In vivo combination of human anti-envelope glycoprotein E2 and -Claudin-1 monoclonal antibodies for prevention of hepatitis C virus infection

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Highlights

- **Synergistic inhibition of HCV entry by anti-E2 and anti-CLDN1 monoclonal antibodies**
- ***In vivo* proof of concept of anti-E2 and anti-CLDN1 combinations to prevent HCV infection**

- **Neutralization of difficult to neutralize variants**
- **Perspective for prevention of HCV infection in transplantations of HCV-positive organs**

Abstract

Despite the development of direct-acting antivirals (DAAs), hepatitis C virus (HCV) infection remains a major cause for liver disease and cancer worldwide. Entry inhibitors block virus host cell entry and, therefore, prevent establishment of chronic infection and liver disease. Due to their unique mechanism of action, entry inhibitors provide an attractive antiviral strategy in organ transplantation. In this study, we developed an innovative approach in preventing HCV infection using a synergistic combination of a broadly neutralizing human monoclonal antibody (HMAb) targeting the HCV E2 protein and a host-targeting anti-claudin 1 (CLDN1) humanized monoclonal antibody. An *in vivo* proof-of-concept study in human liver-chimeric FRG-NOD mice proved the efficacy of the combination therapy at preventing infection by an HCV genotype 1b infectious serum. While administration of individual antibodies at lower doses only showed a delay in HCV infection, the combination therapy was highly protective. Furthermore, the combination proved to be effective in preventing infection of primary human hepatocytes by neutralization-resistant HCV escape variants selected during liver transplantation, suggesting that a combination therapy is suited for the neutralization of difficult-to-treat variants. In conclusion, our findings suggest that the combination of two HMAbs targeting different steps of virus entry improves treatment efficacy while simultaneously reducing treatment duration and costs. Our approach not only provides a clinical perspective to employ HMAb combination therapies to prevent graft re-infection and its associated liver disease but may also help to alleviate the urgent demand for organ transplants by allowing the transplantation of organs from HCV-positive donors.

Keywords:

Antivirals, HCV, entry inhibitors, synergy, humanized liver-chimeric mice, transplantation

Main Text

Hepatitis C virus (HCV) infection is a leading risk factor for liver disease that ultimately leads to liver failure and hepatocellular carcinoma (HCC) (Akinyemiju et al., 2017), which is the second leading cause of cancer mortality. Direct-acting antivirals (DAAs), with sustained virological response rates of more than 95% depending on the genotype (Li and De Clercq, 2017) have dramatically improved disease outcomes. However, reinfection remains possible and WHO estimates annual increase by two million cases per year, mainly due to injection drug use (Zibbell et al., 2015). Moreover, DAAs show limitations that include high cost and limited access to therapy, treatment failure, adverse effects and HCC-risk persistence in patients with advanced liver disease (Baumert et al., 2017). Finally, resistance-associated mutations are rapidly occurring worldwide against current DAAs (Palanisamy et al., 2018). Therefore, therapeutic alternatives are needed, especially for HCV-infected patients with end-stage liver disease like decompensated cirrhosis and HCC (Cheung et al, 2016; Lens et al, 2017). Because DAAs act after infection has been established, they do not inhibit primary infection of the graft. Inhibitors of virus entry could be an attractive strategy to prevent liver graft reinfection and could also allow for the transplantation of organs from HCV positive donors such as kidney, heart, and liver (Colpitts et al., 2017) helping to alleviate the urgent demand for donor organs with more than six times as many people on the waiting list than actual transplantations performed (UNOS, 2016). In a very early pre-DAA study, 50% of recipients of transplanted organs from HCV positive donors became HCV positive (Pereira et al., 1991). A very recent study using DAAs reported promising results but long term effects, including the risk of HCC, still need to be determined (Reese et al., 2018). A complementary approach that eliminates the risks and disease-burden associated with HCV infection could be a very useful addition to existing therapy options.

HCV host cell entry is a well-characterized process that involves the two viral envelope glycoproteins, E1 and E2, as well as several cellular factors that can be targeted by small molecule inhibitors or

antibodies. Entry inhibitors offer several advantages. Due to different modes of action they can synergize with DAAs to reduce treatment duration and costs (Xiao et al., 2015). In contrast to DAAs, they block viral infection before persistence is established and thus also prevent potential long-term effects of HCV infection. In addition, several reports suggest that they might be able to cure established HCV infection (Colpitts et al., 2018; Mailly et al., 2015). Host-targeting agents (HTAs) are also expected to have a high genetic barrier to resistance due to the low mutation rate of host proteins. Combinations of two inhibitors targeting virus entry will further increase the barrier for viral escape rendering treatment failure highly unlikely (Khera et al., 2017; Vercauteren et al., 2014). Currently, several approaches targeting both viral as well as host proteins have reached early stages of clinical development (Crouchet et al., 2018; Zeisel and Baumert, 2017; Zeisel et al., 2015). Here we developed a complementary strategy using broadly neutralizing antibodies (bNAbs) and HTAs to prevent HCV infection. A combination therapy using bNAbs targeting the virus and MAbs against HCV entry factors would efficiently prevent re-infection of the liver graft in HCV-infected transplant recipients and could allow for the transplantation of organs from HCV positive donors into HCV-negative recipients. We previously described a neutralizing IgG_1 human monoclonal antibody (HMAb) named HC84.26.WH.5DL to a conserved epitope on the surface of the HCV E2 protein (Keck et al., 2016). This HMAb conferred broad protection against different HCV genotypes and is not associated with viral escape. Furthermore, we developed a humanized IgG_4 MAb to the extracellular domain of claudin-1 (CLDN1) named H3L3, which prevents and cures persistent HCV infection *in vivo* (Colpitts et al., 2018; Mailly et al., 2015).

To assess synergistic effects of HC84.26.WH.5DL and H3L3, the individual and the combined antiviral activities of these two HMAbs were determined as described previously (Keck et al., 2016, Colpitts et al 2018) (Figure 1A). Although each antibody blocked recombinant cell culture-derived HCV (HCVcc) Luc-Jc1 infection of Huh7.5.1 cells, the combination proved to be more effective even at low doses. The combination of low concentrations of H3L3 with 0.002 to 20 µg HC84.26.W.5DL greatly increased

the antiviral activity compared to the individual antibody doses (Figure 1A). To assess synergy, we compared the combination indices (CI) (Zhao et al., 2004) of H3L3 and HC84.26.WH.5DL at different concentrations. CI values <0.9 indicate synergy, 0.9 – 1.1 = additive and values >1.1 indicate antagonism. The model employed to calculate synergistic, additive or antagonistic effect is most apparent at the linear portion of the curve and not possible at saturation. Combinations of sub-neutralizing combinations of H3L3 (0.001 – 0.1 µg) and HC84.26.WH.5DL (0.002 – 2 µg) showed CI values that were well below 0.9, indicating strong synergistic inhibition (Figure 1B). Combinations of H3L3 and HC84.26.WH.5DL reached CI values of as low as 0.03 for 0.001 µg/ml H3L3 and 0.002 or 0.02 µg/ml HC84.26.WH.5DL, indicating a synergistic inhibition of infection at low antibody concentrations. Synergy was further confirmed using the Prichard-Shipman method that compares the calculated expected inhibition with the observed values (Prichard and Shipman, 1990). Observed values that are >20% above the calculated inhibition are considered synergistic, while calculated values >20% but below the calculated inhibition are considered antagonistic. In line with the CI plot, combinations of low concentrations of anti-E2 and H3L3 showed potent synergistic effects, with observed inhibition >30% above the expected values (Figure 1C). Taken together, these data indicate that even though high antibody concentrations are required to get full inhibition, a synergistic effect could persist at low concentration while antibody concentrations decline.

To assess the suitability of the combination for antiviral therapy *in vivo*, we employed human liver chimeric mice, which are the most relevant *in vivo* model to explore the efficacy of bNAbs (Desombere et al., 2016; Desombere et al., 2017; Law et al., 2008; Meuleman et al., 2011; O'Shea et al., 2016) as well as antibodies targeting host proteins, such as anti-CD81 (Meuleman et al., 2008), anti-SR-BI (Lacek et al., 2012; Meuleman et al., 2012; Vercauteren et al., 2014), anti-CLDN1 (Colpitts et al., 2018; Fukasawa et al., 2015; Mailly et al., 2015) and anti-OCLN (Shimizu et al., 2018) antibodies. Six-week old FRG-NOD Ad-uPa transduced (Wilson et al., 2014) mice kept under NTBC were intrasplenically transplanted with 10^6 cryopreserved primary human hepatocytes (PHH) (Corning).

Liver repopulation was determined using a human albumin-specific ELISA (Bethyl), as previously described (Mailly et al., 2015). The mice were intraperitoneally injected with 500 µg of HC84.26.WH.5DL (Keck et al., 2016) alone or in combination with either 50, 100, 250 or 500 µg of H3L3 (Colpitts et al., 2018) or with the human IgG4 control Motavizumab (Figure 2A,B; Evitria) or the IgG_1 control HMAb R04 (Figure 2C, D; Keck et al., 2012) to a combined mAb dose of 1000 µg per mouse. H3L3 was also used alone at the same concentrations. The mice were then inoculated with 10^5 infectious units (IU) of genotype 1b HCV infectious serum (isolated from a chronically HCV infected patient, one mouse passage) at day 0. Viral loads were quantified using the Abbott RealTime™ HCV assay (Abbott).

We found that H3L3 reduced viral loads with doses starting at 100 µg per injection and complete protection was observed at doses ≥ 250 µg H3L3 (Figure 2A), which is lower than previously reported (Colpitts et al., 2018; Fukasawa et al., 2015). Five hundred µg of anti-E2 delayed infection of humanized liver-chimeric mice with viral loads reaching the levels of the mock control three weeks post-infection (Figures 2C and 2D). To assess the combination of H3L3 and HC84.26.WH.5DL, 50 µg H3L3 was tested alone and in combination with 500 µg HC84.26.WH.5DL (Figures 2C and 2D). However, no additional antiviral effect was observed (Figure 2B) and as expected, 50 µg H3L3 alone also had no antiviral activity (Figures 2C and 2D). The combination of 100 µg H3L3 and 500 µg HC84.26.WH.5DL, conferred complete protection to four out of five mice three weeks post-infection indicating a strong beneficial effect of the combined HMAbs (Figure 2B). The single mouse that was not completely protected increased the average viral load (Figure 2A). Higher doses of H3L3 and anti-E2 conferred complete protection from infection with the GT1b infectious serum without any observed escape, confirming their suitability for antiviral therapy (Figure 2A). Analysis of human albumin levels by ELISA showed that protection was achieved without apparent cytotoxic effects (Figure 2E). Analysis of HMAb levels by IgG specific ELISA (human IgG1 Ready-Set Go kit, Invitrogen) revealed

that lower HMAb titers were not responsible for the viral relapse. as there were no obvious differences between the mice (Figure 2F).

Another key challenge of HCV antivirals is the rapid development of neutralization resistant variants. Compared to the high variability of the viral entry machinery, the variability of host factors required for HCV infection is low. To assess the suitability of anti-E2 and H3L3 antibodies to prevent HCV infection during liver transplantation, we analyzed their antiviral activity against a difficult-to-neutralize HCV variant, VL, that was isolated post-liver transplantation from a chronically HCV-infected patient (Fafi-Kremer et al., 2010). In order to directly confirm the efficacy of the combined HMAbs in the most clinically relevant cell culture system, PHH were infected with HCV pseudoparticles bearing the envelope proteins of the VL strain (Fafi-Kremer et al., 2010), alongside a second partially resistant isolate, HCV-J GT1b (Kato et al., 1990), as well as a well characterized GT3 isolate (S52). (Figure 3). We show that the combination also proved to be effective in blocking host cell entry of the difficult-to-neutralize VL variant, of the HCV-J isolate and of the GT3 strain into PHH. All three variants, in particular the transplant escape variant VL and the GT3 isolate S52, were most efficiently neutralized, thus confirming the validity of our approach. A potential limitation of our study is that only a GT1b infectious serum was tested in the humanized liver-chimeric mice. However, both HC84.26.WH.5DL and H3L3 were shown to be effective against multiple genotypes, including GT3 and different neutralization-resistant strains (Colpitts et al., 2018; Keck et al., 2016). This suggests that the combination of HC84.26.WH.5DL and H3L3 will be effective even against strains escaping from the neutralizing response, as regularly observed during liver transplantation.

An exploratory clinical study employing an anti-envelope HMAb and a single DAA demonstrated that peri-transplant immunoprophylaxis combined with a single oral DAA immediately after transplantation can efficiently prevent HCV recurrence (Smith et al., 2017). This study provided a proof-of-concept for the use of an HCV entry inhibitor in combination with a DAA during an organ transplantation setting. In addition, the risks of DAA failure, the emergence of resistance mutations and the remaining risk for

HCC development (El-Serag et al., 2016) dictate the need to investigate complementary approaches to prevent HCV infection in organ transplantation settings (Colpitts et al., 2017). Prevention of infection rather than curative treatment will improve treatment outcomes and significantly reduce treatment duration and associated costs (Colpitts and Baumert, 2016). Combinations of DAAs with entry inhibitors have shown to greatly reduce the risk of viral escape, highlighting the importance of combination therapies (Khera et al., 2017; Vercauteren et al., 2016; Xiao et al., 2015) Thus, combinations of HCV entry inhibitors open up a promising perspective to shorten waiting times of patients requiring transplantation by allowing for transplantation of organs previously deemed unsuitable. In conclusion, the combination of two or more entry inhibitors could be a highly valuable strategy not only to prevent post-transplantation reinfection events but might also help to alleviate the urgent demand for organ transplants by allowing organ graft from HCV positive donors while drastically reducing the risk of viral escape.

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Figure Legends

Figure 1. Synergistic inhibition of HCV infection by anti-E2 and anti-CLDN1 HMAbs (A) Huh7.5.1 cells were preincubated for 1h with serial concentrations of anti-CLDN1(0.001, 0.01, 0.1, 1 or 10µg/ml) and anti-E2 HMAbs (0.002, 0.02, 0.2, 2 or 20 µg/ml) before incubation with HCVcc Luc-Jc1 in the presence of both compounds. Anti-E2 HMAb was also used alone at the same concentrations as anti-CLDN1 HMAb. After 72h, HCVcc infection was analyzed by luciferase activity as described (Fofana et al., 2012). HCVcc infections in presence of anti-CLDN1 HMAb combined with anti-E2 HMAb are presented. Results are expressed as percentage of infection relative to Ctrl. Means ± SD from three independent experiments performed in duplicate are shown. Fitting curves were determined using Graphpad Prism 6 software. (B) Huh7.5.1 cells were preincubated for 1h with serial concentrations of anti-CLDN1 (0.001, 0.01, 0.1 or 1 µg/ml) and anti-E2 HMAbs (0.002, 0.02, 0.2, 2 or 20 µg/ml) before incubation with HCVcc Luc-Jc1 in the presence of both compounds. After 72h, HCVcc infection was analyzed by luciferase activity as described (Fafi-Kremer et al., 2010) and combination indexes (CI) were determined as described (Xiao et al., 2015). CI <0.9, 0.9–1.1 and >1.1 indicates synergy, additivity and antagonism, respectively (Zhao et al., 2004). Means from two independent experiments performed in duplicate are shown. (C) Synergy was confirmed using the Prichard and Shipman method. Means from three independent experiments performed in duplicate are presented. A surface >20% above the zero plane indicates synergy and a surface <20% below the zero plane indicates antagonism.

Figure 2. Prevention of HCV infection in humanized liver-chimeric FRG-NOD mice by a combination of anti-E2 and anti-CLDN1 HMAbs. Six-week old $Fah^{-/-}$ $Rag2^{-/-}$ IL2Rγc non-obese diabetic (FRG-NOD) Ad-uPa transduced (Wilson et al., 2014) mice kept under NTBC (Nitisinone, SOBI) were intrasplenically transplanted with 10^6 cryopreserved primary human hepatocytes (Corning). Mice selected for experiments were cycled with NTBC at 8 mg/L for 3

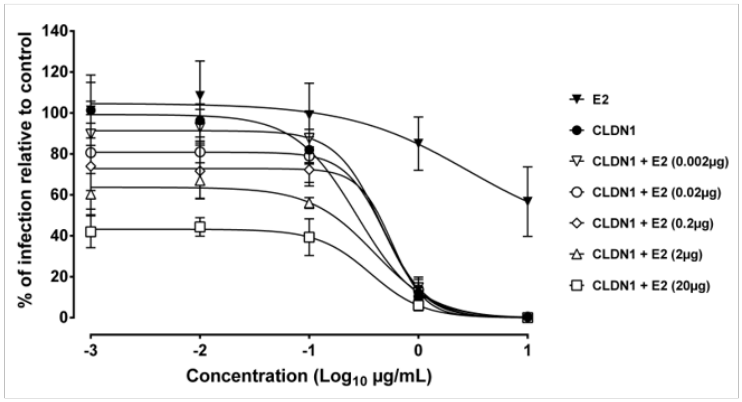
days every 3 weeks. Humanized liver chimeric FRG-NOD mice were intraperiteonally injected at day -1, 1 and 4 (indicated by the arrows) with 500 µg of HC.84.26.WH.5DL anti-E2 HMAb (Keck et al., 2016) alone or in combination with either 50, 100, 250 or 500 µg of H3L3 humanized anti-CLDN1 (Colpitts et al., 2018) or a hIgG4 control MAb (Motavizumab, A-B) or hIgG1 (R04, C-D) up to a combined dose of 1000 µg. Anti-CLDN1 HMAb was also used alone at the same concentrations. The mice were then inoculated with 10^5 IU of genotype 1b HCV infectious serum at day 0 (*). Viral loads were analyzed using the Abbott RealTime™ HCV assay (Abbott). Given the 1:100 serum dilution, the limit of quantification (LOQ) is 1200 UI/ml (i.e 5160 copies/ml). (A) and (C) show the average viral loads of all mice within one group while (B) and (D) show data for individual mice. In (C) and (D), H3L3 (CLDN1) was tested at 50 µg either alone or in combination with 500 µg of HC.84.26.WH.5DL. Control MAb was at 1000 µg/mouse. (E) Albumin levels of mice receiving the highest anti-CLDN1 HMAb doses were analyzed using an albumin-specific ELISA (Bethyl). (F) The anti-E2 hIgG1serum concentration was determined in the group E2 + CLDN1-100, showing a relapsing animal, using a hIgG1-specific ELISA (Invitrogen). The star (*) indicates the anti-E2 HMAb serum concentration for the relapsing mouse. Data below LOQ (grey area) are considered HCV RNA negative. (F) Analysis of hIgG1 levels by specific ELISA of mice receiving 100 µg H3L3 in combination with 500 µg HC84.26.WH.5DL. Stars indicate mice with viral breakthrough.

Figure 3. Efficient neutralization of difficult-to-neutralize variants by anti-E2 and anti-CLDN1 HMAbs. Primary human hepatocytes (PHH) were incubated with combination of anti-CLDN1 HMAb at 10 µg/ml and anti-E2 HMAb at 2 µg/ml for 1h at 37°C before infection with HCV pseudoparticles (HCVpp) of genotype 1b (HCV-J), genotype 3a (S52) and of an HCV escape variant (VL) selected during liver transplantation and characterized by efficient cell entry and poor neutralization. HCVpp infection of PHH was assessed by measurement of luciferase

activity 72h post-infection. Results represent means ± SEM of three experiments performed in triplicate. ** = $p<0.01$, ns = non-significant. One-sided student's t-test.

Figure 1

A


% of infection relative to control
140
120
100
80
60
40
20
0
-3
-2
-1
0
1
Concentration (Log10 µg/mL)
E2
CLDN1
CLDN1 + E2 (0.002µg)
CLDN1 + E2 (0.02µg)
CLDN1 + E2 (0.2µg)
CLDN1 + E2 (2µg)
CLDN1 + E2 (20µg)

B

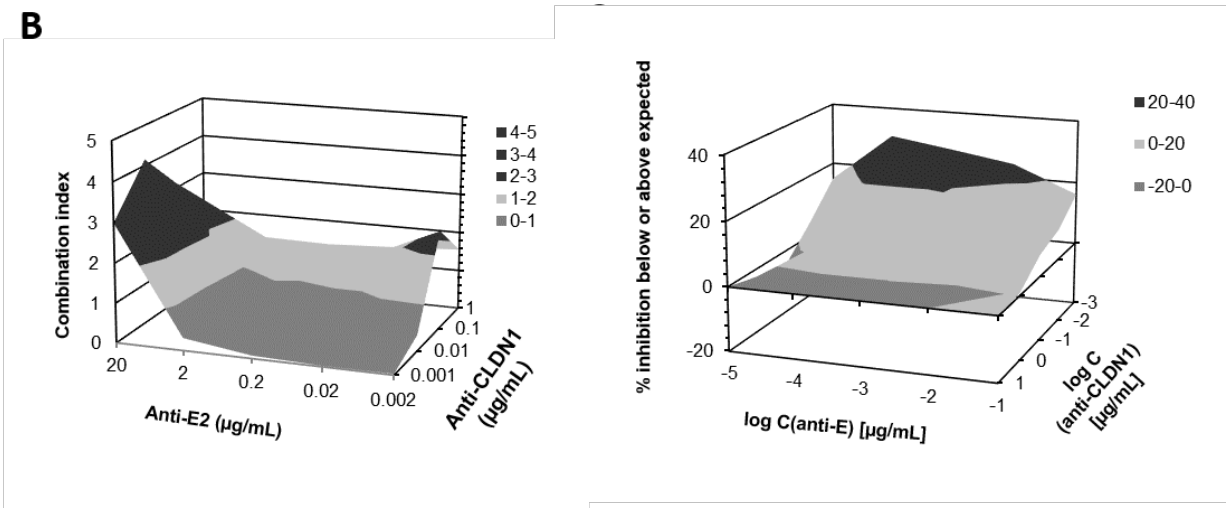

Combination index
5
4
3
2
1
0
4-5
3-4
2-3
1-2
0-1
20
2
0.2
0.02
0.002
Anti-E2 (µg/mL)
1
0.1
0.01
0.001
Anti-CLDN1 (µg/mL)
% inhibition below or above expected
40
20
0
-20
20-40
0-20
-20-0
-5
-4
-3
-2
-1
log C(anti-E) [µg/mL]
-3
-2
-1
0
1
log C (anti-CLDN1) [µg/mL]

Figure 2

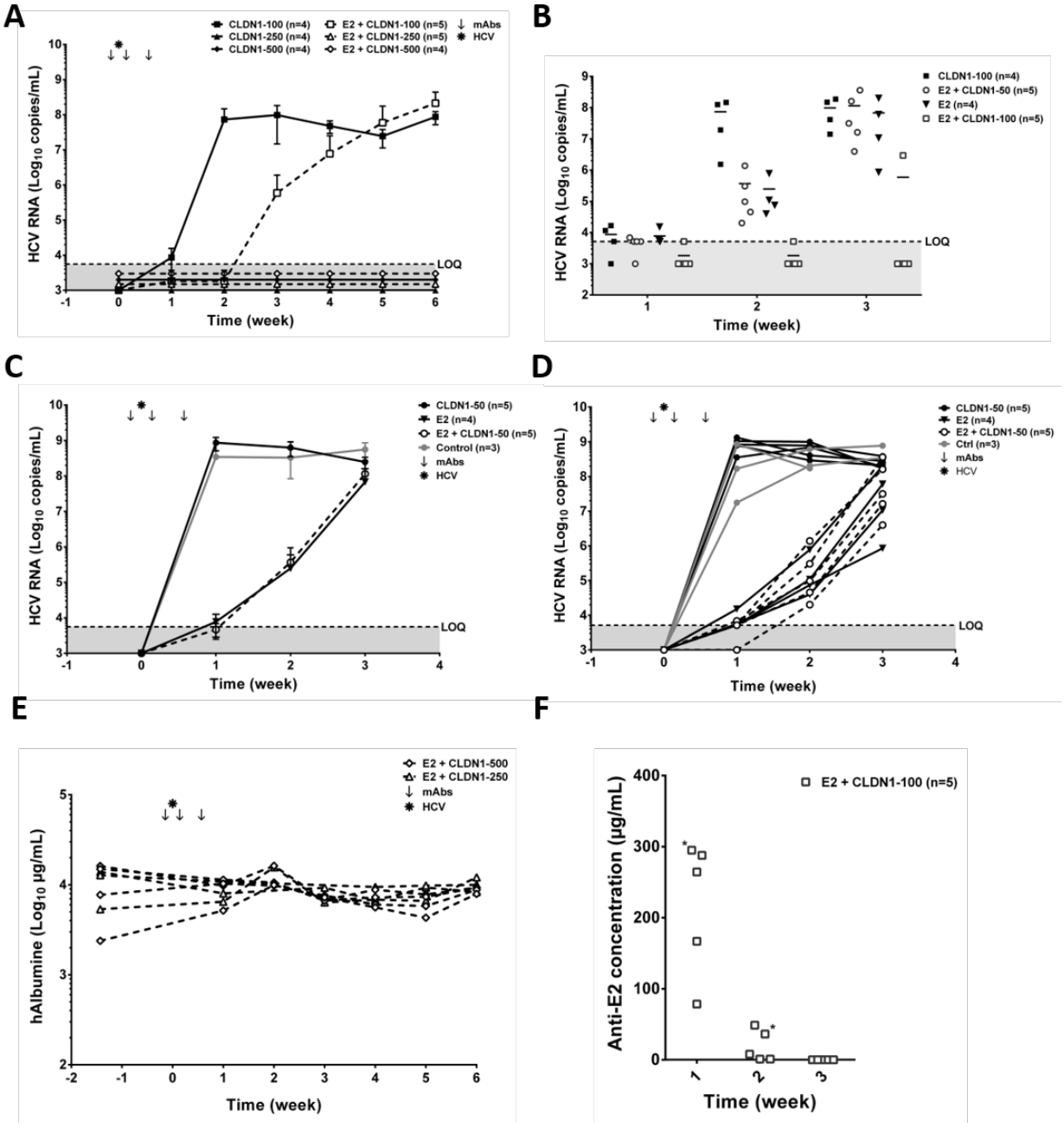
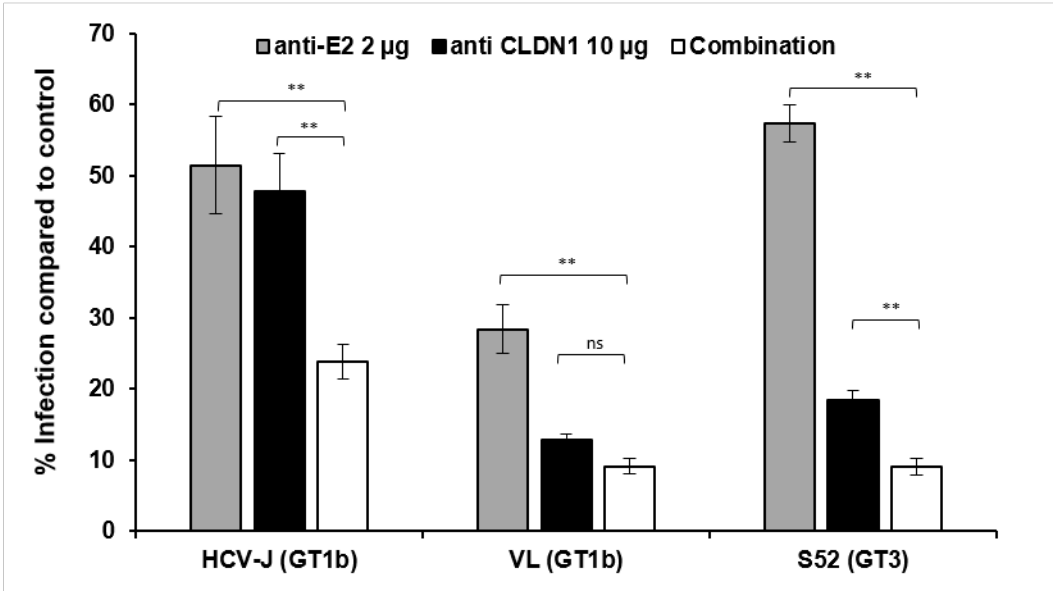

A
CLDN1-100 (n=4)
CLDN1-250 (n=4)
CLDN1-500 (n=4)
E2 + CLDN1-100 (n=5)
E2 + CLDN1-250 (n=5)
E2 + CLDN1-500 (n=4)
mAbs
HCV
HCV RNA (Log10 copies/mL)
LOQ
Time (week)
B
CLDN1-100 (n=4)
E2 + CLDN1-50 (n=5)
E2 (n=4)
E2 + CLDN1-100 (n=5)
HCV RNA (Log10 copies/mL)
LOQ
Time (week)
C
CLDN1-50 (n=5)
E2 (n=4)
E2 + CLDN1-50 (n=5)
Control (n=3)
mAbs
HCV
HCV RNA (Log10 copies/mL)
LOQ
Time (week)
D
CLDN1-50 (n=5)
E2 (n=4)
E2 + CLDN1-50 (n=5)
Ctrl (n=3)
mAbs
HCV
HCV RNA (Log10 copies/mL)
LOQ
Time (week)
E
E2 + CLDN1-500
E2 + CLDN1-250
mAbs
HCV
hAlbumine (Log10 µg/mL)
Time (week)
F
E2 + CLDN1-100 (n=5)
Anti-E2 concentration (µg/mL)
Time (week)

Figure 3


anti-E2 2 µg
anti CLDN1 10 µg
Combination
% Infection compared to control
70
60
50
40
30
20
10
0
**
**
**
ns
**
HCV-J (GT1b)
VL (GT1b)
S52 (GT3)