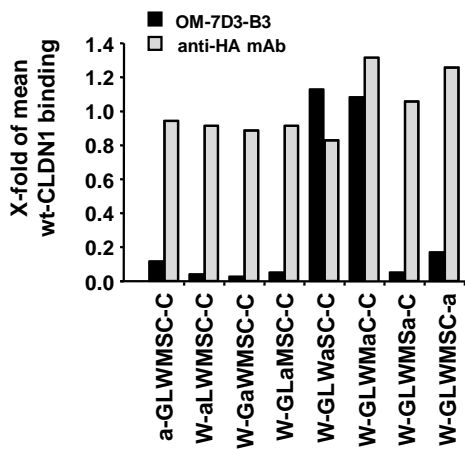




Figure 3

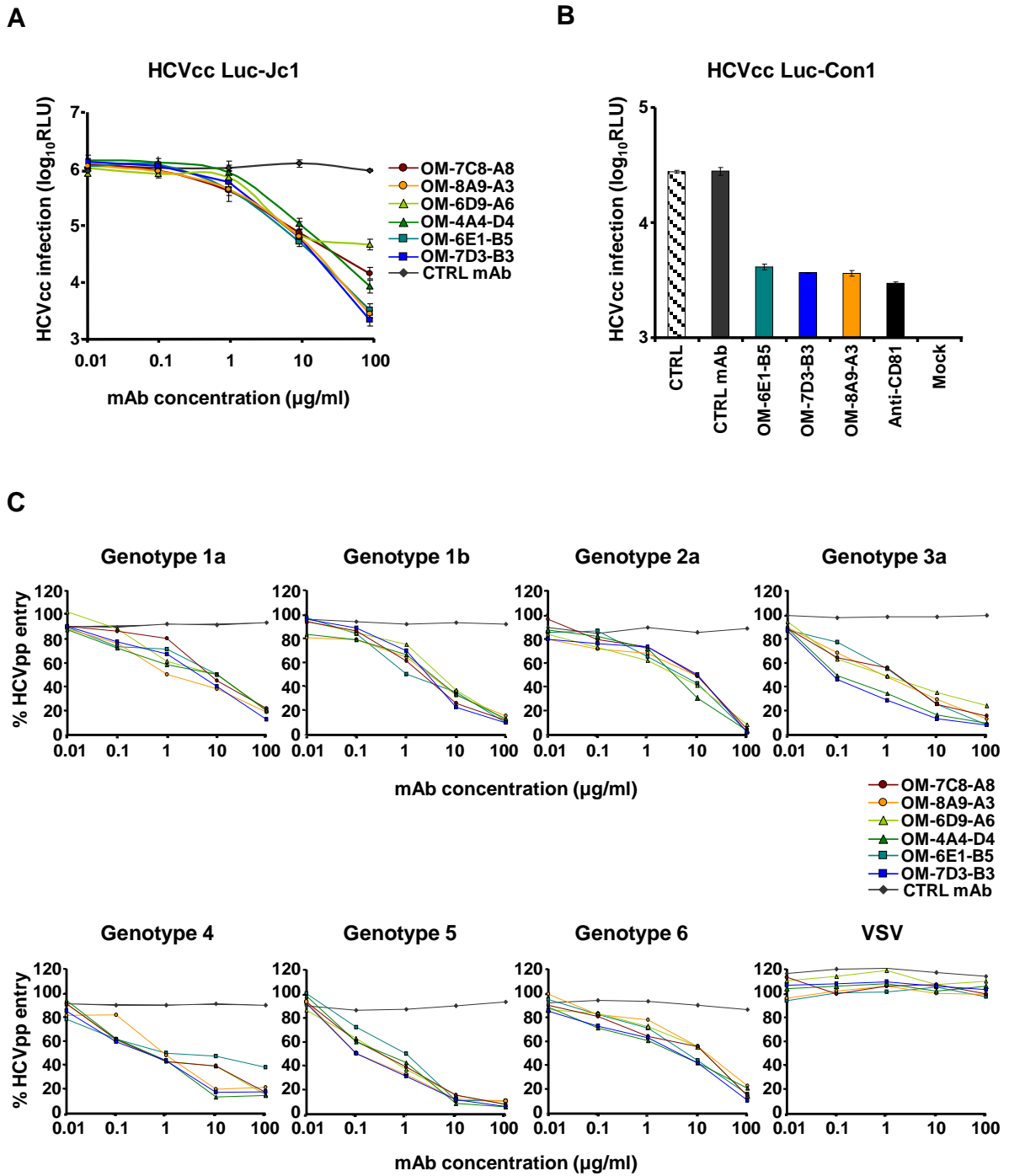
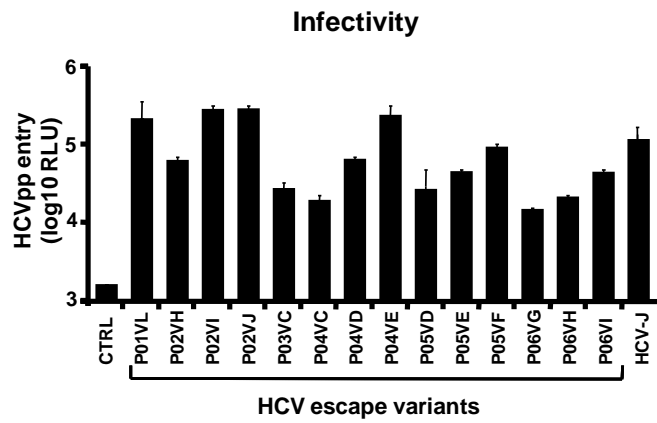
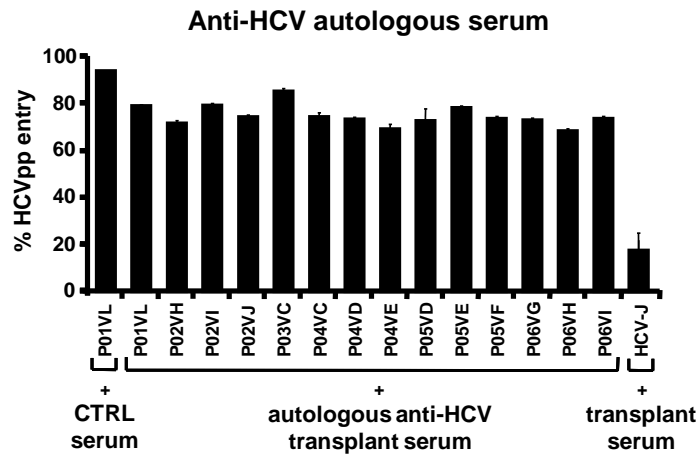


Figure 4

A



B



C

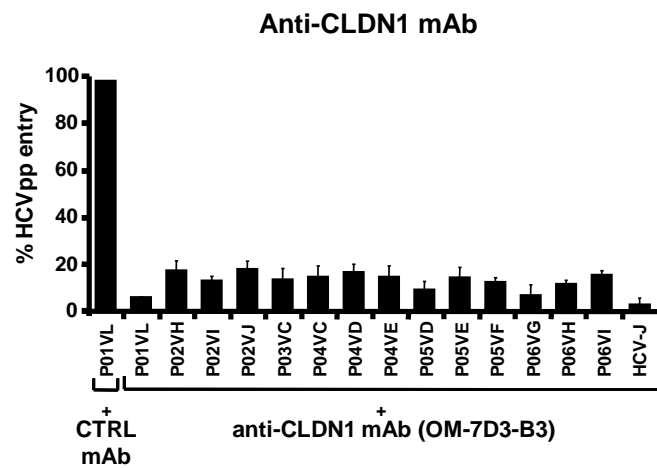
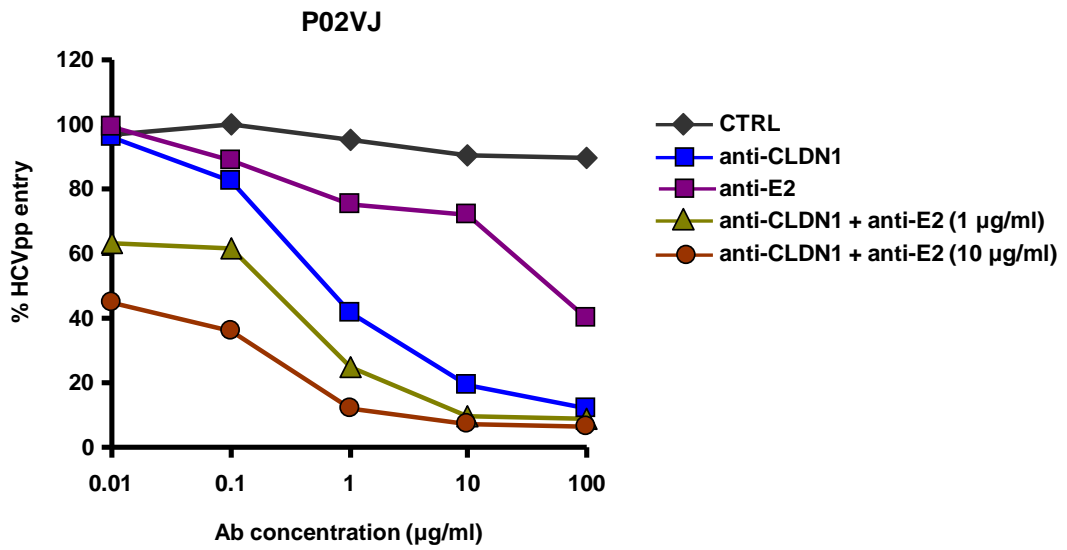


Figure 5

A



B

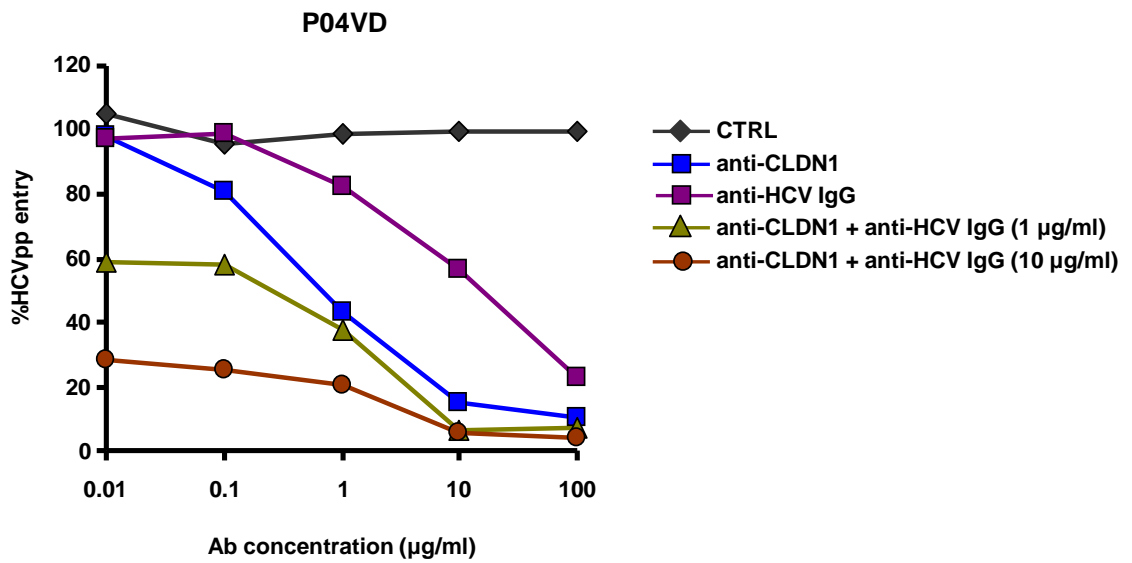
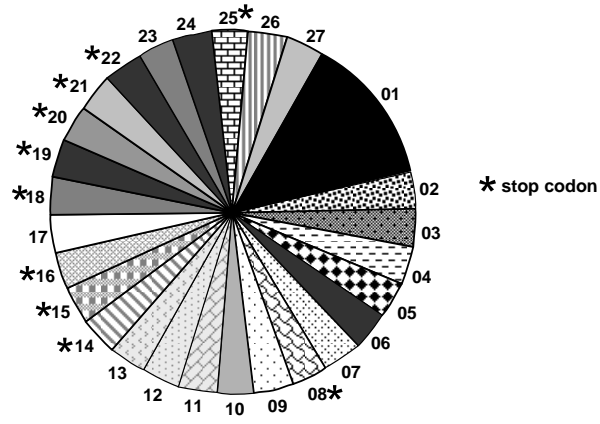
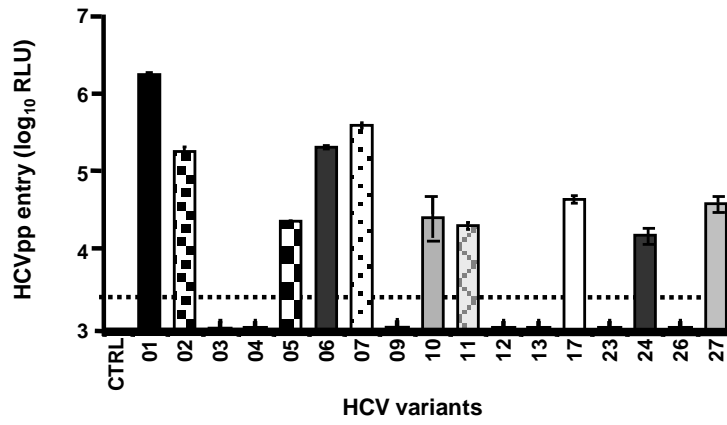


Figure 6

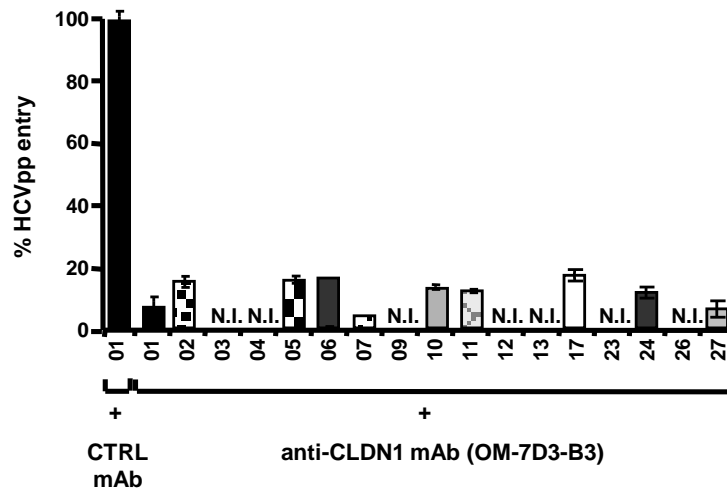
A



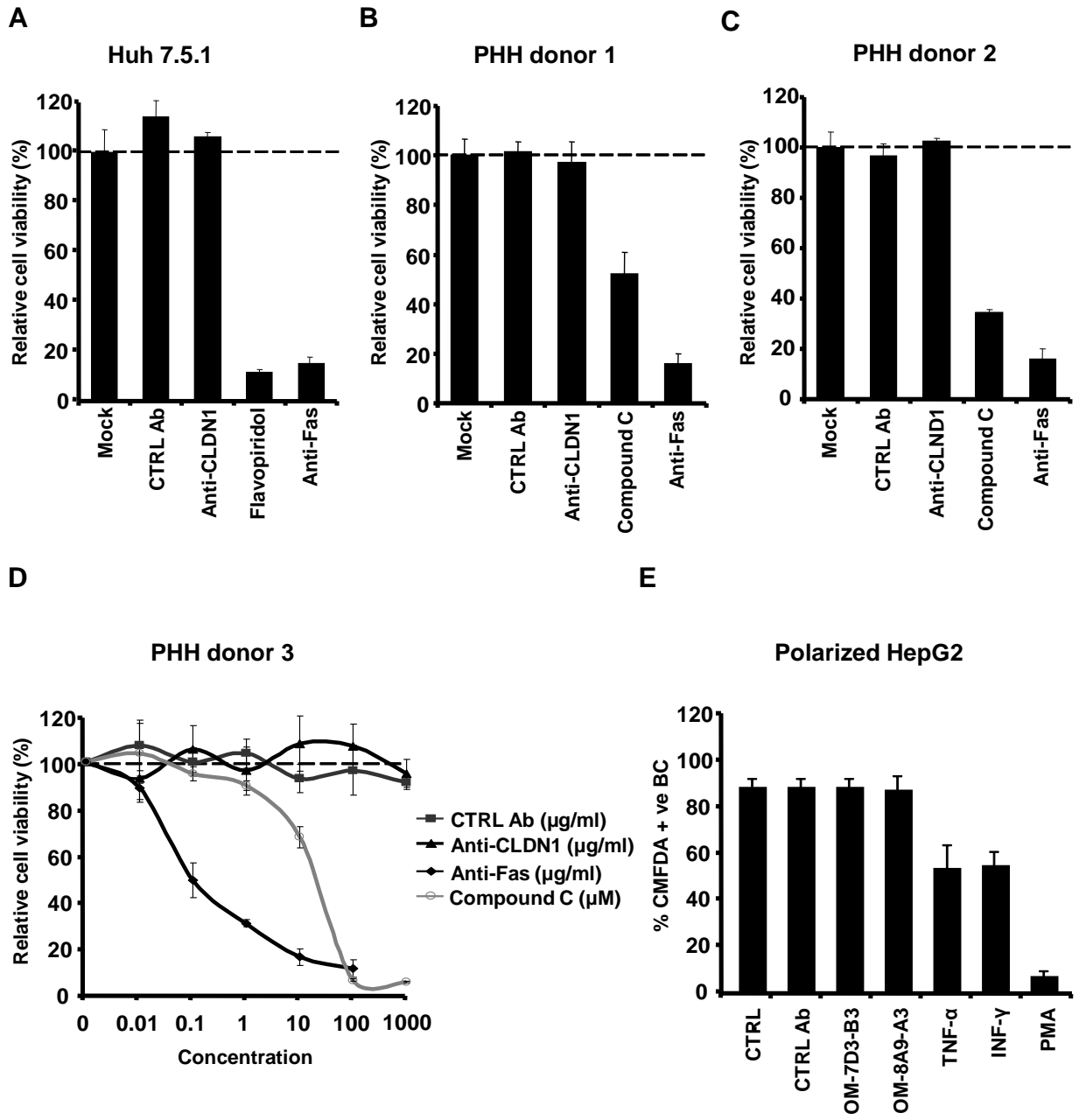
B



C



**Figure 7**



## SUPPLEMENTARY DATA

### SUPPLEMENTARY MATERIALS AND METHODS

**Infection of primary human hepatocytes by serum-derived HCV.** To identify HCV positive serum samples resulting in productive infection of PHH, randomly selected  
5 serum samples from eighteen patients with chronic HCV infection from diverse genotypes were screened for infectivity in PHH. The sera were obtained in the framework of a clinical trial (ClinicalTrials.gov Identifier: NCT00638144) and were characterized by a viral load ranging between  $1.27 \times 10^6$  and  $8.8 \times 10^6$  IU/ml (determined by Abbott RealTime™ HCV) and sero-negativity for HAV, HBV and HIV.

10 PHH ( $3 \times 10^5$  cells, isolated and cultured in collagen-coated 24 well-plates as described)<sup>1,2</sup> were incubated with 25  $\mu$ l of HCV-positive serum. The cells were then washed three times and cultured as described.<sup>3</sup> Five days post-inoculation, cells were washed three times and total cellular RNA was extracted using the RNAeasy kit (Qiagen). Five hundred ng of total RNA were analyzed by quantitative RT-PCR using  
15 the SensiMix™ Probe One-Step Kit (BIOLINE), using primers Con259F (5'AGYGTTGGGTGCGAAAG3'), Con312R (5'GTGAGCGTTCGYGGGA3') and a probe (5'AGTCCGTCATGGTGTTC 3') corresponding to nucleotides 260-270, 298-313 and 279-296 as described.<sup>4</sup> GAPDH mRNA in each sample was quantified in parallel.<sup>3, 5</sup> The measured amounts of HCV RNA were normalized to the amount of  
20 GAPDH mRNA in each sample, and the results were expressed per culture plate ( $3 \times 10^5$  cells) as described recently.<sup>3, 5</sup> Positive infection was confirmed by de-novo detection of intracellular HCV RNA as previously described.<sup>3, 5</sup> Using this experimental set-up, incubation of PHH with two sera resulted in reproducible productive HCV infection. The infectious samples comprised serum #08 (45 year old  
25 male patient infected with genotype 2a and a viral load of  $2.51 \times 10^6$  IU/ml) and

serum #10 (54 year old female patient infected with HCV genotype 2b and a viral load of  $1.37 \times 10^6$  IU/ml). The infectious serum samples were then used to assess inhibition of HCV infection by anti-CLDN1 mAbs. One day after plating, PHH were incubated with anti-CLDN1 OM-7D3-B3 or rat isotype control mAbs (25  $\mu$ g/ml) for 1 h and incubated overnight at 37°C with 25  $\mu$ l of HCV-positive serum #08 and #10 or HCV negative control serum (CTRL), respectively. The apparent multiplicity of infection (estimated by the ratio of HCV RNA copies divided by the number of hepatocytes per well) ranged between 0.5 and 1, respectively. Following quantification of intracellular HCV RNA on day 5 post-infection, the HCV RNA levels were normalized to the amount of GAPDH mRNA in each sample as described,<sup>3, 5</sup> and the results were expressed as percentage of HCV infection compared to control mAb treated PHH as described.<sup>3, 5</sup>

15

## REFERENCES

1. Krieger SE, Zeisel MB, Davis C, et al. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-Claudin-1 associations. *Hepatology* 2010;51:1144-57.
- 5 2. Zeisel MB, Koutsoudakis G, Schnober EK, et al. Scavenger receptor class B type I is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81. *Hepatology* 2007;46:1722-31.
3. Castet V, Fournier C, Soulier A, et al. Alpha interferon inhibits hepatitis C virus replication in primary human hepatocytes infected in vitro. *J Virol* 10 2002;76:8189-99.
4. Engle RE, Russell RS, Purcell RH, et al. Development of a TaqMan assay for the six major genotypes of hepatitis C virus: comparison with commercial assays. *J Med Virol* 2008;80:72-9.
5. Molina S, Castet V, Pichard-Garcia L, et al. Serum-derived hepatitis C virus 15 infection of primary human hepatocytes is tetraspanin CD81 dependent. *J Virol* 2008;82:569-74.
6. Harris HJ, Davis C, Mullins JG, et al. Claudin association with CD81 defines hepatitis C virus entry. *J Biol Chem* [Epub ahead of print] 2010.

20

25



## FIGURE LEGENDS

**Fig. S1. Absent reactivity of anti-CLDN1 mAbs with non type 1 members of the human CLDN family.** CLDN1-deficient 293T cells were transfected with a panel of AcGFP tagged human CLDNs (1, 3, 4, 6, 7, 9, 11, 12, 15, 17) as described.<sup>1,6</sup> Cells transfected with an unrelated vector (pcDNA3.1) served as a negative control. Binding of anti-CLDN1 mAb OM-7D3-B3 to cell surface CLDN was analyzed as described in Fig. 2A. Specific binding of anti-CLDN1 mAb to AcGFP tagged CLDNs is shown as the difference of the mean fluorescence intensity ( $\Delta$ MFI) of cells stained with anti-CLDN1 mAb OM-7D3-B3 and cells stained with isotype control rat mAb (upper panel). Proper expression of AcGFP tagged CLDN proteins was confirmed by flow cytometric analysis of AcGFP expression. Specific fluorescence of the respective AcGFP tags is shown as the difference of the mean fluorescence intensity ( $\Delta$ MFI) of cells transfected with each AcGFP tagged expression construct and cells transfected with non-tagged CLDN1 (lower panel). Means  $\pm$  SD of a representative experiment performed in triplicate are shown.

**Fig. S2. Inhibition of HCVpp infection in PHH by anti-CLDN1 mAbs.** HIV-based HCVpp bearing envelope glycoproteins of strains (A) H77 (genotype 1a), (B) HCV-J (1b), (C) JFH1 (2a), (D) UKN3A1.28 (3) (E) UKN4.21.16 (4), (F) UKN5.14.4 (5), (G) UKN6.5.340 (6) and (H) VSVpp were produced as described in Methods. PHH were pre-incubated with anti-CLDN1 or isotype control mAbs (25  $\mu$ g/ml) for 1 h at 37°C before infection with HCVpp for 4 h at 37°C. (I) Dose-dependent inhibition of infection by HCVpp bearing envelope glycoproteins of strain H77 using anti-CLDN1 mAb OM-8A9-A3. Experiments were performed as described in Fig. 3A. HCVpp infection was

assessed as described in Methods. Means  $\pm$  SD from a representative experiment performed in triplicate are shown.

**Fig. S3. Anti-CLDN1 mAbs inhibit infection of PHH by serum-derived HCV.** PHH  
5 were pre-incubated with anti-CLDN1 OM-7D3-B3 or isotype control mAbs (25  $\mu$ g/ml)  
for 1 h at 37°C before infection with HCV-positive serum (HCVser) obtained from two  
patients chronically infected with HCV genotype 2a (A) and 2b (B) or HCV-negative  
serum (CTRL). Following quantification of newly synthesized intracellular HCV RNA  
on day 5 postinfection by RT-PCR, HCV RNA levels were normalized to the amount  
10 of GAPDH mRNA in each sample as described.<sup>3</sup> The results are expressed as  
percentage of HCV infection in the presence of anti-CLDN1 antibody compared to  
infection in the presence of control isotype mAb (=100%). Means  $\pm$  SD from a  
representative experiment performed in duplicate are shown.

Figure S1

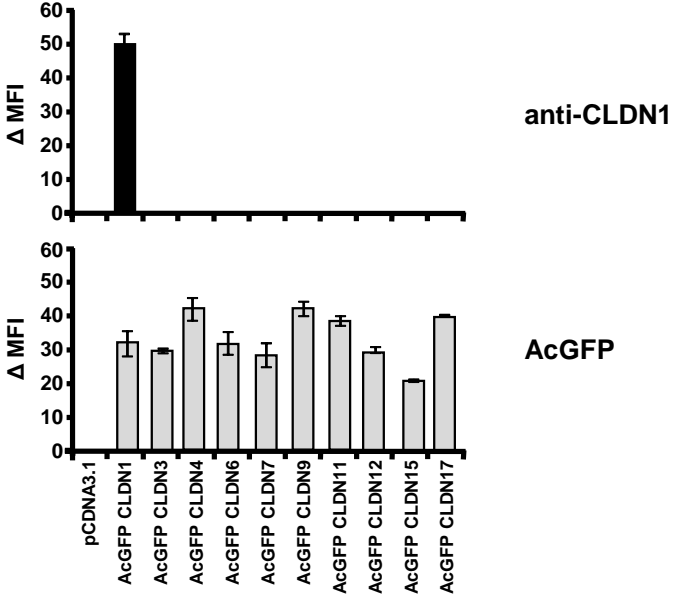


Figure S2

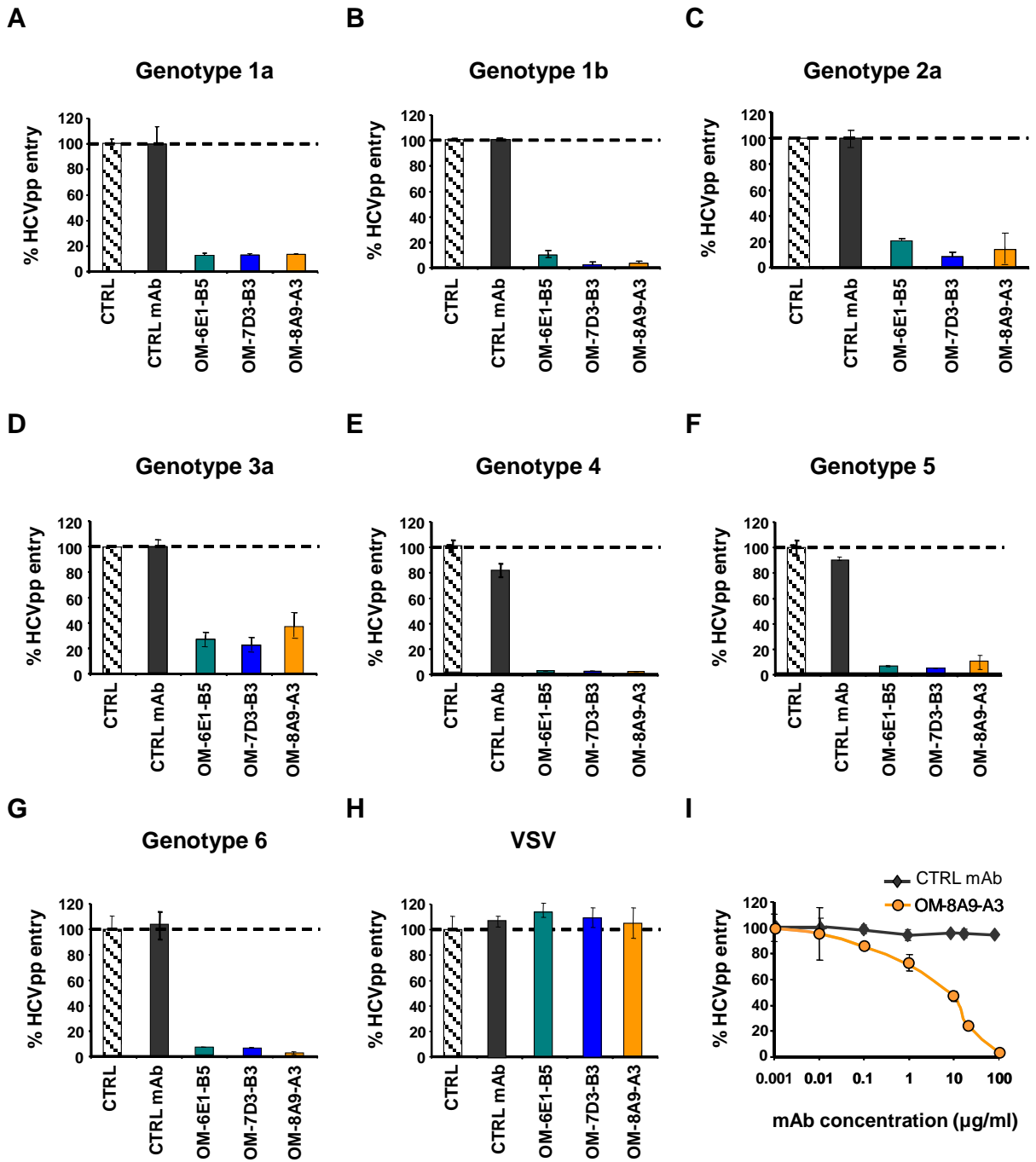
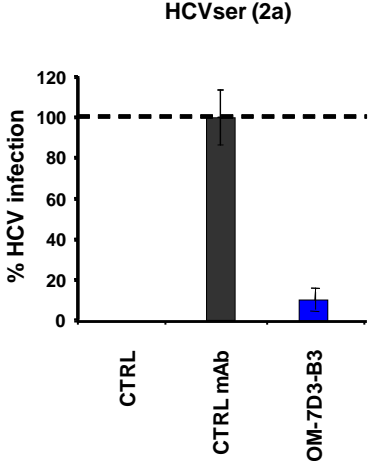


Figure S3

A



B

