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3 **Inhibition of hepatitis C virus infection by anti-Claudin antibodies is**
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5 **mediated by neutralization of E2-CD81-Claudin 1 association(s)**
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FOOTNOTES

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Abbreviations: BC - bile canalicular surface; CLDN1 - claudin-1; ConA - concanamycin A, CTRL - control; EL1 and 2 - extracellular loops 1 and 2; IFN γ - interferon-gamma; FRET - fluorescence resonance energy transfer; HCV - hepatitis C virus; HCVcc - cell culture-derived HCV; HCVpp - HCV pseudotype particles; RLU - relative light units; PI - pre-immune serum; SR-BI - scavenger receptor class B type I; TJ - tight junction

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ABSTRACT

The tight junction protein claudin-1 (CLDN1) has been shown to be essential for hepatitis C virus (HCV) entry – the first step of viral infection. Due to the lack of neutralizing anti-CLDN1 antibodies, the role of CLDN1 in the viral entry process is poorly understood. In this study, we produced antibodies directed against the human CLDN1 extracellular loops by genetic immunization and used these antibodies to investigate the mechanistic role of CLDN1 for HCV entry in an infectious HCV cell culture system and human hepatocytes. Antibodies specific for cell surface expressed CLDN1 specifically inhibit HCV infection in a dose-dependent manner. Antibodies specific for CLDN1, scavenger receptor B1 and CD81 show an additive neutralizing capacity compared to when either agent was used alone. Kinetic studies with anti-CLDN1 and anti-CD81 antibodies demonstrate that HCV interaction(s) with both entry factors occur at a similar time in the internalization process. Anti-CLDN1 antibodies inhibit the binding of envelope glycoprotein E2 to HCV permissive cell lines in the absence of detectable CLDN1-E2 interaction. Using fluorescent labelled entry factors and fluorescence resonance energy transfer (FRET) methodology, we demonstrate that anti-CLDN1 antibodies inhibit CD81-CLDN1 association. In contrast, CLDN1-CLDN1 and CD81-CD81 associations were not modulated. Taken together, our results demonstrate that antibodies targeting CLDN1 neutralize HCV infectivity by reducing E2 association with the cell surface and disrupting CD81-CLDN1 interactions. In conclusion, these results further define the function of CLDN1 in the HCV entry process and highlight new antiviral strategies targeting E2-CD81-CLDN1 interactions.

INTRODUCTION

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6 With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a
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8 major impact on public health. HCV is a hepatotropic virus that causes persistent
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10 infection in the majority of infected individuals (1). Therapeutic options for chronic
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13 5 infection are limited and a vaccine is not available (2).
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18 HCV entry into hepatocytes is the first step of the viral life cycle resulting in
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20 productive viral infection (3, 4). Furthermore, HCV entry is a major target of host
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22 neutralizing responses (5-7) and a target for antiviral immunopreventive and
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24 10 therapeutic strategies (for review see (4, 8)). Viral entry is believed to be mediated by
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26 the viral envelope glycoproteins E1 and E2 and several host entry factors. These
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28 include heparan sulfate, tetraspanin CD81, scavenger receptor class B type I (SR-BI)
29
30 (3) and the tight junction (TJ) proteins claudin-1 (CLDN1) (9) and occludin (10, 11).
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32 As none of these host cell surface factors alone is able to promote HCV entry, the
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34 15 interaction of HCV and its target cells leading to the internalization of the virus is
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36 believed to be a multistep process involving the interplay of several host cell factors
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41 (3, 4, 8).
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47 Evans and colleagues reported that CLDN1 is essential for HCV infection (9).
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49 20 Subsequent studies demonstrated that CLDN-6 and -9 are also able to mediate HCV
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51 entry in non permissive cell lines (12, 13). CLDNs are critical components of TJs that
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53 regulate paracellular permeability and polarity and have a tetraspanin topology with
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55 four transmembrane domains, two extracellular and one intracellular loops, and N-
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57 and C-terminal cytoplasmic domains (14). CLDN1 extracellular loop 1 (EL1) is
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60 25 required for HCV entry (9) and is involved in barrier function and contributes to pore

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3 formation between polarized cells (15). Mutagenesis studies in non-polarized 293T
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5 cells demonstrate that CLDN1 enrichment at cell-cell contacts may be important for
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7 HCV entry (16). We (17, 18) and others (16, 19, 20) using a variety of imaging and
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9 biochemical techniques reported that CLDN1 associates with CD81. However, due to
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12 5 the lack of neutralizing anti-CLDN1 antibodies targeting extracellular epitopes, the
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14 exact role of CLDN1 in the viral entry process is poorly understood.
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20 In this study, for the first time we produced anti-CLDN1 antibodies that bound
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22 to cell surface expressed CLDN1 and inhibited HCV infection. The anti-CLDN1
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24 10 antibodies inhibit HCV E2 glycoprotein interaction with permissive hepatoma cells
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26 and fluorescence resonance energy transfer (FRET) between CD81-CLDN1 co-
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28 receptor complexes supporting a model where CLDN1 potentiates CD81 interaction
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30 with the virus and facilitates particle internalization.
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MATERIALS AND METHODS

Cells. Human Huh7 (5), Huh7.5.1 (21), HepG2 (18), 293T (5), Bosc (22), Caco-2 (23), and rat BRL-3A hepatocyte-derived cell lines (24) were propagated in DMEM/10% foetal bovine serum. 293T/CLDN1 cells were obtained by stable transfection of 293T cells with a pcDNA3.1 vector encoding CLDN1 cDNA. DMSO-mediated differentiation of Huh7.5.1 cells was performed as described (25). Primary human hepatocytes were isolated from liver resections from patients at the Strasbourg University Hospitals with approval from the Institutional Review Board (26, 27). In brief, liver specimen were perfused with calcium-free HEPES buffer supplemented with 0.5 mM EGTA (Fluka) followed by perfusion with HEPES buffer supplemented with 0.05% collagenase (Sigma) and 0.075% CaCl₂ at 37 °C. Following washing of cells with PBS and removal of nonviable cells by Percoll (Sigma) gradient centrifugation, freshly isolated hepatocytes (3 x 10⁵ cells/well) were plated in 24-well-plates pre-coated with collagen (Biocoat, BD Biosciences) and allowed to adhere in William's E medium (Sigma Aldrich) containing 1 % Glutamax (Gibco), 1 % ITS (insulin transferrin selenium, Gibco), 10⁻⁷ M dexamethasone (Sigma), 0.15% bovine serum albumine (Sigma) and 10% fetal calf serum (PAN Biotec).

Antibodies. Anti-CLDN1 antibodies were raised by genetic immunization of Wistar rats using a human CLDN1 cDNA expression vector. For screening, Bosc cells transfected with pCMV-SPORT6 or pCMV-SPORT6/CLDN1 were incubated with anti-CLDN1 or pre-immune serum and analyzed for cell surface CLDN1 expression by flow cytometry as described (28). Purified IgG from rat anti-CLDN1 serum were obtained by MAbTrapTM kit (GE Healthcare). To analyze cross-reactivity of antibodies with other members of the CLDN family, 293T cells were transfected to express

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3 AcGFP tagged CLDN1, 4, 6, 7, 9, 11, 15 and 17 or chimeric CLDN1/7 (described in
4 (9)) and 48 h later stained with rat anti-CLDN1 antibodies and Alexa-633 coupled
5 anti-Rat Ig (Invitrogen). Cells were imaged by flow cytometry and data analyzed by
6 FLOWJo. Polyclonal rat anti-SR-BI or CD81 antibodies were obtained by genetic
7 immunization as described (26). R-phycoerythrin-conjugated and Cy5-conjugated
8 anti-rat IgG were from Jackson ImmunoResearch Laboratories, mouse IgG from
9 Caltag, mouse anti-CD81 (JS-81) from BD Biosciences.

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12 **Imaging studies of cell surface CLDN1.** Living Huh7.5.1 cells were incubated with
13 pre-immune or anti-CLDN1 serum (1/50) and a Cy5-conjugated anti-rat secondary
14 antibody (1/300; Jackson ImmunoResearch). Polarized Caco-2 cells, as described in
15 (23), were fixed in 3% paraformaldehyde, permeabilized with saponin and stained
16 with polyclonal anti-CLDN1 (1/50) or control serum. Following staining, cells were
17 fixed, mounted and observed using a Leica TCS SP2 CLSM (for Huh7.5.1) or a Zeiss
18 Cell Axio Observer Z1 microscope (for Caco-2).

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21 **Determination of tight junction barrier function.** To determine the functionality of
22 TJs and whether they restrict the paracellular diffusion of solutes from the bile-
23 canalicular (BC) lumen to the basolateral medium (barrier function), HepG2 cells
24 were treated with either control (PBS), rat anti-CLDN1, rat control serum or IFN γ and
25 incubated with 5 mM 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) at
26 37°C for 10 minutes to allow internalization and translocation to BC lumen by MRP2.
27 After washing with PBS, the capacity of BC lumens to retain CMFDA was analyzed
28 as described (18).

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3 **HCVcc production and infection.** HCVcc (Luc-Jc1 or Jc1 strains) were generated
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5 as described (6, 26, 29). For infection experiments, Huh7.5.1 cells were pre-
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7 incubated in the presence or absence of antibodies for 1 h at 37°C and infected at
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9 37°C for 4 h with HCVcc. 48 h later HCV infection was analyzed in cell lysates by
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11 quantification of luciferase activity or viral RNA (6, 26, 29, 30). Kinetic studies in the
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13 5 presence of antibodies or inhibitors were performed as described (6, 26, 29, 30).
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20 **HCV pseudoparticle (HCVpp) production and infection.** Infection of 293T/CLDN1
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22 or Huh7.5.1 cells with MLV-based HCVpp in kinetic assays was performed as
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24 described (5, 6). Primary hepatocytes were infected with HIV-based HCVpp
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26 expressing envelope glycoproteins of strains HCV-J (genotype 1b), JFH-1 (genotype
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28 2a), UKN3A.1.28 (genotype 3a) and UKN4.21.16 (genotype 4) (described in (5))
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30 containing a luciferase reporter element. One day following hepatocyte isolation and
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32 plating, hepatocytes were washed with PBS and pre-incubated with rat anti-CLDN1
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34 or control serum (1/50) for 1 hour at 37°C in William's E medium. Then, HCVpp were
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36 15 added for 3 hours at 37°C. Following infection, the supernatant was removed and
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38 replaced by fresh William's E medium. HCVpp infection was assessed by
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40 measurement of luciferase activity 72 hours post-infection as described (6, 26).
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48 20 **Cellular binding of HCV envelope glycoproteins E1 and E2.** Production and
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50 binding of C-terminally truncated envelope glycoproteins has been described (6, 24).
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52 For the study of E2-entry factor interaction, CHO cells were transfected with pcDNA3
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54 based expression vectors encoding SR-BI, CD81 or CLDN1 as described (31).
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56 Expression of entry factors was assessed by flow cytometry using anti-receptor
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58 antibodies as described (31). For the study of envelope glycoprotein binding in the
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3 presence of anti-receptor antibodies, Huh7.5.1 cells (21) or rat BRL-3A cells stably
4 expressing human SR-BI, CD81 and CLDN1 (24) were pre-incubated 1 h at RT with
5 rat anti-SR-BI, -CLDN1, -CD81 serum (1/100) or mouse anti-human CD81 (JS-81; 5
6 $\mu\text{g}/\text{mL}$) or control antibodies (1/100 or 5 $\mu\text{g}/\text{mL}$). Recombinant E2 (30 μL cell culture
7 supernatant) or E1 (10 $\mu\text{g}/\text{mL}$) was added to cells for 1 h at RT. Following washing
8 with PBS, bound envelope glycoproteins were detected using flow cytometry and
9 human anti-E1 (IGH526 (6)) or mouse anti-His (RGS-His, Qiagen) and PE-
10 conjugated secondary antibodies (24, 28). For quantitation of HCVcc binding,
11 Huh7.5.1 cells were pre-incubated with heparin (250 $\mu\text{g}/\text{mL}$), rat anti-CLDN1 (1/50) or
12 control serum (1/50) for 1 hour at 37°C prior to incubation with HCVcc (Jc1 strain)
13 which had been partially purified from cell culture supernatants using sucrose
14 gradient ultracentrifugation. Following incubation with HCVcc, non bound HCVcc
15 were removed by washing of cells with PBS. Binding of HCVcc was then quantified
16 by RT-PCR of cell bound HCV RNA as described (9).
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39 **Receptor association using fluorescence resonance energy transfer (FRET).**
40 Homotypic and heterotypic interactions of CD81 and CLDN1 were analyzed as
41 described (17, 18). Briefly, 293T cells transduced to express AcGFP and DsRED
42 tagged CD81 and CLDN1 were grown on glass coverslips and fixed in ice-cold
43 methanol. The cells were imaged on a Zeiss meta head LSCM, with microscope
44 settings optimized for each fluorescent protein to obtain the highest signal-to-noise
45 ratio. For FRET analysis, the gradual acceptor photobleaching method of FRET was
46 used, which entailed photobleaching the DsRED fluorophore gradually over time
47 while monitoring AcGFP fluorescent intensity (17). After background and cross-talk
48 correction, any increase in AcGFP intensity following DsRED photobleaching is due
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3 to FRET between proteins, implying a distance of less than 10 nm. The percent
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5 FRET is defined as the number of pixels that display FRET over the total number of
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7 pixels analyzed at the plasma membrane of the cells (17). The data from 10 cells
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9 were normalized and the localized expression calculated.
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15 **Statistical analysis.** Results are expressed the mean \pm standard deviation (SD).
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17 Statistical analyses were performed using Student's *t* test with a *P* value of <0.05
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19 being considered statistically significant.
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RESULTS

Production of antibodies directed against cell surface CLDN1. To investigate the role of CLDN1 in HCV infection, we produced polyclonal anti-CLDN1 antibodies by genetic immunization and screened for reactivity with cell surface expressed CLDN1.

5 Antibodies were selected for their ability to bind non-permeabilized Bosc cells transfected to express human CLDN1. Bosc cells are 293T-derived ecotropic packaging cells (22) which do not express endogenous CLDN1 (data not shown). As shown in Fig. 1A, incubation of Bosc cells expressing human CLDN1 with polyclonal anti-CLDN1 sera resulted in a specific interaction with CLDN1 extracellular domains (Fig. 1A). To confirm the specific interaction of anti-sera with CLDN1 we generated 293T cells stably expressing human CLDN1 (Fig. 1B). Incubation of 293T/CLDN1 cells with rat polyclonal anti-CLDN1 antibodies resulted in a specific interaction of these antibodies with human CLDN1 (Fig. 1B). These data demonstrate that anti-CLDN1 antibodies obtained by genetic immunization specifically bind to the extracellular loops of human CLDN1 expressed on the cell surface. Using 293T cells transfected with tagged AcGFP tagged CLDN1, 4, 6, 7, 9, 11, 15 and 17 or chimeric CLDN1/7, we show that anti-CLDN1 antibodies demonstrate minimal or absent cross-reactivity against other members of the CLDN family (Table 1).

20 Analysis of anti-CLDN1 reactivity to chimeric CLDN1/7 expressed on the cell surface of 293T cells demonstrated that the antibodies interact strongly with CLDN7 where the N-terminal third (N1/3) or half (N1/2) was replaced with the corresponding coding region of CLDN1 (Table 1). In contrast, the antibodies did not exhibit any detectable interaction with CLDN7 where the C-terminal half (C1/2) of EL1 was replaced with the corresponding coding region of CLDN1. A reduced interaction was

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3 observed for CLDN7 expressing the entire EL2 of CLDN1 (Table 1). These data
4 demonstrate that anti-CLDN1 antibodies recognize epitopes in the N-terminal half of
5 the CLDN1 EL1 which has been shown to be required for HCV entry (9) as well as
6 EL2 epitopes (Table 1). Since antibodies failed to recognize overlapping peptides
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13 5 encoding for linear epitopes comprising the CLDN1 EL1 and 2 in an ELISA or an
14 infection assay using peptides as capture antigens (data not shown), it is likely that
15 epitopes targeted by anti-CLDN1 antibodies are conformation-dependent.
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22 To study whether anti-CLDN1 antibodies bind to CLDN1 on the cell surface of
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24 10 HCV permissive cells, Huh7.5.1 and primary human hepatocytes were incubated with
25 anti-CLDN1 and analyzed by flow cytometry. Positive staining of human Huh7.5.1
26 hepatoma cells and human hepatocytes with polyclonal anti-CLDN1 antibodies in the
27 absence of permeabilizing reagents demonstrated that these antibodies bind to
28 CLDN1 expressed on the surface of primary hepatocytes and HCV permissive cell
29 lines (Fig. 1C). To further address the specificity of antibodies, we performed CLDN1
30 knock-down experiments in Huh7.5.1 cells using a pool of three siRNAs described by
31 Evans et al. (9). CLDN1 silencing resulted in a decrease of anti-CLDN1 staining in
32 immunoblot analyses (data not shown) further confirming the specificity of the
33 antibodies.
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51 Positive staining of native cell surface CLDN1 in living, non-permeabilized
52 Huh7.5.1 cells with anti-CLDN1 antibodies was confirmed using imaging studies.
53 Interestingly, in living native Huh7.5.1 cells, the antibody appeared to localize to
54 certain areas of cell-cell contact (Fig. 1D), whereas in permeabilized Huh7.5.1 or
55 Caco-2 cells antibody staining showed a polygonal web-like structure (Fig. 1D) which
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3 was similar to previous studies using non-neutralizing anti-CLDN1 antibodies (23).
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5 CLDN1 staining appeared to be more pronounced in polarized Caco-2 cells than in
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7 non-polarized Huh7.5.1 cells (Fig. 1D). Further imaging studies are ongoing to
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9 determine the detailed subcellular localization of CLDN1 recognized by neutralizing
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11 anti-CLDN1 antibodies in HCV permissive cells.
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18 Taken together, these data demonstrate that anti-CLDN1 serum produced by
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20 genetic immunization specifically binds to the CLDN1 extracellular loops expressed
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22 on the cell surface of HCV permissive cell lines and human hepatocytes.
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27 **Anti-CLDN1 antibodies do not affect tight-junction integrity.** We previously
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29 reported that TJs impose a physical barrier and restrict viral access to receptors (23)
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31 and that complex hepatocyte-like polarity limits HCV entry (18). To investigate
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33 whether binding of anti-CLDN1 antibodies to polarized human hepatoma cells
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35 perturbed TJ integrity, we assessed the ability of TJs to restrict the paracellular
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37 diffusion of 5-chloromethylfluorescein diacetate (CMFDA) from the bile-canalicular
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39 (BC) lumen to the basolateral medium (barrier function) as previously described (18).
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41 As shown in Fig. 2, the capacity of BC lumens to retain CMFDA was similar in
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43 polarized HepG2 cells treated with rat anti-CLDN1 antibodies, rat control serum or
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45 PBS whereas CMFDA retention was reduced in IFN γ -treated HepG2 cells (Fig. 2B).
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These data suggest that anti-CLDN1 antibodies have no effect on TJ integrity.

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Neutralization of HCV infection by anti-CLDN1 antibodies. To investigate whether
anti-CLDN1 antibodies could inhibit HCV infection, Huh7.5.1 cells were infected with
chimeric J6/CF-JFH1 firefly luciferase reporter virus (Luc-Jc1) (26, 29) in the

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3 presence of anti-CLDN1 or control antibodies. Fig. 3A shows that anti-CLDN1 serum
4 inhibits Luc-Jc1 infection of Huh7.5.1 cells in a dose-dependent manner whereas the
5 control pre-immune serum had no inhibitory effect. Neutralization of HCVcc infection
6 correlated with binding of antibodies to the target cell line (Fig. 3B). To confirm that
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13 5 inhibition of Luc-Jc1 infection was mediated by anti-CLDN1 antibodies, we purified
14 IgG from rat anti-CLDN1 and pre-immune serum. As shown in Fig. 3C, anti-CLDN1
15 IgG but not control IgG markedly inhibited Luc-Jc1 HCVcc infection in a dose-
16 dependent manner. These data demonstrate that the inhibitory effect of anti-CLDN1
17 serum was mediated by anti-CLDN1 IgG and not by other substances present in the
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24 10 serum. Infection experiments using primary human hepatocytes and HCVpp
25 packaged with envelope glycoproteins from genotypes 1-4 demonstrated that anti-
26 CLDN1 blocking activity was similar for infection with HCV bearing envelope proteins
27 of other genotypes (Fig. 3D). Taken together, these findings demonstrate that
28 antibodies directed against the CLDN1 extracellular loops inhibit HCV infection in
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36 15 HCV permissive cell lines and human hepatocytes.
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41 **CLDN1 acts cooperatively with CD81 and SR-BI in HCV entry.** We previously
42 demonstrated that CD81 and SR-BI act in concert to mediate HCV entry (26). To
43 investigate whether the three host factors CLDN1, CD81 and SR-BI act in a
44 cooperative manner, we added low concentrations of anti-receptor antibodies
45 simultaneously prior to HCV infection. The use of antibody concentrations that sub-
46 maximally blocked HCV infection allowed us to observe additive or synergistic
47 effects. First, we determined the ability of combinations of two out of the three
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60 25 concomitant blocking of both CD81 and CLDN1 (Fig. 4B), SR-BI and CLDN1 (Fig.

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3 4C) or CD81 and SR-BI (Fig. 4D). This effect was not observed when control IgG or
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5 control serum was used in combination with anti-CLDN1 antibodies (data not shown).
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8 Next, we assessed the impact of synchronously blocking all three host cell factors on
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10 HCVcc infection. Fig. 4E shows an additive effect of the three antibodies used.
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13 5 Indeed, Luc-Jc1 HCVcc infection was inhibited by more than 90% after simultaneous
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15 blocking of three host cell factors at antibody concentrations that inhibited HCVcc
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17 infection between 15% and 60% when used individually. Taken together, these
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19 results suggest that CLDN1 mediates HCV entry in cooperation with CD81 and SR-
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21 BI.
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27 **CLDN1 mediates an HCV entry step closely linked to HCV-CD81 interaction.** To
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29 investigate the role of CLDN1 in the entry process, we investigated the inhibitory
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31 capacity of anti-CLDN1 antibodies in kinetic studies (26, 29). To discriminate
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33 between virus binding and post-binding events, Luc-Jc1 HCVcc binding to Huh7.5.1
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35 cells was performed for 1 h at 4°C in the presence or absence of inhibitors before the
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37 15 temperature was shifted to 37°C to initiate synchronous infection (Fig. 5A). Fig. 5B
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39 shows that similarly to anti-CD81 and anti-SR-BI, rat anti-CLDN1 inhibited Luc-Jc1
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41 HCVcc infection when added following binding of the virus to the target cell (Fig. 5B).
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43 To fine-map the entry step mediated by CLDN1, we added antibodies in side-by-side
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48 20 experiments every 20 min for up to 120 min after viral binding (Fig. 5C). The half-
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50 maximal times ($t_{1/2}$) required for anti-CD81 and anti-CLDN1 antibodies to inhibit HCV
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52 entry were +30 and +33 minutes (Fig. 5C-E; Table 2), whereas the half-maximal
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54 times for heparin was -60 minutes and for concanamycin A was +60 minutes (Fig.
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57 5C; Table 2). The time-course of anti-CLDN1 and anti-CD81-antibody-mediated
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60 25 inhibition was not significantly different, and both differed from those observed with

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3 heparin and concanamycin A (Table 2). Similar results were obtained in DMSO
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5 differentiated Huh7.5.1 (27) cells (Fig. 5E). These data support a model where
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7 CLDN1 and CD81 exert their effects at a similar time in the viral internalization
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9 process.
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14 Using Flag-tagged CLDN1 transfected 293T cells, Evans et al. reported that
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16 anti-Flag inhibition of HCVpp infection occurred at later time points compared to a
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18 CD81 specific antibody (9). These results differ from those obtained in this study that
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20 may be attributable to the experimental systems used in the two studies, including:
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24 10 293T/CLDN1 versus Huh7.5.1 cell lines, HCVpp versus HCVcc, the strain of HCV
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26 envelope glycoproteins H77 versus J6/JFH1 and the blocking antibodies (anti-
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28 CLDN1 versus anti-Flag). To further address this question, we studied the kinetics of
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30 anti-CLDN1 and anti-CD81 neutralization of HCVpp infection of 293T/CLDN1.
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33 Inhibition of HCVpp infection of 293T/CLDN1 cells by anti-CLDN1 and anti-CD81
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36 15 demonstrated a similar kinetics (Fig. 5F) to those observed for HCVcc infection of
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38 Huh7.5.1 cells (Fig. 5D, E). Thus, the different kinetic results described by Evans et
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40 al. and us are most likely not related to the experimental model system but rather
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42 related to the insertion of a Flag tag into CLDN1 (9).
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48 **Anti-CLDN1 inhibits binding of envelope glycoprotein E2 to HCV**
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50 **permissive cells in the absence of CLDN1-E2 interactions.** Next, we investigated
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52 whether anti-CLDN1 antibodies could interfere with E2 binding to permissive cell
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54 lines. Binding studies were performed using recombinant E1 and E2 glycoproteins in
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56 the presence of anti-receptor or control antibodies. As shown in Fig. 6B, anti-CD81,
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59 25 anti-SR-BI and anti-CLDN1 antibodies inhibited the binding of E2 to Huh7.5.1 cells.
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3 In contrast, pre-immune or unrelated control serum had no effect (Fig. 6A-C). Similar
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5 results were obtained for antibody inhibition of E2 binding to BRL-3A rat hepatocyte-
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8 derived cells engineered to express the three human entry co-factors, SR-BI, CD81
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10 and CLDN1 (24) (Fig. 6E). Expression of SR-BI, CD81 and CLDN1 on the cell
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13 5 surface of stably transfected BRL-3A cells was confirmed by flow cytometry and
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15 expression levels were comparable to Huh7 cells (data not shown and (24)).
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17 Interestingly, the magnitude of inhibition of E2 binding to Huh7.5.1 cells (Fig. 6C)
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19 correlated with the magnitude of inhibition of HCV infection (Fig. 3B), suggesting that
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21 inhibition of binding of E2-cell surface interactions provides a mechanism of action for
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24 10 the neutralizing activity of the anti-CLDN1 antibodies. In contrast, E1 binding was not
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26 affected by anti-CLDN1 (Fig. 6D). To investigate whether inhibition of E2 binding
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28 resulted in an inhibition of binding of infectious virions, we studied cellular binding of
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30 Jc1 HCVcc in the presence of anti-CLDN1 antibodies. Although HCVcc binding
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32 analyses were characterized by a higher inter-assay variability compared to E2
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35 15 binding studies, anti-CLDN1 antibodies markedly and significantly inhibited HCVcc
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37 binding to Huh7.5.1 cells (Fig. 6F).
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44 To study whether antibody inhibition of E2 binding to permissive cell lines was
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46 attributable to CLDN1 interactions with E2 we investigated whether CLDN1 was able
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48 20 to bind recombinant truncated glycoprotein E2. To address this question CHO cells
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50 were engineered to express human CLDN1, SR-BI or CD81 (Fig. 7A). Cell surface
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52 expression of human CD81 or human SR-BI conferred E2 binding to CHO cells (Fig.
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54 7B), whereas CLDN1 expression had no effect (Fig. 7B). These data suggest that
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56 CLDN1 does not interact directly with HCV envelope glycoprotein E2 and that
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3 antibody blocking of E2-cell surface interactions may be mediated by indirect
4 mechanisms.
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10 **Anti-CLDN1 antibodies inhibit CLDN1-CD81 co-receptor association(s).** Since

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12 5 anti-CLDN1 inhibits E2 binding to HCV permissive cells in the absence of a direct
13 CLDN1-E2 interaction (Fig. 7B), we hypothesized that anti-CLDN1 antibodies may
14 interfere with CD81-CLDN1 co-receptor complexes. To assess whether anti-CLDN1
15 alter CLDN1-CD81 association, 293T cells were transfected to express AcGFP-CD81
16 and DsRED-CD81 or AcGFP-CLDN1 and DsRED-CD81 or AcGFP-CLDN1 and
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18 10 DsRED-CLDN1 (17), incubated with pre-immune and anti-CLDN1 serum (1/100 and
19 1/400) and co-receptor interaction(s) analyzed by FRET. As shown in Fig. 8, anti-
20 CLDN1 antibodies significantly reduced FRET between CD81 and CLDN1 in a dose-
21 dependent manner. Pre-incubation of cells with control serum did not modify CD81-
22 CLDN1 co-receptor interaction(s). Inhibition of CD81-CLDN1 co-receptor interaction
23
24 15 was specific as shown by the unchanged FRET between CD81-CD81 and CLDN1-
25 CLDN1 following pre-incubation with anti-CLDN1 serum. Taken together, these data
26 suggest that anti-CLDN1 antibodies interfere with CD81-CLDN1 heterodimer
27 association.
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DISCUSSION

For the first time we report the genesis and characterization of antibodies directed against the extracellular loops of human CLDN1 which inhibit HCV infection. CLDN1 showed no evidence for a direct association with the viral envelope E1E2 glycoproteins and yet anti-CLDN1 serum inhibited E2 association with the cell surface and disrupted CD81-CLDN1 interactions. These data suggest a role for CD81-CLDN1 complexes in viral entry and highlight new antiviral strategies targeting co-receptor complex formation.

CLDN1 is an essential co-factor conferring HCV entry (9), however, the precise role of CLDN1 in the multi-step entry process remains poorly understood. Using antibodies directed against CLDN1 EL, we demonstrate a dose-dependent inhibition of viral envelope association with HCV permissive cell lines. Using transfected CHO cells expressing human HCV entry factors, we demonstrate that in contrast to CD81 and SR-BI, CLDN1 does not directly interact with envelope glycoprotein E2 at the cell surface.

Using a recent FRET-based system to study CD81-CLDN1 co-receptor association (17), we demonstrate that neutralizing anti-CLDN1 antibodies specifically disrupt CD81-CLDN1 FRET (Fig. 8). These data suggest that CD81-CLDN1 co-receptor complexes are critical for HCV entry and CLDN1 may potentiate CD81 association with HCV particles via E2 interactions. The functional relevance of the CD81-CLDN1 co-receptor complex for HCV entry is further corroborated by kinetic studies demonstrating that CD81 and CLDN1 act at a similar time point during HCV entry (Fig. 5). Although the magnitude of antibody-mediated inhibition of HCVcc

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3 infection was slightly different, the kinetics of inhibition by anti-CLDN1 and anti-CD81
4 were similar (Fig. 5C-F and Table 2).
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10 Using a HCVpp kinetic assay in 293T cells expressing Flag-tagged CLDN1
11 and anti-Flag antibody, Evans et al. observed anti-Flag antibody inhibition of HCVpp
12 5 infection at a later time point than anti-CD81, suggesting that CLDN1 has a role in
13 infection at a later time point than anti-CD81, suggesting that CLDN1 has a role in
14 late stages of the viral internalization process (9). Evans et al., reported that the
15 inhibitory activity of anti-CD81 antibody was lost much earlier than the anti-Flag
16 antibody (half-maximal inhibition at 18 and 73 min post temperature shift,
17 respectively). However, we observed a loss of anti-CLDN1 and anti-CD81 inhibitory
18 activity at similar times (half-maximal inhibition for both antibodies at +30 and +33
19 min post temperature shift respectively). Comparable results using HCVpp infection
20 of 293T/CLDN1 cells (Fig. 5F) suggest that the differences between the two studies
21 relate to the inserted Flag epitope in CLDN1 sequence or the use of an anti-Flag
22 antibody. It is conceivable that insertion of a triple Flag epitope into CLDN1 EL1 (9)
23 may alter CLDN1 trafficking and possible association with CD81 resulting in a
24 delayed inhibition of infection by anti-Flag antibody (9) compared to antibodies
25 targeting native CLDN1. We conclude that CLDN1 and CD81 entry factors act in a
26 cooperative manner in a closely linked step during HCV entry, consistent with earlier
27 reports on CD81-CLDN1 association (17-19).
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53 Taken together, our findings support a model in which viral attachment and
54 interaction with glycosaminoglycans and SR-BI promote or facilitate viral interaction
55 with CD81-CLDN1 complexes. Since anti-CLDN1 inhibit envelope glycoprotein E2
56 and virion binding to permissive cells in the absence of any detectable CLDN1-E2
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3 interactions, it is conceivable that CLDN1 association with CD81 enhances viral
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5 glycoprotein association(s) to the HCV co-receptor complex that are required for
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7 virus internalization. These results define the function of CLDN1 in the HCV entry
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9 process and highlight new antiviral strategies targeting E2-CD81-CLDN1 interactions.
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15 The development of neutralizing anti-CLDN1 antibodies may provide new
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17 therapeutic options for the prevention of HCV infection. Our data clearly demonstrate
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19 that CLDN1 is a target for HCV therapeutic intervention that may complement
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21 ongoing efforts to block intracellular replication events with inhibitors of the HCV
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23 proteases and polymerase (9). The observation that anti-CLDN1 had no effect on
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25 HepG2 permeability and TJ integrity (Fig. 2) merits further investigation into the use
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27 of anti-CLDN1 antibodies as a therapeutic for HCV infection. The production of
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29 antibodies directed against HCV entry factors such as CLDN1 may widen the future
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31 preventive and therapeutic strategies for HCV infection and ultimately be used for the
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33 prevention of HCV infection following needle stick injury or during liver
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35 transplantation. Further efforts are underway to produce monoclonal anti-CLDN1
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37 antibodies for that strategy.
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47 In conclusion, our results suggest that viral entry requires the formation of a
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49 virus-co-receptor complex including HCV E2, CD81 and CLDN1. The functional
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51 mapping of E2-CD81-CLDN1 association and its impact for HCV entry has important
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53 implications for the understanding of the very first steps of HCV infection and the
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55 development of novel antiviral strategies targeting viral entry.
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16
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For Peer Review

FIGURE LEGENDS

Fig. 1. Production of antibodies directed against human CLDN1 extracellular

domains using genetic immunization. (A) Specific binding of rat anti-human CLDN1 serum to CLDN1 expressed on the cell surface of transfected Bosc cells.

- 5 Bosc cells were transfected with pCMV-SPORT6-CLDN1 (red histograms) or control vector (pCMV-SPORT6; black histograms). Flow cytometry of cells incubated with control serum (left panel) or anti-CLDN1 serum (right panel) demonstrated specific interaction of anti-CLDN1 antibodies with human CLDN1 (red histograms). The x and y axes show mean fluorescence intensities and relative numbers of stained cells, respectively. (B) Staining of cell surface CLDN1 on 293T/CLDN1 cells stably expressing CLDN1 by anti-CLDN1 antibodies. Flow cytometry of 293T/CLDN1 (clone IIIA6) cells (red histograms) or 293T parental cells (black histograms) incubated with control (left panel) or anti-CLDN1 IgG (right panel) demonstrated specific interaction of anti-CLDN1 antibodies with human CLDN1. (C) Cell surface expression of CLDN1 on hepatoma cells lines or primary hepatocytes was determined by flow cytometry in the absence of permeabilization. Histograms corresponding to cell surface expression of CLDN1 (open curves) are overlaid with histograms of cells incubated with rat control serum (black shaded curves). (D) Imaging of cell surface CLDN1 on living Huh7.5.1 cells (left panels), permeabilized Huh7.5.1 (middle panels) and Caco-2 (right panels) cells by anti-CLDN1 antibodies (upper panels). Cells incubated with control serum are depicted in the lower panels. Cells were incubated with pre-immune serum or anti-CLDN1 and analyzed as described in Materials and Methods. Cell nuclei were stained with DAPI.

Fig. 2. Anti-CLDN1 antibody does not alter tight junction integrity in polarized

HepG2 cells. (A) The bile canalicular (BC) lumen in polarized HepG2 cells was assessed for TJ “barrier” function. Cells were incubated with CMFDA, with restriction of the compound to the BC indicating that polarized HepG2 cells have functional TJs.

(B) Polarized HepG2 cells grown for 3 days were treated with serum free DMEM for 4h before being exposed to either control (PBS), irrelevant IgG control (1:100), anti-CLDN1 Ab (1:100) or 10 ng/mL IFN γ for 1 h. TJ barrier function was measured by quantifying the number of BC retaining CMFDA compared to the total BC in a minimum of three fields of view on three replicate coverslips. *** $P < 0.0001$ (t test).

Fig. 3. Dose-dependent inhibition of HCVcc infection by anti-CLDN1 antibodies.

(A) Inhibition of Luc-Jc1 HCVcc infection by rat anti-CLDN1 serum. Huh7.5.1 cells were pre-incubated with serial dilutions of rat anti-CLDN1 serum or control rat serum for 1 h at 37°C before infection with Luc-Jc1 HCVcc for 4 h at 37°C. HCV infection was assessed by measurement of luciferase activity 48 h post-infection. Mean \pm SD from a representative experiment performed in triplicate are shown. (B) Binding of anti-CLDN1 antibody to Huh7.5.1 cells. Huh7.5.1 cells were incubated with decreasing dilutions of anti-CLDN1 antibody and binding of anti-CLDN1 was determined by flow cytometry as described in Fig. 1. (C) Jc1 HCVcc infection in the

presence of purified rat anti-CLDN1 IgG. Huh7.5.1 cells were pre-incubated for 1 h at 37°C with serial dilutions of IgG isolated from rat anti-CLDN1 or control serum before infection with Jc1 HCVcc. 48 h later, HCV infection was analyzed by quantitation of HCV RNA using RT-PCR in intracellular lysates. Results are expressed as percent of HCVcc infectivity in the absence of antibody. Mean \pm SD from a representative experiment performed in triplicate are shown. (D) Inhibition of HCVpp infection in

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3 primary human hepatocytes by anti-CLDN1 antibodies. One day after isolation and
4 plating, hepatocytes were washed and pre-incubated with rat anti-CLDN1 or control
5 serum (1/50) for 1 hour at 37°C in medium. Then, HIV-based HCVpp bearing
6 envelope glycoproteins of strains HCV-J (genotype 1b), JFH-1 (genotype 2a),
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8 UKN3A.1.28 (genotype 3a) and UKN4.21.16 (genotype 4) were added for 3 hours at
9 37°C. Following infection, the supernatant was removed and replaced by fresh
10 medium. HCVpp infection was assessed by measurement of luciferase activity 72
11 hours post-infection. Inhibition of HCVpp infection is shown as % infection compared
12 to hepatocytes incubated with control serum (=100%). *** $P < 0.0001$.
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27 **Fig. 4. CLDN1, CD81 and SR-BI act in concert to mediate HCVcc entry.** (A) Dose-
28 dependent inhibition of Luc-Jc1 HCVcc infection by anti-CLDN1, anti-CD81, and anti-
29 SR-BI antibodies. Huh7.5.1 cells were pre-incubated for 1 h at 37°C with control
30 mouse IgG (0.1 µg/mL), control rat pre-immune serum (PI) (1/200), anti-CD81
31 antibody JS-81 (0.1 and 0.05 µg/mL), rat anti-CLDN1 serum (1/100, 1/200, 1/400) or
32 rat anti-SR-BI serum (1/200, 1/400, and 1/800) before infection with Luc-Jc1 HCVcc
33 for 4 h at 37°C. HCV infection was assessed by measurement of luciferase activity
34 48 h post-infection. Data are expressed as percent of Luc-Jc1 HCVcc infectivity in
35 the absence of antibody. (B-E) Additive effects of anti-CD81 and anti-CLDN1 (panel
36 B), anti-SR-BI and anti-CLDN1 (panel C), anti-CD81 and anti-SR-BI (panel D) and
37 anti-CD81, anti-CLDN1 and anti-SR-BI antibodies (panel E) resulting in inhibition of
38 HCVcc entry. Huh7.5.1 cells were pre-incubated for 1 h at 37°C with rat anti-CLDN1
39 (1/200 and 1/400) mouse anti-CD81 JS-81 (0.1 µg/mL and 0.05 µg/mL) and rat anti-
40 SR-BI (1/400 and 1/800) antibodies either alone (black bars) or in combination before
41 infection (grey bars) with Luc-Jc1 HCVcc for 4 h at 37°C. HCV infection was
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3 assessed as described in (A). Data are expressed as percent of Luc-Jc1 HCVcc
4 infectivity in the absence of antibody. Means \pm SD of four independent experiments
5 performed in duplicate are shown. *** $P < 0.0001$, ** $P < 0.001$ *, $P < 0.01$ (t test).
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12 **5 Fig. 5. Kinetics of HCVcc and HCVpp entry demonstrate that CLDN1 mediates**
13 **an HCV entry step closely linked to CD81.** (A) Schematic drawing of the
14 experimental setup. Inhibition of Luc-Jc1 HCVcc entry into Huh7.5.1 cells by heparin
15 (250 $\mu\text{g}/\text{mL}$), control monoclonal mouse IgG (5 $\mu\text{g}/\text{mL}$), control rat pre-immune serum
16 (1/100), rat anti-CLDN1 serum (1/100), mouse monoclonal anti-CD81 antibody JS-81
17 (5 $\mu\text{g}/\text{mL}$) and rat anti-SR-BI serum (1/100) was compared using different protocols
18 as described in Materials and Methods. Dashed lines indicate the time intervals
19 where inhibitors or antibodies were present. All results are expressed as percent Luc-
20 Jc1 HCVcc infectivity in the absence of inhibitory compound or antibody (CTRL).
21 Virus binding to target cells was performed in the presence (protocol I) or absence
22 (protocol II) of compounds as described in Materials and Methods. (B) Kinetics of
23 HCVcc entry into human hepatoma cells by compared protocol I and II. Means \pm SD
24 of three independent experiments performed in duplicate are shown. (C) Kinetics of
25 HCVcc infection into human hepatoma cells in the presence of heparin – an
26 attachment inhibitor, anti-CD81 antibody and concanamycin A - an inhibitor of
27 endocytosis. Kinetics of HCVcc entry in the presence heparin (\bullet), anti-CD81 (JS-81)
28 antibody (\blacksquare), Concanamycin A (\blacklozenge) (conA; 25nM) or control rat pre-immune serum
29 (CTRL) (Δ) was determined as described (6, 26, 29, 30). Means of three independent
30 experiments performed in triplicate are shown. (D, E) Kinetics of HCVcc entry into
31 non-differentiated (D) or DMSO-differentiated (E) Huh7.5.1 cells. The efficiency of
32 infection using rat anti-CLDN1 serum (\blacktriangle), anti-CD81 (JS-81) antibody (\blacksquare), or control
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3 rat pre-immune serum (CTRL) (Δ) was measured by luciferase assay 48 h later.
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5 Means of three independent experiments performed in triplicate are shown. (F)
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7 Kinetics of HCVpp entry in stably transfected 293T/CLDN1+ cells expressing CLDN1
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9 (as shown in Fig. 1B) using anti-CD81 (JS-81), anti-CLDN1 or control antibodies.
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13 5 Means of three independent experiments performed in triplicate are shown.
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18 **Fig. 6. Dose-dependent inhibition of E2 binding to permissive cell lines by anti-**

19 **CLDN1 antibodies.** (A) Binding of recombinant E2 glycoprotein to permissive

20 Huh7.5.1 cells. Huh7.5.1 cells were pre-incubated with control rat pre-immune serum

21 (black lined histograms) or rat anti-CLDN1 antibodies (blue lined histograms) diluted

22 1/100 for 1 h at RT. Binding of E2 was detected by flow cytometry as described in

23 Materials and Methods. Cells incubated in the absence of antibody and E2 (PBS)

24 served as negative control ("NC" – light shaded histograms). A representative

25 experiment is shown. (B) Binding of recombinant E2 glycoprotein to permissive

26 Huh7.5.1 cells. Huh7.5.1 cells were pre-incubated with rat anti-CD81, rat anti-SR-BI

27 and rat anti-CLDN1 antibodies or control rat pre-immune serum (all diluted 1/100) for

28 1 h at RT. Binding of E2 was detected by flow cytometry as described in Materials

29 and Methods. Results are expressed as percent E2 binding in the absence of

30 antibody (PBS). Means \pm SD of four independent experiments performed in duplicate

31 are shown. (C) Dose-dependent inhibition of E2 binding to Huh7.5.1 cells by anti-

32 CLDN1. Huh7.5.1 cells were pre-incubated with different dilutions of anti-CLDN1 (\blacksquare)

33 antibodies or control rat pre-immune serum (\blacklozenge). Results are expressed as percent E2

34 binding in the absence of antibody. Means \pm SD of four independent experiments

35 performed in duplicate are shown. (D) Binding of recombinant E1 glycoprotein to

36 permissive Huh7.5.1 cells. Huh7.5.1 cells were pre-incubated with heparin, mouse

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3 anti-CD81 (JS-81; 5 $\mu\text{g}/\text{mL}$), control mouse IgG (5 $\mu\text{g}/\text{mL}$), rat anti-CLDN1 (1/100),
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5 rat pre-immune serum (1/100) for 1 h at RT. Binding of E1 was detected by flow
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7 cytometry as described (6, 26, 29). Results are expressed as percent E1 binding in
8
9 the absence of antibody (PBS). Means \pm SD of two independent experiments
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11 performed in duplicate are shown. (E) Binding of recombinant E2 glycoprotein to rat
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13 BRL-3A cells stably expressing human SR-BI, CD81 and CLDN1 (24). Cells were
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15 pre-incubated with mouse anti-CD81 (JS-81, 5 $\mu\text{g}/\text{mL}$), control mouse IgG (5 $\mu\text{g}/\text{mL}$),
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17 rat anti-SR-BI, rat anti-CLDN1 or rat control serum (all diluted at 1/100) and binding
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19 of E2 was detected using FITC-conjugated anti-His antibody and flow cytometry as
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21 described in panel (B). Results are expressed as percent E2 binding in the absence
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23 of antibody (PBS). Means \pm SD of two independent experiments performed in
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25 duplicate are shown. (F) Inhibition of HCVcc binding to permissive Huh7.5.1 cells by
26
27 anti-CLDN1. Huh7.5.1 cells were pre-incubated with heparin (250 $\mu\text{g}/\text{mL}$), rat anti-
28
29 CLDN1 or control (CTRL) serum (all diluted 1/50) for 1 hour at 37°C prior to
30
31 incubation with HCVcc (Jc1 strain) which had been partially purified from cell culture
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33 supernatants using gradient ultracentrifugation. Following incubation with HCVcc,
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35 non-bound HCVcc were removed by washing of cells with PBS. Binding of HCVcc
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37 was then quantified by RT-PCR of cell bound HCV RNA as described (11) which is
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39 indicated on the y-axis. Means \pm SD of five independent experiments performed in
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41 triplicate are shown. *** $P < 0.0001$.
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53 **Fig. 7. Cellular binding of envelope glycoprotein E2 to CHO cells expressing**
54 **CD81 and SR-BI but not CLDN1.** (A) Expression of human entry factors in
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56 transfected CHO cell. CHO cells were transfected with expression plasmids encoding
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58 human CLDN1, SR-BI or CD81 as described in Materials and Methods. Transfected
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3 CHO cells were analyzed by flow cytometry using rat control (CTRL), rat anti-CLDN1
4 (left panel), rat anti-SR-BI (middle panel) or mouse control IgG and anti-CD81 (JS-
5 81; right panel) (B) Binding of envelope glycoprotein E2 to CHO cells expressing
6 human HCV entry factors. CHO cells were transfected with individual expression
7 plasmids encoding for human CLDN1, SR-BI or CD81 as indicated. Cellular E2
8 binding was analyzed by flow cytometry as described (31). A representative
9 experiment performed in duplicate is shown.
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22 **Fig. 8. Anti-CLDN1 inhibition of CD81-CLDN1 co-receptor association using**

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24 **FRET analysis.** 293T cells co-transfected to express AcGFP-CD81 and DsRED-
25 CD81, AcGFP-CLDN1 and DsRED-CD81, or AcGFP-CLDN1 and DsRED-CLDN1
26 were seeded onto glass coverslips and treated with pre-immune or anti-CLDN1 sera
27 for 1 h. Cells were fixed, imaged by laser scanning confocal microscopy and FRET
28 between AcGFP donor and DsRED acceptor proteins measured. % FRET is defined
29 as the frequency of pixels demonstrating FRET relative to the total number of pixels
30 analyzed at the plasma membrane of ten cells. *** $P < 0.0001$, * $P < 0.01$. AcGFP-
31 CLDN1 and DsRED-CD81 at intracellular (black) and plasma membrane (white)
32 locations in untreated and anti-CLDN1 treated cells were quantified and the
33 percentage of CLDN1 at each location determined.
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Table 1. Anti-CLDN1 antibody reactivity with members of the human CLDN family. 293T cells were transfected with plasmids encoding a panel of AcGFP tagged human CLDNs (1, 4, 6, 7, 9, 11, 15, 17) and CLDN7/1 chimeric proteins, where the N-terminal third (N1/3), half (N1/2) or C-terminal half (C1/2) of EL1 or the entire EL2 is replaced with the corresponding coding region of CLDN1 as described in (9). The frequency of cells expressing AcGFP-CLDN and binding anti-CLDN1 antiserum was determined by flow cytometry. Data are presented as the frequency of AcGFP-CLDN positive cells and their reactivity with anti-CLDN1 serum relative to mock transfected cells.

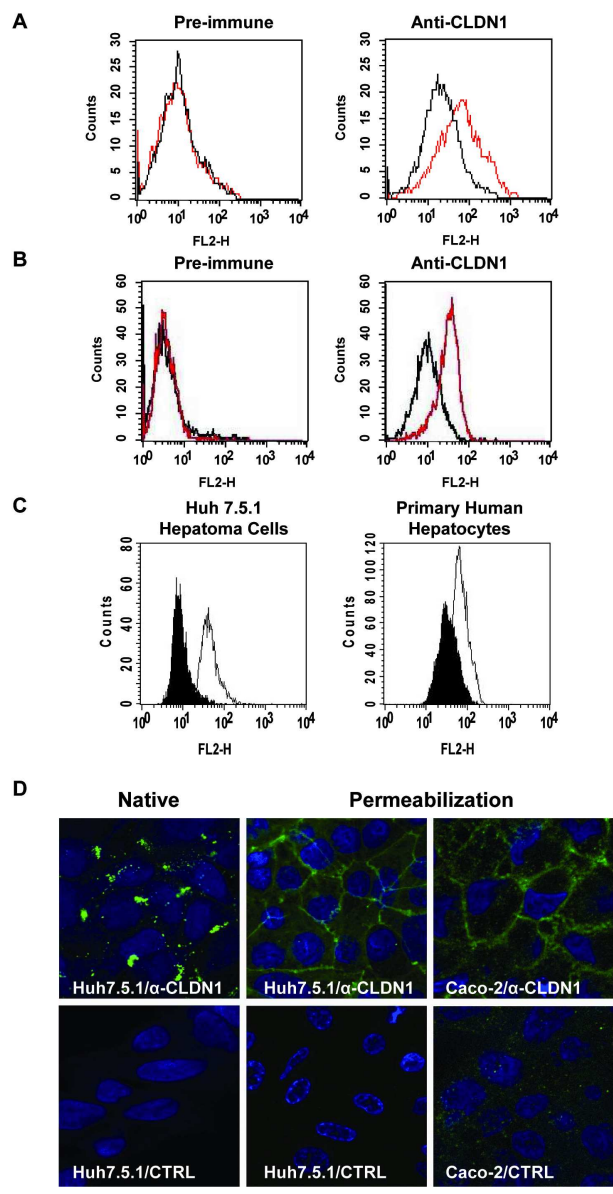
AcGFP CLDN expression construct	Frequency of AcGFP CLDN positive cells (%)	Frequency of anti-CLDN1 positive cells (%)
Mock	0	0
CLDN1	51.3	47.5
CLDN4	53.7	1.14
CLDN6	18.4	0.27
CLDN7	46.6	0.91
CLDN9	21.8	0.41
CLDN11	38.2	0.52
CLDN15	27.5	0.42
CLDN17	24.4	3.4
CLDN7/1 N1/3	26.1	16.7
CLDN7/1 N1/2	12.6	7.33
CLDN7/1 C1/2	14.5	0.29
CLDN7/1 EL2	27.6	7.1

Table 2. Half-maximal times ($t_{1/2}$) required for heparin, concanamycin A and anti-receptor antibodies to inhibit HCVcc entry in kinetic studies. Half-maximal times $t_{1/2}$ derived from kinetic assays shown in Fig. 5 are indicated. Values are means from three independent experiments \pm SD. Student's t-test was used to analyze differences in $t_{1/2}$ for heparin, CD81 and concanamycin A compared to $t_{1/2}$ for anti-CLDN1 antibodies. A *P* value of <0.05 was considered statistically significant.

Compound	Half-maximal time (min)	Significance (P-value)
Heparin	-60 ± 0	<0.05
Anti-CD81 (JS-81)	$+30 \pm 10$	0.643
Anti-CLDN1	$+33 \pm 6$	reference
Concanamycin A	$+60 \pm 10$	<0.05

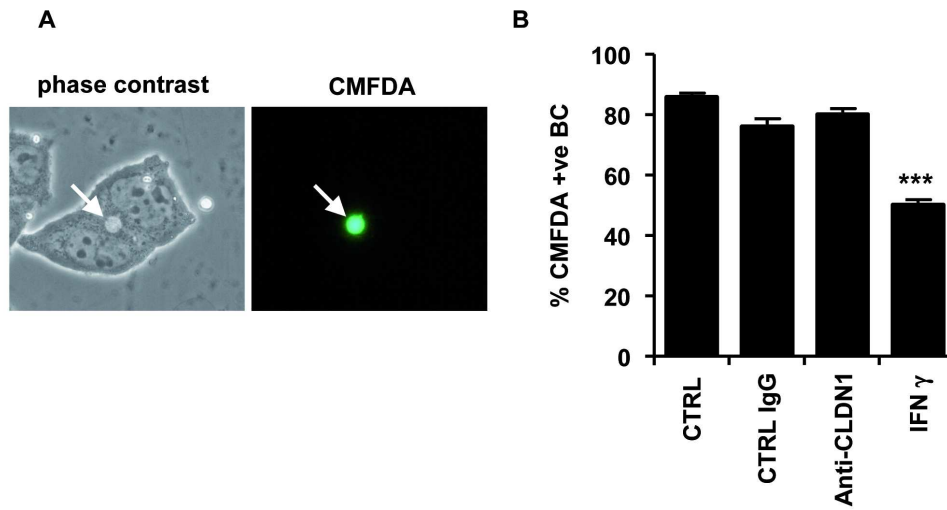
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Figure 1



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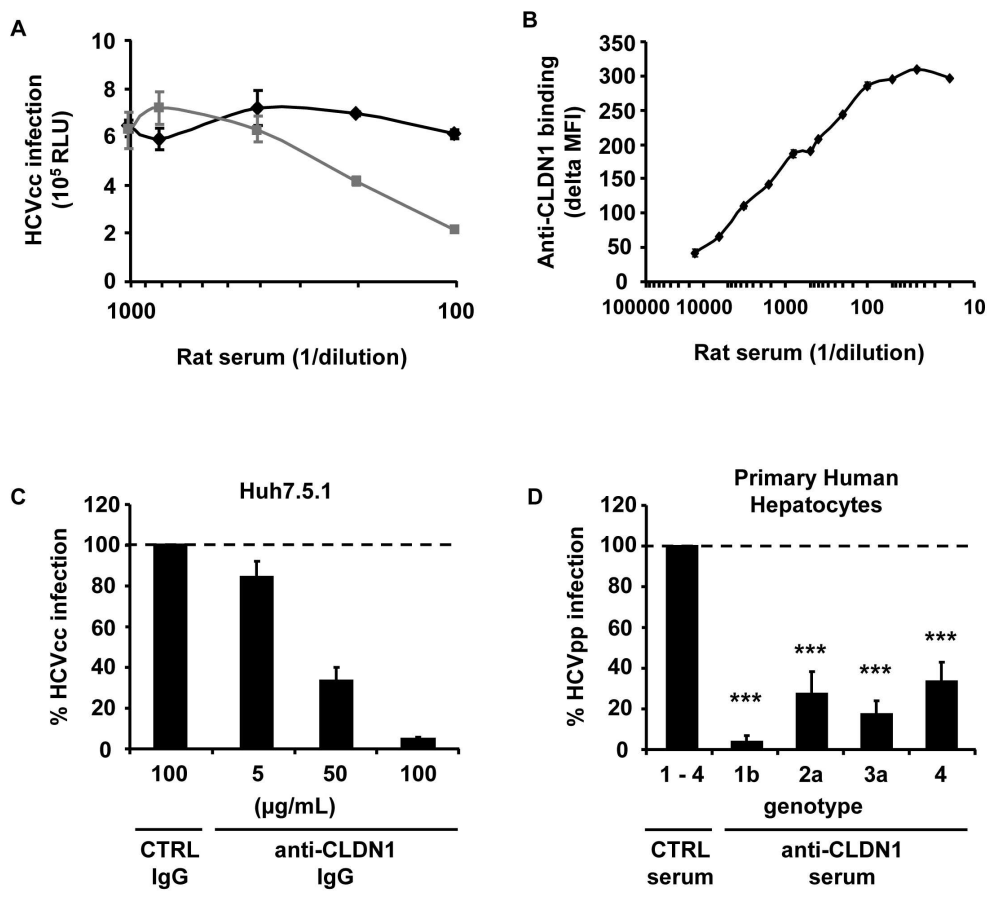
Figure 2



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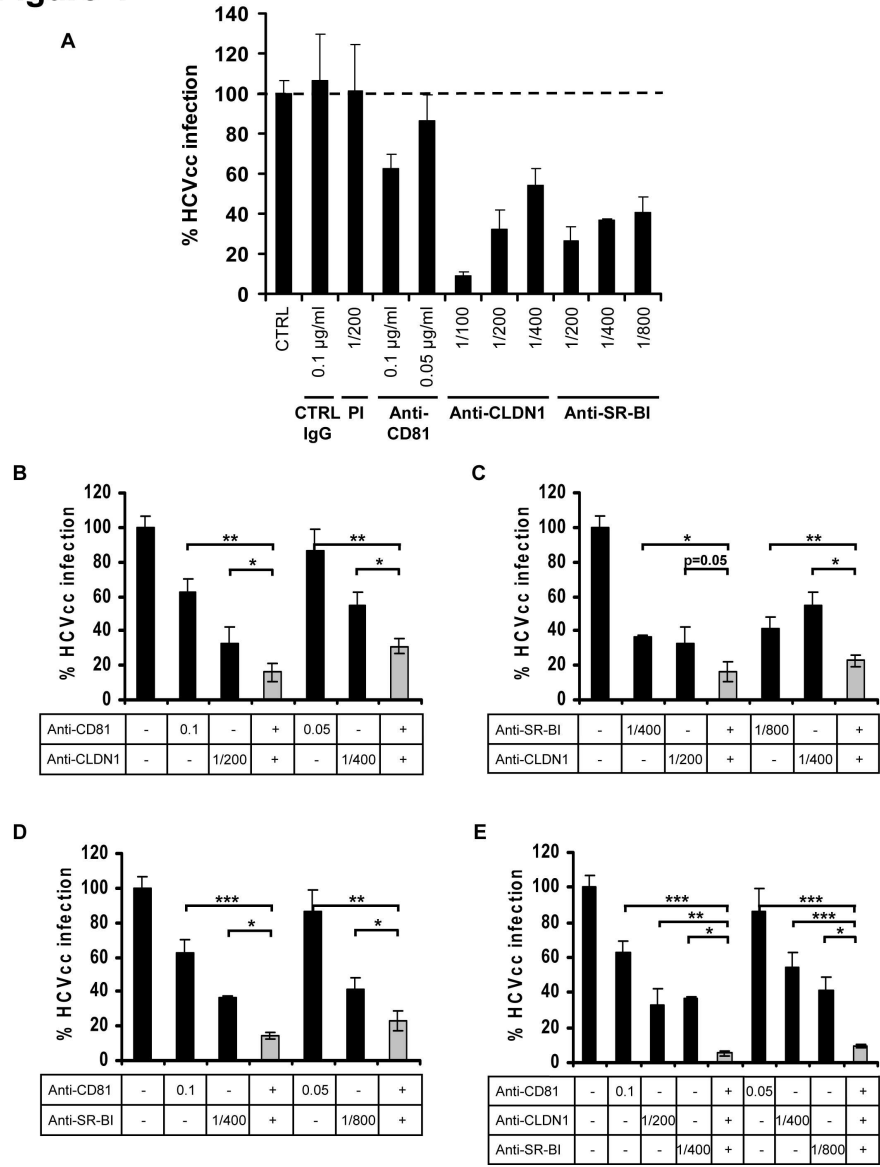
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Figure 3



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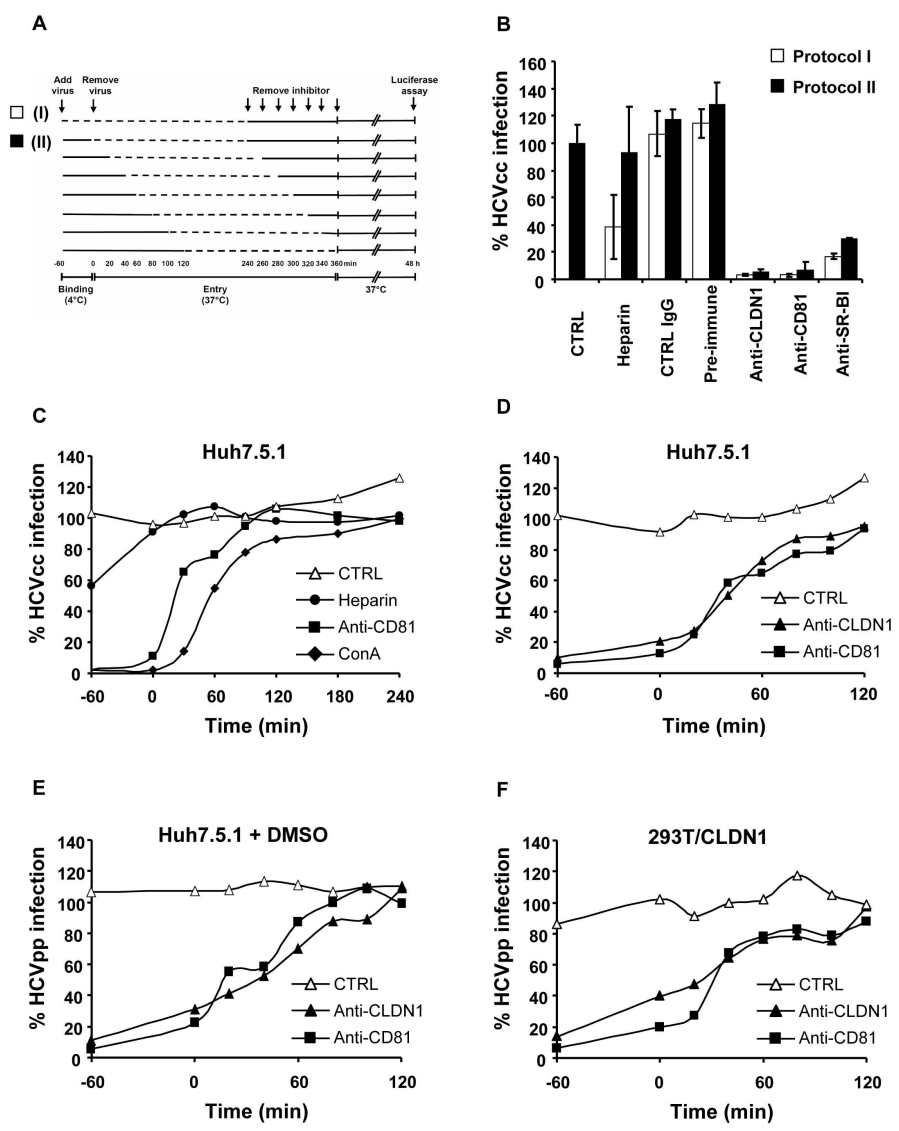
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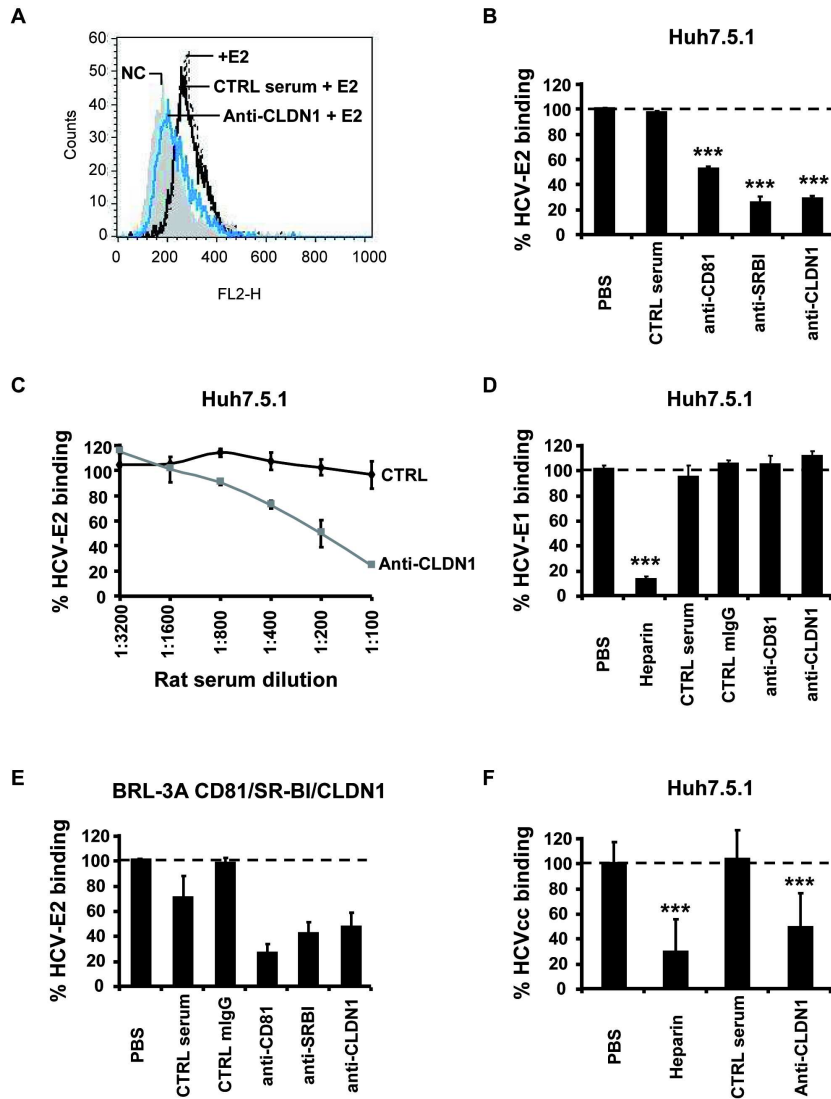
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Figure 5



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Figure 6



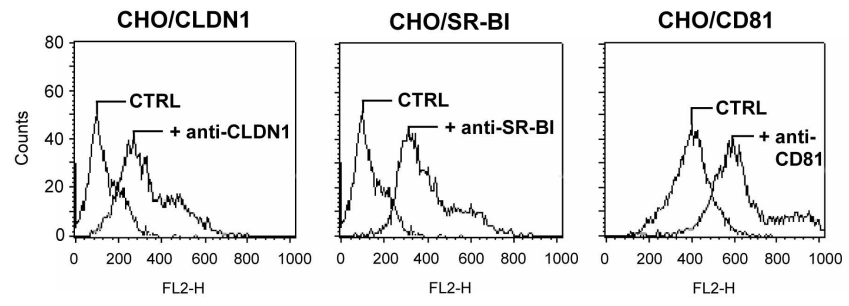
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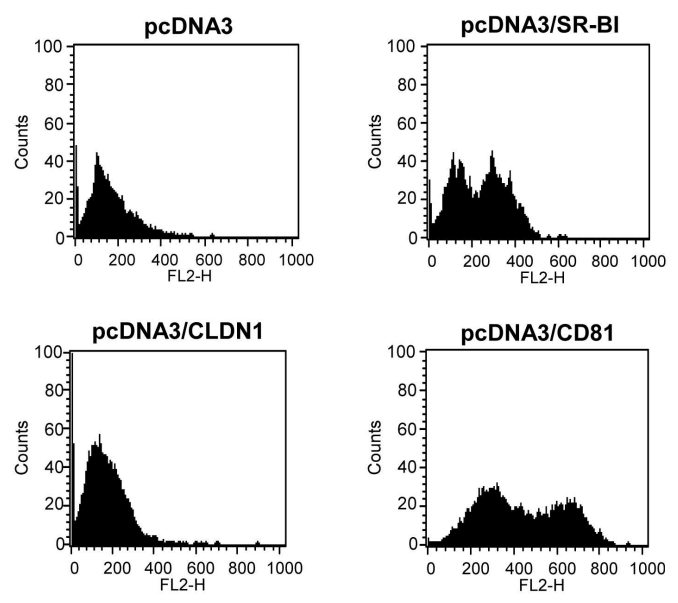
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Figure 7

A

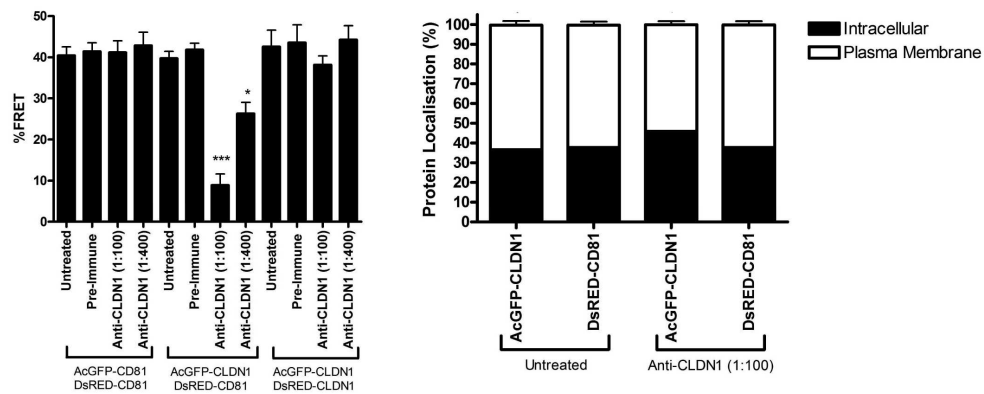


B



171x219mm (300 x 300 DPI)

Figure 8



182x111mm (300 x 300 DPI)

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3 **Inhibition of hepatitis C virus infection by anti-Claudin antibodies is**
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5 **mediated by neutralization of E2-CD81-Claudin 1 association(s)**
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13 5 Sophie E. Krieger^{1,2}, Mirjam B. Zeisel^{1,2}, Christopher Davis³, Christine Thumann^{1,2},
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Keywords: hepatocyte, infection, polarization, receptor, tight junction

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FOOTNOTES

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20 Abbreviations: BC - bile canalicular surface; CLDN1 - claudin-1; ConA -
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22 concanamycin A, CTRL - control; EL1 and 2 - extracellular loops 1 and 2; IFN γ -
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24 10 interferon-gamma; FRET - fluorescence resonance energy transfer; HCV - hepatitis
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26 C virus; HCVcc - cell culture-derived HCV; HCVpp - HCV pseudotype particles; RLU
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28 - relative light units; PI - pre-immune serum; SR-BI - scavenger receptor class B type
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30 I; TJ - tight junction
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ABSTRACT

The tight junction protein claudin-1 (CLDN1) has been shown to be essential for hepatitis C virus (HCV) entry – the first step of viral infection. Due to the lack of neutralizing anti-CLDN1 antibodies, the role of CLDN1 in the viral entry process is poorly understood. In this study, we produced antibodies directed against the human CLDN1 extracellular loops by genetic immunization and used these antibodies to investigate the mechanistic role of CLDN1 for HCV entry in an infectious HCV cell culture system and human hepatocytes. Antibodies specific for cell surface expressed CLDN1 specifically inhibit HCV infection in a dose-dependent manner. Antibodies specific for CLDN1, scavenger receptor B1 and CD81 show an additive neutralizing capacity compared to when either agent was used alone. Kinetic studies with anti-CLDN1 and anti-CD81 antibodies demonstrate that HCV interaction(s) with both entry factors occur at a similar time in the internalization process. Anti-CLDN1 antibodies inhibit the binding of envelope glycoprotein E2 to HCV permissive cell lines in the absence of detectable CLDN1-E2 interaction. Using fluorescent labelled entry factors and fluorescence resonance energy transfer (FRET) methodology, we demonstrate that anti-CLDN1 antibodies inhibit CD81-CLDN1 association. In contrast, CLDN1-CLDN1 and CD81-CD81 associations were not modulated. Taken together, our results demonstrate that antibodies targeting CLDN1 neutralize HCV infectivity by reducing E2 association with the cell surface and disrupting CD81-CLDN1 interactions. In conclusion, these results further define the function of CLDN1 in the HCV entry process and highlight new antiviral strategies targeting E2-CD81-CLDN1 interactions.

INTRODUCTION

With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public health. HCV is a hepatotropic virus that causes persistent infection in the majority of infected individuals (1). Therapeutic options for chronic infection are limited and a vaccine is not available (2).

HCV entry into hepatocytes is the first step of the viral life cycle resulting in productive viral infection (3, 4). Furthermore, HCV entry is a major target of host neutralizing responses (5-7) and a target for antiviral immunopreventive and therapeutic strategies (for review see (4, 8)). Viral entry is believed to be mediated by the viral envelope glycoproteins E1 and E2 and several host entry factors. These include heparan sulfate, tetraspanin CD81, scavenger receptor class B type I (SR-BI) (3) and the tight junction (TJ) proteins claudin-1 (CLDN1) (9) and occludin (10, 11). As none of these host cell surface factors alone is able to promote HCV entry, the interaction of HCV and its target cells leading to the internalization of the virus is believed to be a multistep process involving the interplay of several host cell factors (3, 4, 8).

Evans and colleagues reported that CLDN1 is essential for HCV infection (9). Subsequent studies demonstrated that CLDN-6 and -9 are also able to mediate HCV entry in non permissive cell lines (12, 13). CLDNs are critical components of TJs that regulate paracellular permeability and polarity and have a tetraspanin topology with four transmembrane domains, two extracellular and one intracellular loops, and N- and C-terminal cytoplasmic domains (14). CLDN1 extracellular loop 1 (EL1) is required for HCV entry (9) and is involved in barrier function and contributes to pore

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3 formation between polarized cells (15). Mutagenesis studies in non-polarized 293T
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5 cells demonstrate that CLDN1 enrichment at cell-cell contacts may be important for
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7 HCV entry (16). We (17, 18) and others (16, 19, 20) using a variety of imaging and
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9 biochemical techniques reported that CLDN1 associates with CD81. However, due to
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12 5 the lack of neutralizing anti-CLDN1 antibodies targeting extracellular epitopes, the
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14 exact role of CLDN1 in the viral entry process is poorly understood.
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20 In this study, for the first time we produced anti-CLDN1 antibodies that bound
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22 to cell surface expressed CLDN1 and inhibited HCV infection. The anti-CLDN1
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24 10 antibodies inhibit HCV E2 glycoprotein interaction with permissive hepatoma cells
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26 and fluorescence resonance energy transfer (FRET) between CD81-CLDN1 co-
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28 receptor complexes supporting a model where CLDN1 potentiates CD81 interaction
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30 with the virus and facilitates particle internalization.
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MATERIALS AND METHODS

Cells. Human Huh7 (5), Huh7.5.1 (21), HepG2 (18), 293T (5), Bosc (22), Caco-2 (23), and rat BRL-3A hepatocyte-derived cell lines (24) were propagated in DMEM/10% foetal bovine serum. 293T/CLDN1 cells were obtained by stable transfection of 293T cells with a pcDNA3.1 vector encoding CLDN1 cDNA. DMSO-mediated differentiation of Huh7.5.1 cells was performed as described (25). Primary human hepatocytes were isolated from liver resections from patients at the Strasbourg University Hospitals with approval from the Institutional Review Board (26, 27). In brief, liver specimen were perfused with calcium-free HEPES buffer supplemented with 0.5 mM EGTA (Fluka) followed by perfusion with HEPES buffer supplemented with 0.05% collagenase (Sigma) and 0.075% CaCl₂ at 37 °C. Following washing of cells with PBS and removal of nonviable cells by Percoll (Sigma) gradient centrifugation, freshly isolated hepatocytes (3 x 10⁵ cells/well) were plated in 24-well-plates pre-coated with collagen (Biocoat, BD Biosciences) and allowed to adhere in William's E medium (Sigma Aldrich) containing 1 % Glutamax (Gibco), 1 % ITS (insulin transferrin selenium Gibco), 10⁻⁷ M dexamethasone (Sigma), 0.15% bovine serum albumine (Sigma) and 10% fetal calf serum (PAN Biotec).

Antibodies. Anti-CLDN1 antibodies were raised by genetic immunization of Wistar rats using a human CLDN1 cDNA expression vector. For screening, Bosc cells transfected with pCMV-SPORT6 or pCMV-SPORT6/CLDN1 were incubated with anti-CLDN1 or pre-immune serum and analyzed for cell surface CLDN1 expression by flow cytometry as described (28). Purified IgG from rat anti-CLDN1 serum were obtained by MAbTrapTM kit (GE Healthcare). To analyze cross-reactivity of antibodies with other members of the CLDN family, 293T cells were transfected to express

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3 AcGFP tagged CLDN1, 4, 6, 7, 9, 11, 15 and 17 or chimeric CLDN1/7 (described in
4 (9)) and 48 h later stained with rat anti-CLDN1 antibodies and Alexa-633 coupled
5 anti-Rat Ig (Invitrogen). Cells were imaged by flow cytometry and data analyzed by
6 FLOWJo. Polyclonal rat anti-SR-BI or CD81 antibodies were obtained by genetic
7 immunization as described (26). R-phycoerythrin-conjugated and Cy5-conjugated
8 anti-rat IgG were from Jackson ImmunoResearch Laboratories, mouse IgG from
9 Caltag, mouse anti-CD81 (JS-81) from BD Biosciences.

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12 **Imaging studies of cell surface CLDN1.** Living Huh7.5.1 cells were incubated with
13 pre-immune or anti-CLDN1 serum (1/50) and a Cy5-conjugated anti-rat secondary
14 antibody (1/300; Jackson ImmunoResearch). Polarized Caco-2 cells, as described in
15 (23), were fixed in 3% paraformaldehyde, permeabilized with saponin and stained
16 with polyclonal anti-CLDN1 (1/50) or control serum. Following staining, cells were
17 fixed, mounted and observed using a Leica TCS SP2 CLSM (for Huh7.5.1) or a Zeiss
18 Cell Axio Observer Z1 microscope (for Caco-2).

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21 **Determination of tight junction barrier function.** To determine the functionality of
22 TJs and whether they restrict the paracellular diffusion of solutes from the bile-
23 canalicular (BC) lumen to the basolateral medium (barrier function), HepG2 cells
24 were treated with either control (PBS), rat anti-CLDN1, rat control serum or IFN γ and
25 incubated with 5 mM 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) at
26 37°C for 10 minutes to allow internalization and translocation to BC lumen by MRP2.
27 After washing with PBS, the capacity of BC lumens to retain CMFDA was analyzed
28 as described (18).

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3 **HCVcc production and infection.** HCVcc (Luc-Jc1 or Jc1 strains) were generated
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5 as described (6, 26, 29). For infection experiments, Huh7.5.1 cells were pre-
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7 incubated in the presence or absence of antibodies for 1 h at 37°C and infected at
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9 37°C for 4 h with HCVcc. 48 h later HCV infection was analyzed in cell lysates by
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13 5 quantification of luciferase activity or viral RNA (6, 26, 29, 30). Kinetic studies in the
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15 presence of antibodies or inhibitors were performed as described (6, 26, 29, 30).
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20 **HCV pseudoparticle (HCVpp) production and infection.** Infection of 293T/CLDN1
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22 or Huh7.5.1 cells with MLV-based HCVpp in kinetic assays was performed as
23
24
25 10 described (5, 6). Primary hepatocytes were infected with HIV-based HCVpp
26
27 expressing envelope glycoproteins of strains HCV-J (genotype 1b), JFH-1 (genotype
28
29 2a), UKN3A.1.28 (genotype 3a) and UKN4.21.16 (genotype 4) (described in (5))
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31 containing a luciferase reporter element. One day following hepatocyte isolation and
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33 plating, hepatocytes were washed with PBS and pre-incubated with rat anti-CLDN1
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36 15 or control serum (1/50) for 1 hour at 37°C in William's E medium. Then, HCVpp were
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38 added for 3 hours at 37°C. Following infection, the supernatant was removed and
39
40 replaced by fresh William's E medium. HCVpp infection was assessed by
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42 measurement of luciferase activity 72 hours post-infection as described (6, 26).
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48 20 **Cellular binding of HCV envelope glycoproteins E1 and E2.** Production and
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50 binding of C-terminally truncated envelope glycoproteins has been described (6, 24).
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52 For the study of E2-entry factor interaction, CHO cells were transfected with pcDNA3
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54 based expression vectors encoding SR-BI, CD81 or CLDN1 as described (31).
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56 Expression of entry factors was assessed by flow cytometry using anti-receptor
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59 25 antibodies as described (31). For the study of envelope glycoprotein binding in the
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3 presence of anti-receptor antibodies, Huh7.5.1 cells (21) or rat BRL-3A cells stably
4 expressing human SR-BI, CD81 and CLDN1 (24) were pre-incubated 1 h at RT with
5 rat anti-SR-BI, -CLDN1, -CD81 serum (1/100) or mouse anti-human CD81 (JS-81; 5
6 $\mu\text{g}/\text{mL}$) or control antibodies (1/100 or 5 $\mu\text{g}/\text{mL}$). Recombinant E2 (30 μL cell culture
7 supernatant) or E1 (10 $\mu\text{g}/\text{mL}$) was added to cells for 1 h at RT. Following washing
8 with PBS, bound envelope glycoproteins were detected using flow cytometry and
9 human anti-E1 (IGH526 (6)) or mouse anti-His (RGS-His, Qiagen) and PE-
10 conjugated secondary antibodies (24, 28). For quantitation of HCVcc binding,
11 Huh7.5.1 cells were pre-incubated with heparin (250 $\mu\text{g}/\text{mL}$), rat anti-CLDN1 (1/50) or
12 control serum (1/50) for 1 hour at 37°C prior to incubation with HCVcc (Jc1 strain)
13 which had been partially purified from cell culture supernatants using sucrose
14 gradient ultracentrifugation. Following incubation with HCVcc, non bound HCVcc
15 were removed by washing of cells with PBS. Binding of HCVcc was then quantified
16 by RT-PCR of cell bound HCV RNA as described (9).
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39 **Receptor association using fluorescence resonance energy transfer (FRET).**
40 Homotypic and heterotypic interactions of CD81 and CLDN1 were analyzed as
41 described (17, 18). Briefly, 293T cells transduced to express AcGFP and DsRED
42 tagged CD81 and CLDN1 were grown on glass coverslips and fixed in ice-cold
43 methanol. The cells were imaged on a Zeiss meta head LSCM, with microscope
44 settings optimized for each fluorescent protein to obtain the highest signal-to-noise
45 ratio. For FRET analysis, the gradual acceptor photobleaching method of FRET was
46 used, which entailed photobleaching the DsRED fluorophore gradually over time
47 while monitoring AcGFP fluorescent intensity (17). After background and cross-talk
48 correction, any increase in AcGFP intensity following DsRED photobleaching is due
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3 to FRET between proteins, implying a distance of less than 10 nm. The percent
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5 FRET is defined as the number of pixels that display FRET over the total number of
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7 pixels analyzed at the plasma membrane of the cells (17). The data from 10 cells
8
9 were normalized and the localized expression calculated.
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15 **Statistical analysis.** Results are expressed the mean \pm standard deviation (SD).
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17 Statistical analyses were performed using Student's *t* test with a *P* value of <0.05
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19 being considered statistically significant.
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RESULTS

Production of antibodies directed against cell surface CLDN1. To investigate the role of CLDN1 in HCV infection, we produced polyclonal anti-CLDN1 antibodies by genetic immunization and screened for reactivity with cell surface expressed CLDN1.

5 Antibodies were selected for their ability to bind non-permeabilized Bosc cells
14 transfected to express human CLDN1. Bosc cells are 293T-derived ecotropic
15 packaging cells (22) which do not express endogenous CLDN1 (data not shown). As
16 shown in Fig. 1A, incubation of Bosc cells expressing human CLDN1 with polyclonal
17 anti-CLDN1 sera resulted in a specific interaction with CLDN1 extracellular domains
18 (Fig. 1A). To confirm the specific interaction of anti-sera with CLDN1 we generated
19 293T cells stably expressing human CLDN1 (Fig. 1B). Incubation of 293T/CLDN1
20 cells with rat polyclonal anti-CLDN1 antibodies resulted in a specific interaction of
21 these antibodies with human CLDN1 (Fig. 1B). These data demonstrate that anti-
22 CLDN1 antibodies obtained by genetic immunization specifically bind to the
23 extracellular loops of human CLDN1 expressed on the cell surface. Using 293T cells
24 transfected with tagged AcGFP tagged CLDN1, 4, 6, 7, 9, 11, 15 and 17 or chimeric
25 CLDN1/7, we show that anti-CLDN1 antibodies demonstrate minimal or absent
26 cross-reactivity against other members of the CLDN family (Table 1).

20 Analysis of anti-CLDN1 reactivity to chimeric CLDN1/7 expressed on the cell
21 surface of 293T cells demonstrated that the antibodies interact strongly with CLDN7
22 where the N-terminal third (N1/3) or half (N1/2) was replaced with the corresponding
23 coding region of CLDN1 (Table 1). In contrast, the antibodies did not exhibit any
24 detectable interaction with CLDN7 where the C-terminal half (C1/2) of EL1 was
25 replaced with the corresponding coding region of CLDN1. A reduced interaction was

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3 observed for CLDN7 expressing the entire EL2 of CLDN1 (Table 1). These data
4 demonstrate that anti-CLDN1 antibodies recognize epitopes in the N-terminal half of
5 the CLDN1 EL1 which has been shown to be required for HCV entry (9) as well as
6 EL2 epitopes (Table 1). Since antibodies failed to recognize overlapping peptides
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13 5 encoding for linear epitopes comprising the CLDN1 EL1 and 2 in an ELISA or an
14 infection assay using peptides as capture antigens (data not shown), it is likely that
15 epitopes targeted by anti-CLDN1 antibodies are conformation-dependent.
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22 To study whether anti-CLDN1 antibodies bind to CLDN1 on the cell surface of
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25 10 HCV permissive cells, Huh7.5.1 and primary human hepatocytes were incubated with
26 anti-CLDN1 and analyzed by flow cytometry. Positive staining of human Huh7.5.1
27 hepatoma cells and human hepatocytes with polyclonal anti-CLDN1 antibodies in the
28 absence of permeabilizing reagents demonstrated that these antibodies bind to
29 CLDN1 expressed on the surface of primary hepatocytes and HCV permissive cell
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36 15 lines (Fig. 1C). To further address the specificity of antibodies, we performed CLDN1
37 knock-down experiments in Huh7.5.1 cells using a pool of three siRNAs described by
38 Evans et al. (9). CLDN1 silencing resulted in a decrease of anti-CLDN1 staining in
39 immunoblot analyses (data not shown) further confirming the specificity of the
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20 antibodies.

51 Positive staining of native cell surface CLDN1 in living, non-permeabilized
52 Huh7.5.1 cells with anti-CLDN1 antibodies was confirmed using imaging studies.
53 Interestingly, in living native Huh7.5.1 cells, the antibody appeared to localize to
54 certain areas of cell-cell contact (Fig. 1D), whereas in permeabilized Huh7.5.1 or
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60 25 Caco-2 cells antibody staining showed a polygonal web-like structure (Fig. 1D) which

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3 was similar to previous studies using non-neutralizing anti-CLDN1 antibodies (23).
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5 CLDN1 staining appeared to be more pronounced in polarized Caco-2 cells than in
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7 non-polarized Huh7.5.1 cells (Fig. 1D). Further imaging studies are ongoing to
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9 determine the detailed subcellular localization of CLDN1 recognized by neutralizing
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11 anti-CLDN1 antibodies in HCV permissive cells.
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18 Taken together, these data demonstrate that anti-CLDN1 serum produced by
19
20 genetic immunization specifically binds to the CLDN1 extracellular loops expressed
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22 on the cell surface of HCV permissive cell lines and human hepatocytes.
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27 **Anti-CLDN1 antibodies do not affect tight-junction integrity.** We previously
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29 reported that TJs impose a physical barrier and restrict viral access to receptors (23)
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31 and that complex hepatocyte-like polarity limits HCV entry (18). To investigate
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33 whether binding of anti-CLDN1 antibodies to polarized human hepatoma cells
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35 perturbed TJ integrity, we assessed the ability of TJs to restrict the paracellular
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37 diffusion of 5-chloromethylfluorescein diacetate (CMFDA) from the bile-canalicular
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39 (BC) lumen to the basolateral medium (barrier function) as previously described (18).
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41 As shown in Fig. 2, the capacity of BC lumens to retain CMFDA was similar in
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43 polarized HepG2 cells treated with rat anti-CLDN1 antibodies, rat control serum or
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45 PBS whereas CMFDA retention was reduced in IFN γ -treated HepG2 cells (Fig. 2B).
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These data suggest that anti-CLDN1 antibodies have no effect on TJ integrity.

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Neutralization of HCV infection by anti-CLDN1 antibodies. To investigate whether
anti-CLDN1 antibodies could inhibit HCV infection, Huh7.5.1 cells were infected with
chimeric J6/CF-JFH1 firefly luciferase reporter virus (Luc-Jc1) (26, 29) in the

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3 presence of anti-CLDN1 or control antibodies. Fig. 3A shows that anti-CLDN1 serum
4 inhibits Luc-Jc1 infection of Huh7.5.1 cells in a dose-dependent manner whereas the
5 control pre-immune serum had no inhibitory effect. Neutralization of HCVcc infection
6 correlated with binding of antibodies to the target cell line (Fig. 3B). To confirm that
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13 5 inhibition of Luc-Jc1 infection was mediated by anti-CLDN1 antibodies, we purified
14 IgG from rat anti-CLDN1 and pre-immune serum. As shown in Fig. 3C, anti-CLDN1
15 IgG but not control IgG markedly inhibited Luc-Jc1 HCVcc infection in a dose-
16 dependent manner. These data demonstrate that the inhibitory effect of anti-CLDN1
17 serum was mediated by anti-CLDN1 IgG and not by other substances present in the
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24 10 serum. Infection experiments using primary human hepatocytes and HCVpp
25 packaged with envelope glycoproteins from genotypes 1-4 demonstrated that anti-
26 CLDN1 blocking activity was similar for infection with HCV bearing envelope proteins
27 of other genotypes (Fig. 3D). Taken together, these findings demonstrate that
28 antibodies directed against the CLDN1 extracellular loops inhibit HCV infection in
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36 15 HCV permissive cell lines and human hepatocytes.

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41 **CLDN1 acts cooperatively with CD81 and SR-BI in HCV entry.** We previously
42 demonstrated that CD81 and SR-BI act in concert to mediate HCV entry (26). To
43 investigate whether the three host factors CLDN1, CD81 and SR-BI act in a
44 cooperative manner, we added low concentrations of anti-receptor antibodies
45 simultaneously prior to HCV infection. The use of antibody concentrations that sub-
46 maximally blocked HCV infection allowed us to observe additive or synergistic
47 effects. First, we determined the ability of combinations of two out of the three
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60 25 concomitant blocking of both CD81 and CLDN1 (Fig. 4B), SR-BI and CLDN1 (Fig.

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3 4C) or CD81 and SR-BI (Fig. 4D). This effect was not observed when control IgG or
4
5 control serum was used in combination with anti-CLDN1 antibodies (data not shown).
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8 Next, we assessed the impact of synchronously blocking all three host cell factors on
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10 HCVcc infection. Fig. 4E shows an additive effect of the three antibodies used.
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13 5 Indeed, Luc-Jc1 HCVcc infection was inhibited by more than 90% after simultaneous
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15 blocking of three host cell factors at antibody concentrations that inhibited HCVcc
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17 infection between 15% and 60% when used individually. Taken together, these
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19 results suggest that CLDN1 mediates HCV entry in cooperation with CD81 and SR-
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26 **CLDN1 mediates an HCV entry step closely linked to HCV-CD81 interaction.** To
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28 investigate the role of CLDN1 in the entry process, we investigated the inhibitory
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30 capacity of anti-CLDN1 antibodies in kinetic studies (26, 29). To discriminate
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32 between virus binding and post-binding events, Luc-Jc1 HCVcc binding to Huh7.5.1
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34 cells was performed for 1 h at 4°C in the presence or absence of inhibitors before the
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36 15 cells was performed for 1 h at 4°C in the presence or absence of inhibitors before the
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38 temperature was shifted to 37°C to initiate synchronous infection (Fig. 5A). Fig. 5B
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40 shows that similarly to anti-CD81 and anti-SR-BI, rat anti-CLDN1 inhibited Luc-Jc1
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42 HCVcc infection when added following binding of the virus to the target cell (Fig. 5B).
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44 To fine-map the entry step mediated by CLDN1, we added antibodies in side-by-side
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48 20 experiments every 20 min for up to 120 min after viral binding (Fig. 5C). The half-
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50 maximal times ($t_{1/2}$) required for anti-CD81 and anti-CLDN1 antibodies to inhibit HCV
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52 entry were +30 and +33 minutes (Fig. 5C-E; Table 2), whereas the half-maximal
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54 times for heparin was -60 minutes and for concanamycin A was +60 minutes (Fig.
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57 5C; Table 2). The time-course of anti-CLDN1 and anti-CD81-antibody-mediated
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60 25 inhibition was not significantly different, and both differed from those observed with

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3 heparin and concanamycin A (Table 2). Similar results were obtained in DMSO
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5 differentiated Huh7.5.1 (27) cells (Fig. 5E). These data support a model where
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7 CLDN1 and CD81 exert their effects at a similar time in the viral internalization
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9 process.
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15 Using Flag-tagged CLDN1 transfected 293T cells, Evans et al. reported that
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17 anti-Flag inhibition of HCVpp infection occurred at later time points compared to a
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19 CD81 specific antibody (9). These results differ from those obtained in this study that
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21 may be attributable to the experimental systems used in the two studies, including:
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24 10 293T/CLDN1 versus Huh7.5.1 cell lines, HCVpp versus HCVcc, the strain of HCV
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26 envelope glycoproteins H77 versus J6/JFH1 and the blocking antibodies (anti-
27
28 CLDN1 versus anti-Flag). To further address this question, we studied the kinetics of
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30 anti-CLDN1 and anti-CD81 neutralization of HCVpp infection of 293T/CLDN1.
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33 Inhibition of HCVpp infection of 293T/CLDN1 cells by anti-CLDN1 and anti-CD81
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36 15 demonstrated a similar kinetics (Fig. 5F) to those observed for HCVcc infection of
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38 Huh7.5.1 cells (Fig. 5D, E). Thus, the different kinetic results described by Evans et
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40 al. and us are most likely not related to the experimental model system but rather
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42 related to the insertion of a Flag tag into CLDN1 (9).
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48 **Anti-CLDN1 inhibits binding of envelope glycoprotein E2 to HCV**
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50 **permissive cells in the absence of CLDN1-E2 interactions.** Next, we investigated
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52 whether anti-CLDN1 antibodies could interfere with E2 binding to permissive cell
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54 lines. Binding studies were performed using recombinant E1 and E2 glycoproteins in
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56 the presence of anti-receptor or control antibodies. As shown in Fig. 6B, anti-CD81,
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59 25 anti-SR-BI and anti-CLDN1 antibodies inhibited the binding of E2 to Huh7.5.1 cells.
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3 In contrast, pre-immune or unrelated control serum had no effect (Fig. 6A-C). Similar
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5 results were obtained for antibody inhibition of E2 binding to BRL-3A rat hepatocyte-
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8 derived cells engineered to express the three human entry co-factors, SR-BI, CD81
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10 and CLDN1 (24) (Fig. 6E). Expression of SR-BI, CD81 and CLDN1 on the cell
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13 5 surface of stably transfected BRL-3A cells was confirmed by flow cytometry and
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15 expression levels were comparable to Huh7 cells (data not shown and (24)).
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17 Interestingly, the magnitude of inhibition of E2 binding to Huh7.5.1 cells (Fig. 6C)
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19 correlated with the magnitude of inhibition of HCV infection (Fig. 3B), suggesting that
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21 inhibition of binding of E2-cell surface interactions provides a mechanism of action for
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24 10 the neutralizing activity of the anti-CLDN1 antibodies. In contrast, E1 binding was not
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26 affected by anti-CLDN1 (Fig. 6D). To investigate whether inhibition of E2 binding
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28 resulted in an inhibition of binding of infectious virions, we studied cellular binding of
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30 Jc1 HCVcc in the presence of anti-CLDN1 antibodies. Although HCVcc binding
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32 analyses were characterized by a higher inter-assay variability compared to E2
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35 15 binding studies, anti-CLDN1 antibodies markedly and significantly inhibited HCVcc
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37 binding to Huh7.5.1 cells (Fig. 6F).
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44 To study whether antibody inhibition of E2 binding to permissive cell lines was
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46 attributable to CLDN1 interactions with E2 we investigated whether CLDN1 was able
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48 20 to bind recombinant truncated glycoprotein E2. To address this question CHO cells
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50 were engineered to express human CLDN1, SR-BI or CD81 (Fig. 7A). Cell surface
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52 expression of human CD81 or human SR-BI conferred E2 binding to CHO cells (Fig.
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54 7B), whereas CLDN1 expression had no effect (Fig. 7B). These data suggest that
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57 CLDN1 does not interact directly with HCV envelope glycoprotein E2 and that
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3 antibody blocking of E2-cell surface interactions may be mediated by indirect
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5 mechanisms.
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10 **Anti-CLDN1 antibodies inhibit CLDN1-CD81 co-receptor association(s).** Since

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12 5 anti-CLDN1 inhibits E2 binding to HCV permissive cells in the absence of a direct
13 CLDN1-E2 interaction (Fig. 7B), we hypothesized that anti-CLDN1 antibodies may
14 interfere with CD81-CLDN1 co-receptor complexes. To assess whether anti-CLDN1
15 alter CLDN1-CD81 association, 293T cells were transfected to express AcGFP-CD81
16 and DsRED-CD81 or AcGFP-CLDN1 and DsRED-CD81 or AcGFP-CLDN1 and
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24 10 DsRED-CLDN1 (17), incubated with pre-immune and anti-CLDN1 serum (1/100 and
25 1/400) and co-receptor interaction(s) analyzed by FRET. As shown in Fig. 8, anti-
26 CLDN1 antibodies significantly reduced FRET between CD81 and CLDN1 in a dose-
27 dependent manner. Pre-incubation of cells with control serum did not modify CD81-
28 CLDN1 co-receptor interaction(s). Inhibition of CD81-CLDN1 co-receptor interaction
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36 15 was specific as shown by the unchanged FRET between CD81-CD81 and CLDN1-
37 CLDN1 following pre-incubation with anti-CLDN1 serum. Taken together, these data
38 suggest that anti-CLDN1 antibodies interfere with CD81-CLDN1 heterodimer
39 association.
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DISCUSSION

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6 For the first time we report the genesis and characterization of antibodies directed
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8 against the extracellular loops of human CLDN1 which inhibit HCV infection. CLDN1
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10 showed no evidence for a direct association with the viral envelope E1E2
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12 glycoproteins and yet anti-CLDN1 serum inhibited E2 association with the cell
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14 surface and disrupted CD81-CLDN1 interactions. These data suggest a role for
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16 CD81-CLDN1 complexes in viral entry and highlight new antiviral strategies targeting
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18 co-receptor complex formation.
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25 10 CLDN1 is an essential co-factor conferring HCV entry (9), however, the
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27 precise role of CLDN1 in the multi-step entry process remains poorly understood.
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29 Using antibodies directed against CLDN1 EL, we demonstrate a dose-dependent
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31 inhibition of viral envelope association with HCV permissive cell lines. Using
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33 transfected CHO cells expressing human HCV entry factors, we demonstrate that in
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35 contrast to CD81 and SR-BI, CLDN1 does not directly interact with envelope
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37 glycoprotein E2 at the cell surface.
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44 Using a recent FRET-based system to study CD81-CLDN1 co-receptor
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46 association (17), we demonstrate that neutralizing anti-CLDN1 antibodies specifically
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48 20 disrupt CD81-CLDN1 FRET (Fig. 8). These data suggest that CD81-CLDN1 co-
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50 receptor complexes are critical for HCV entry and CLDN1 may potentiate CD81
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52 association with HCV particles via E2 interactions. The functional relevance of the
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54 CD81-CLDN1 co-receptor complex for HCV entry is further corroborated by kinetic
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56 studies demonstrating that CD81 and CLDN1 act at a similar time point during HCV
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58 entry (Fig. 5). Although the magnitude of antibody-mediated inhibition of HCVcc
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3 infection was slightly different, the kinetics of inhibition by anti-CLDN1 and anti-CD81
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5 were similar (Fig. 5C-F and Table 2).
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10 Using a HCVpp kinetic assay in 293T cells expressing Flag-tagged CLDN1
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12 5 and anti-Flag antibody, Evans et al. observed anti-Flag antibody inhibition of HCVpp
13 infection at a later time point than anti-CD81, suggesting that CLDN1 has a role in
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15 late stages of the viral internalization process (9). Evans et al., reported that the
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17 inhibitory activity of anti-CD81 antibody was lost much earlier than the anti-Flag
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19 antibody (half-maximal inhibition at 18 and 73 min post temperature shift,
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21 respectively). However, we observed a loss of anti-CLDN1 and anti-CD81 inhibitory
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23 activity at similar times (half-maximal inhibition for both antibodies at +30 and +33
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25 10 min post temperature shift respectively). Comparable results using HCVpp infection
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27 of 293T/CLDN1 cells (Fig. 5F) suggest that the differences between the two studies
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29 relate to the inserted Flag epitope in CLDN1 sequence or the use of an anti-Flag
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31 antibody. It is conceivable that insertion of a triple Flag epitope into CLDN1 EL1 (9)
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33 may alter CLDN1 trafficking and possible association with CD81 resulting in a
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35 delayed inhibition of infection by anti-Flag antibody (9) compared to antibodies
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37 15 targeting native CLDN1. We conclude that CLDN1 and CD81 entry factors act in a
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39 cooperative manner in a closely linked step during HCV entry, consistent with earlier
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41 reports on CD81-CLDN1 association (17-19).
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53 Taken together, our findings support a model in which viral attachment and
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55 interaction with glycosaminoglycans and SR-BI promote or facilitate viral interaction
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57 with CD81-CLDN1 complexes. Since anti-CLDN1 inhibit envelope glycoprotein E2
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59 and virion binding to permissive cells in the absence of any detectable CLDN1-E2
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3 interactions, it is conceivable that CLDN1 association with CD81 enhances viral
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5 glycoprotein association(s) to the HCV co-receptor complex that are required for
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7 virus internalization. These results define the function of CLDN1 in the HCV entry
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9 process and highlight new antiviral strategies targeting E2-CD81-CLDN1 interactions.
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15 The development of neutralizing anti-CLDN1 antibodies may provide new
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17 therapeutic options for the prevention of HCV infection. Our data clearly demonstrate
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19 that CLDN1 is a target for HCV therapeutic intervention that may complement
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21 ongoing efforts to block intracellular replication events with inhibitors of the HCV
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23 proteases and polymerase (9). The observation that anti-CLDN1 had no effect on
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25 HepG2 permeability and TJ integrity (Fig. 2) merits further investigation into the use
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27 of anti-CLDN1 antibodies as a therapeutic for HCV infection. The production of
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29 antibodies directed against HCV entry factors such as CLDN1 may widen the future
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31 preventive and therapeutic strategies for HCV infection and ultimately be used for the
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33 prevention of HCV infection following needle stick injury or during liver
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35 transplantation. Further efforts are underway to produce monoclonal anti-CLDN1
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37 antibodies for that strategy.
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47 In conclusion, our results suggest that viral entry requires the formation of a
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49 virus-co-receptor complex including HCV E2, CD81 and CLDN1. The functional
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51 mapping of E2-CD81-CLDN1 association and its impact for HCV entry has important
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53 implications for the understanding of the very first steps of HCV infection and the
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55 development of novel antiviral strategies targeting viral entry.
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13 5 for providing plasmids for production of recombinant HCV Jc1 and JFH-1 HCVpp, C.
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For Peer Review

FIGURE LEGENDS**Fig. 1. Production of antibodies directed against human CLDN1 extracellular**

domains using genetic immunization. (A) Specific binding of rat anti-human CLDN1 serum to CLDN1 expressed on the cell surface of transfected Bosc cells.

- 5 Bosc cells were transfected with pCMV-SPORT6-CLDN1 (red histograms) or control vector (pCMV-SPORT6; black histograms). Flow cytometry of cells incubated with control serum (left panel) or anti-CLDN1 serum (right panel) demonstrated specific interaction of anti-CLDN1 antibodies with human CLDN1 (red histograms). The x and y axes show mean fluorescence intensities and relative numbers of stained cells, respectively. (B) Staining of cell surface CLDN1 on 293T/CLDN1 cells stably expressing CLDN1 by anti-CLDN1 antibodies. Flow cytometry of 293T/CLDN1 (clone IIIA6) cells (red histograms) or 293T parental cells (black histograms) incubated with control (left panel) or anti-CLDN1 IgG (right panel) demonstrated specific interaction of anti-CLDN1 antibodies with human CLDN1. (C) Cell surface expression of CLDN1 on hepatoma cells lines or primary hepatocytes was determined by flow cytometry in the absence of permeabilization. Histograms corresponding to cell surface expression of CLDN1 (open curves) are overlaid with histograms of cells incubated with rat control serum (black shaded curves). (D) Imaging of cell surface CLDN1 on living Huh7.5.1 cells (left panels), permeabilized Huh7.5.1 (middle panels) and Caco-2 (right panels) cells by anti-CLDN1 antibodies (upper panels). Cells incubated with control serum are depicted in the lower panels. Cells were incubated with pre-immune serum or anti-CLDN1 and analyzed as described in Materials and Methods. Cell nuclei were stained with DAPI.

Fig. 2. Anti-CLDN1 antibody does not alter tight junction integrity in polarized

HepG2 cells. (A) The bile canalicular (BC) lumen in polarized HepG2 cells was assessed for TJ “barrier” function. Cells were incubated with CMFDA, with restriction of the compound to the BC indicating that polarized HepG2 cells have functional TJs.

(B) Polarized HepG2 cells grown for 3 days were treated with serum free DMEM for 4h before being exposed to either control (PBS), irrelevant IgG control (1:100), anti-CLDN1 Ab (1:100) or 10 ng/mL IFN γ for 1 h. TJ barrier function was measured by quantifying the number of BC retaining CMFDA compared to the total BC in a minimum of three fields of view on three replicate coverslips. *** $P < 0.0001$ (t test).

Fig. 3. Dose-dependent inhibition of HCVcc infection by anti-CLDN1 antibodies.

(A) Inhibition of Luc-Jc1 HCVcc infection by rat anti-CLDN1 serum. Huh7.5.1 cells were pre-incubated with serial dilutions of rat anti-CLDN1 serum or control rat serum for 1 h at 37°C before infection with Luc-Jc1 HCVcc for 4 h at 37°C. HCV infection was assessed by measurement of luciferase activity 48 h post-infection. Mean \pm SD from a representative experiment performed in triplicate are shown. (B) Binding of anti-CLDN1 antibody to Huh7.5.1 cells. Huh7.5.1 cells were incubated with decreasing dilutions of anti-CLDN1 antibody and binding of anti-CLDN1 was determined by flow cytometry as described in Fig. 1. (C) Jc1 HCVcc infection in the

presence of purified rat anti-CLDN1 IgG. Huh7.5.1 cells were pre-incubated for 1 h at 37°C with serial dilutions of IgG isolated from rat anti-CLDN1 or control serum before infection with Jc1 HCVcc. 48 h later, HCV infection was analyzed by quantitation of HCV RNA using RT-PCR in intracellular lysates. Results are expressed as percent of HCVcc infectivity in the absence of antibody. Mean \pm SD from a representative experiment performed in triplicate are shown. (D) Inhibition of HCVpp infection in

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3 primary human hepatocytes by anti-CLDN1 antibodies. One day after isolation and
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5 plating, hepatocytes were washed and pre-incubated with rat anti-CLDN1 or control
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7 serum (1/50) for 1 hour at 37°C in medium. Then, HIV-based HCVpp bearing
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9 envelope glycoproteins of strains HCV-J (genotype 1b), JFH-1 (genotype 2a),
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12 5 UKN3A.1.28 (genotype 3a) and UKN4.21.16 (genotype 4) were added for 3 hours at
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14 37°C. Following infection, the supernatant was removed and replaced by fresh
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16 medium. HCVpp infection was assessed by measurement of luciferase activity 72
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18 hours post-infection. Inhibition of HCVpp infection is shown as % infection compared
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20 to hepatocytes incubated with control serum (=100%). *** $P < 0.0001$.
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26 **Fig. 4. CLDN1, CD81 and SR-BI act in concert to mediate HCVcc entry.** (A) Dose-
27
28 dependent inhibition of Luc-Jc1 HCVcc infection by anti-CLDN1, anti-CD81, and anti-
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30 SR-BI antibodies. Huh7.5.1 cells were pre-incubated for 1 h at 37°C with control
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32 mouse IgG (0.1 µg/mL), control rat pre-immune serum (PI) (1/200), anti-CD81
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34 antibody JS-81 (0.1 and 0.05 µg/mL), rat anti-CLDN1 serum (1/100, 1/200, 1/400) or
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36 15 rat anti-SR-BI serum (1/200, 1/400, and 1/800) before infection with Luc-Jc1 HCVcc
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38 for 4 h at 37°C. HCV infection was assessed by measurement of luciferase activity
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40 48 h post-infection. Data are expressed as percent of Luc-Jc1 HCVcc infectivity in
41
42 the absence of antibody. (B-E) Additive effects of anti-CD81 and anti-CLDN1 (panel
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44 B), anti-SR-BI and anti-CLDN1 (panel C), anti-CD81 and anti-SR-BI (panel D) and
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46 anti-CD81, anti-CLDN1 and anti-SR-BI antibodies (panel E) resulting in inhibition of
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48 20 HCVcc entry. Huh7.5.1 cells were pre-incubated for 1 h at 37°C with rat anti-CLDN1
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50 (1/200 and 1/400) mouse anti-CD81 JS-81 (0.1 µg/mL and 0.05 µg/mL) and rat anti-
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52 SR-BI (1/400 and 1/800) antibodies either alone (black bars) or in combination before
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54 25 infection (grey bars) with Luc-Jc1 HCVcc for 4 h at 37°C. HCV infection was
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3 assessed as described in (A). Data are expressed as percent of Luc-Jc1 HCVcc
4 infectivity in the absence of antibody. Means \pm SD of four independent experiments
5 performed in duplicate are shown. *** $P < 0.0001$, ** $P < 0.001$ *, $P < 0.01$ (t test).
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12 **5 Fig. 5. Kinetics of HCVcc and HCVpp entry demonstrate that CLDN1 mediates**
13 **an HCV entry step closely linked to CD81.** (A) Schematic drawing of the
14 experimental setup. Inhibition of Luc-Jc1 HCVcc entry into Huh7.5.1 cells by heparin
15 (250 μ g/mL), control monoclonal mouse IgG (5 μ g/mL), control rat pre-immune serum
16 (1/100), rat anti-CLDN1 serum (1/100), mouse monoclonal anti-CD81 antibody JS-81
17 (5 μ g/mL) and rat anti-SR-BI serum (1/100) was compared using different protocols
18 as described in Materials and Methods. Dashed lines indicate the time intervals
19 where inhibitors or antibodies were present. All results are expressed as percent Luc-
20 Jc1 HCVcc infectivity in the absence of inhibitory compound or antibody (CTRL).
21 Virus binding to target cells was performed in the presence (protocol I) or absence
22 (protocol II) of compounds as described in Materials and Methods. (B) Kinetics of
23 HCVcc entry into human hepatoma cells by compared protocol I and II. Means \pm SD
24 of three independent experiments performed in duplicate are shown. (C) Kinetics of
25 HCVcc infection into human hepatoma cells in the presence of heparin – an
26 attachment inhibitor, anti-CD81 antibody and concanamycin A - an inhibitor of
27 endocytosis. Kinetics of HCVcc entry in the presence heparin (●), anti-CD81 (JS-81)
28 antibody (■), Concanamycin A (◆) (conA; 25nM) or control rat pre-immune serum
29 (CTRL) (Δ) was determined as described (6, 26, 29, 30). Means of three independent
30 experiments performed in triplicate are shown. (D, E) Kinetics of HCVcc entry into
31 non-differentiated (D) or DMSO-differentiated (E) Huh7.5.1 cells. The efficiency of
32 infection using rat anti-CLDN1 serum (\blacktriangle), anti-CD81 (JS-81) antibody (■), or control
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3 rat pre-immune serum (CTRL) (Δ) was measured by luciferase assay 48 h later.
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5 Means of three independent experiments performed in triplicate are shown. (F)
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7 Kinetics of HCVpp entry in stably transfected 293T/CLDN1+ cells expressing CLDN1
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9 (as shown in Fig. 1B) using anti-CD81 (JS-81), anti-CLDN1 or control antibodies.
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13 5 Means of three independent experiments performed in triplicate are shown.
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18 **Fig. 6. Dose-dependent inhibition of E2 binding to permissive cell lines by anti-**

19 **CLDN1 antibodies.** (A) Binding of recombinant E2 glycoprotein to permissive

20 Huh7.5.1 cells. Huh7.5.1 cells were pre-incubated with control rat pre-immune serum

21 (black lined histograms) or rat anti-CLDN1 antibodies (blue lined histograms) diluted

22 1/100 for 1 h at RT. Binding of E2 was detected by flow cytometry as described in

23 Materials and Methods. Cells incubated in the absence of antibody and E2 (PBS)

24 served as negative control ("NC" – light shaded histograms). A representative

25 experiment is shown. (B) Binding of recombinant E2 glycoprotein to permissive

26 Huh7.5.1 cells. Huh7.5.1 cells were pre-incubated with rat anti-CD81, rat anti-SR-BI

27 and rat anti-CLDN1 antibodies or control rat pre-immune serum (all diluted 1/100) for

28 1 h at RT. Binding of E2 was detected by flow cytometry as described in Materials

29 and Methods. Results are expressed as percent E2 binding in the absence of

30 antibody (PBS). Means \pm SD of four independent experiments performed in duplicate

31 are shown. (C) Dose-dependent inhibition of E2 binding to Huh7.5.1 cells by anti-

32 CLDN1. Huh7.5.1 cells were pre-incubated with different dilutions of anti-CLDN1 (\blacksquare)

33 antibodies or control rat pre-immune serum (\blacklozenge). Results are expressed as percent E2

34 binding in the absence of antibody. Means \pm SD of four independent experiments

35 performed in duplicate are shown. (D) Binding of recombinant E1 glycoprotein to

36 permissive Huh7.5.1 cells. Huh7.5.1 cells were pre-incubated with heparin, mouse

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3 anti-CD81 (JS-81; 5 $\mu\text{g}/\text{mL}$), control mouse IgG (5 $\mu\text{g}/\text{mL}$), rat anti-CLDN1 (1/100),
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5 rat pre-immune serum (1/100) for 1 h at RT. Binding of E1 was detected by flow
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7 cytometry as described (6, 26, 29). Results are expressed as percent E1 binding in
8
9 the absence of antibody (PBS). Means \pm SD of two independent experiments
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11 performed in duplicate are shown. (E) Binding of recombinant E2 glycoprotein to rat
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13 BRL-3A cells stably expressing human SR-BI, CD81 and CLDN1 (24). Cells were
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15 pre-incubated with mouse anti-CD81 (JS-81, 5 $\mu\text{g}/\text{mL}$), control mouse IgG (5 $\mu\text{g}/\text{mL}$),
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17 rat anti-SR-BI, rat anti-CLDN1 or rat control serum (all diluted at 1/100) and binding
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19 of E2 was detected using FITC-conjugated anti-His antibody and flow cytometry as
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21 described in panel (B). Results are expressed as percent E2 binding in the absence
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23 of antibody (PBS). Means \pm SD of two independent experiments performed in
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25 duplicate are shown. (F) Inhibition of HCVcc binding to permissive Huh7.5.1 cells by
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27 anti-CLDN1. Huh7.5.1 cells were pre-incubated with heparin (250 $\mu\text{g}/\text{mL}$), rat anti-
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29 CLDN1 or control (CTRL) serum (all diluted 1/50) for 1 hour at 37°C prior to
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31 incubation with HCVcc (Jc1 strain) which had been partially purified from cell culture
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33 supernatants using gradient ultracentrifugation. Following incubation with HCVcc,
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35 non-bound HCVcc were removed by washing of cells with PBS. Binding of HCVcc
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37 was then quantified by RT-PCR of cell bound HCV RNA as described (11) which is
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39 indicated on the y-axis. Means \pm SD of five independent experiments performed in
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41 triplicate are shown. *** $P < 0.0001$.
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53 **Fig. 7. Cellular binding of envelope glycoprotein E2 to CHO cells expressing**
54 **CD81 and SR-BI but not CLDN1.** (A) Expression of human entry factors in
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56 transfected CHO cell. CHO cells were transfected with expression plasmids encoding
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58 human CLDN1, SR-BI or CD81 as described in Materials and Methods. Transfected
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3 CHO cells were analyzed by flow cytometry using rat control (CTRL), rat anti-CLDN1
4 (left panel), rat anti-SR-BI (middle panel) or mouse control IgG and anti-CD81 (JS-
5 81; right panel) (B) Binding of envelope glycoprotein E2 to CHO cells expressing
6 human HCV entry factors. CHO cells were transfected with individual expression
7 plasmids encoding for human CLDN1, SR-BI or CD81 as indicated. Cellular E2
8 binding was analyzed by flow cytometry as described (31). A representative
9 experiment performed in duplicate is shown.
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22 **Fig. 8. Anti-CLDN1 inhibition of CD81-CLDN1 co-receptor association using**

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24 10 **FRET analysis.** 293T cells co-transfected to express AcGFP-CD81 and DsRED-
25 CD81, AcGFP-CLDN1 and DsRED-CD81, or AcGFP-CLDN1 and DsRED-CLDN1
26 were seeded onto glass coverslips and treated with pre-immune or anti-CLDN1 sera
27 for 1 h. Cells were fixed, imaged by laser scanning confocal microscopy and FRET
28 between AcGFP donor and DsRED acceptor proteins measured. % FRET is defined
29 as the frequency of pixels demonstrating FRET relative to the total number of pixels
30 analyzed at the plasma membrane of ten cells. *** $P < 0.0001$, * $P < 0.01$. AcGFP-
31 CLDN1 and DsRED-CD81 at intracellular (black) and plasma membrane (white)
32 locations in untreated and anti-CLDN1 treated cells were quantified and the
33 percentage of CLDN1 at each location determined.
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