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The relevance of claudin-6 and claudin-9 in hepatitis C virus (HCV) entry remains elusive. We produced claudin-6 or claudin-9 specific monoclonal antibodies that inhibit HCV entry into non-hepatic cells expressing exogenous claudin-6 or claudin-9. These antibodies had no effect on HCV infection of hepatoma cells or primary hepatocytes. Thus although claudin-6 and claudin-9 can serve as entry factors in cell lines, HCV infection into human hepatocytes is not dependent on claudin-6 and claudin-9.

Hepatitis C virus (HCV) enters cells via a multi-step process that requires viral and host cell factors including the viral envelope glycoproteins E1 and E2, tetraspanin CD81, scavenger receptor class B type I (SR-BI), tight junction proteins claudin-1 (CLDN1) and occludin (OCLN), as well as co-entry factors such as epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2), Niemann-Pick C1-Like1 (NPC1L1) and transferrin receptor 1 (1-3). Understanding the mechanisms of viral entry is a prerequisite to defining anti-viral therapies targeting early step(s) in the viral life cycle.

CLDNs are critical components of tight junctions (TJ) and regulate paracellular permeability and polarity. The CLDN superfamily comprises more than 20 members that are expressed in a tissue-specific manner. CLDN1 is an essential host factor defining HCV entry (4) and CLDN1-specific antibodies inhibit HCV infection of human hepatocytes *in vitro* (5, 6). CLDN1 associates with CD81 in a variety of cell types and the resulting receptor complex is essential for HCV infection (5, 7-9). CLDN6 and CLDN9 have been reported to mediate HCV entry in CLDN1-deficient Bel7402 hepatoma cells (10) and CLDN null 293T embryonic kidney-derived cells (11-13). State-of-the-art cell culture models that support HCV replication include human

hepatoma Huh7-derived cell lines and primary human hepatocytes (PHHs). However, the role of CLDN6 and CLDN9 in mediating HCV infection of these cells and their potential as antiviral targets are unknown.

To investigate the functional role of CLDN6 and CLDN9 in HCV entry we generated monoclonal antibodies (mAbs) by genetic immunization using full-length human CLDN6 or CLDN9 cDNA expression vectors as previously described (6, 14). Following lymphocyte fusion, 10 96-well plates were screened for each target using transiently transfected cells expressing specific CLDNs on their cell surface and 54 positive clones were subsequently amplified and subcloned. We selected five CLDN6- (WU-8F5-E7, WU-9E1-G2, WU-5H6-D6, WU-10A4-B9, WU-3C9-B11) and three CLDN9- (YD-4E9-A2, YD-6F9-H2, YD-1C4-A4) specific mAbs that bind specific CLDNs expressed on 293T cells without any cross-reactivity for further studies (6) (Fig. 1A). To characterize these antibodies, we first used well characterized 293T cells that do not endogenously express CLDNs (12, 13, 15) (Fig. 1B) and thus allow to express each target CLDN individually (Fig. 1C). A previously described CLDN1-specific mAb (OM-7D3-B3) was used as control (6). We confirmed that, in contrast to naive 293T cells, HCV pseudoparticles (HCVpp) expressing a diverse panel of glycoproteins (strains H77 (1a), HCV-J (1b), JFH1 (2a), UKN3A1.28 (3a), UKN4.21.16 (4) described in reference (16)) infect 293T cells engineered to express CLDN1, CLDN6 or CLDN9 (Fig. 1D). In contrast to previous reports (10, 11), we noted that HCV-JFH1 only infected 293T cells expressing CLDN1, suggesting that this strain cannot utilize CLDN6 or CLDN9. Interestingly, a recent study also reported that Huh6 cells - expressing CLDN6 but devoid CLDN1 - are resistant to HCVpp expressing genotype 2a glycoproteins in contrast to HCVpp of genotype 1 (17). Next, we assessed the ability of CLDN-specific mAbs to inhibit HCVpp entry into these cells

using CD81-specific antibody as a positive control (18). All of the CLDN6- and CLDN9-specific mAbs inhibited the entry of HCVpp expressing representative genotype 1a and 1b glycoproteins into CLDN-expressing 293T cells (Fig. 1E). The two most potent mAbs were further characterized for their dose-dependent inhibition of entry HCVpp genotype 1a and 1b (Fig. 2A-B) and cross-reactivity to inhibit a panel of HCVpp expressing diverse glycoproteins. In contrast to mouse leukemia virus pseudoparticle (MLVpp) entry (19), CLDN6- and CLDN9-specific mAbs inhibited the entry of HCVpp expressing glycoproteins of genotypes 3a and 4 into 293T-derived cells (Fig. 2C). These data demonstrate that CLDN6- and CLDN9-specific mAbs inhibit HCV entry of 293T cells in a genotype independent manner.

CLDN6 and CLDN9 mRNA have been reported to be expressed in human liver, albeit at low levels (10). All of the CLDN6-specific mAbs bound Huh7.5.1 cells with comparable values to CLDN1-specific mAb, whereas we failed to detect binding of the anti-CLDN9 mAbs (Fig. 3A). These data suggest that CLDN6 is expressed in this hepatoma cell line and that CLDN9 is either not or weakly expressed on Huh7.5.1 cells. This is consistent with CLDN6 and CLDN9 mRNA expression in these cells (Fig. 1B). Then, to investigate whether the antibodies inhibit HCV entry into human hepatoma cells, Huh7.5.1 cells were pre-incubated with CLDN-specific mAbs before infection with HCVpp from different genotypes (strains H77 (1a), HCV-J (1b), JFH1 (2a)). Surprisingly, in contrast to CLDN1-specific mAb and to results obtained with CLDN-expressing 293T cells (Fig. 1-2), none of the CLDN6- or CLDN9-specific mAbs inhibited HCVpp entry into Huh7.5.1 cells even at high concentrations (Fig. 3B). Furthermore, none of the CLDN6- or CLDN9-specific mAbs inhibited infection of cell culture-derived HCV (HCVcc; strains Luc-Con1, genotype 1b/2a and Luc-Jc1, genotype 2a/2a described in reference (20)) in these cells (Fig. 3C).

Exogenous expression of CLDN6 or CLDN9 in CLDN1-silenced Huh7.5 has been reported to confer a small but statistically significant level of HCVpp genotype 1b entry, but to have no effect on HCVcc infection (11). To investigate whether CDLN6 or CLDN9 may be used as a substitute for CLDN1 in Huh7.5.1 cells, we assessed the ability of CLDN-specific mAbs to inhibit HCVpp entry in CLDN1-silenced Huh7.5.1 cells. In contrast to CLDN1-specific mAb, neither CLDN6- nor CLDN9-specific mAbs were able to further decrease HCV entry into CLDN1-silenced Huh7.5.1 cells (Fig. 3D). To further investigate a potential interplay between CLDN1 and other CLDNs during HCV infection, we studied whether the combination of CLDN6- or CLDN9- and CLDN1-specific mAbs provided additive or synergistic inhibitory effect(s) on HCVpp entry (6). Combining CLDN6- or CLDN9-specific mAbs with CLDN1-specific mAb did not result in any additive effect on inhibition of HCVpp entry of different genotypes (Fig. 4). Taken together, these data suggest a limited role of CLDN6 and CLDN9 in HCV entry into Huh7-derived cell lines.

Finally, to further investigate the *in vivo* relevance of CLDN6 and CLDN9 as potential HCV entry factors and antiviral targets, we performed similar experiments on the natural and most relevant target cell of HCV, the human hepatocyte. In comparison to CLDN1-specific mAb, we noted low to negligible binding of CLDN6 or CLDN9-specific mAbs to PHHs (Fig. 5A). These data suggest that CLDN6 and CLDN9 are either not or weakly expressed in PHHs. Most importantly, in contrast to CLDN1-specific mAb, CLDN6- and CLDN9-specific mAbs had minimal or absent effect(s) on HCVpp infection of PHHs (Fig. 5B). These data are consistent with the limited detection of CLDN6 and CLDN9 mRNA in hepatocytes isolated from a range of donors, in contrast to the high level expression of CD81, SR-BI and CLDN1 (Fig. 5C). Another study recently reported high levels of the main HCV entry factors but

126 highly variable CLDN6 mRNA levels in liver biopsies from HCV patients (17).
127 Immunostaining of liver sections also demonstrated that, in contrast to CLDN6-
128 positive seminoma, CLDN6 was not detected in human liver sections (Fig. 5D).

129 The aim of this study was to explore the functional role of CLDN6 and CLDN9
130 in the HCV entry process in PHHs and hepatoma cells. Understanding the
131 mechanism of HCV entry in the most relevant cell culture model will allow the
132 development of more efficient antivirals. We generated novel CLDN6- and CLDN9-
133 specific mAbs that specifically interact with CLDN6 or CLDN9 (Fig. 1A) and inhibit
134 HCVpp entry into human embryonic 293T kidney cells expressing defined CLDN
135 receptors that have been classically used to assess the role of CLDNs in the HCV
136 entry process (Fig. 1-2). These data demonstrate that the antibodies are functional
137 with respect to binding cognate receptors and inhibiting HCV internalization. In line
138 with mRNA expression data (Fig. 1B), antibody binding studies demonstrate
139 expression of CLDN6 on Huh7.5.1 cells (Fig. 3A), albeit with lower binding values
140 than observed with 293T cells. However, CLDN6- or CLDN9-specific mAbs showed
141 low or absent binding to PHHs (Fig. 5A), suggesting minimal expression, a
142 conclusion further supported by the low mRNA levels of these CLDNs in PHHs
143 isolated from multiple donors and absent detection of CLDN6 in liver sections (Fig.
144 5C-D). Noteworthy, functional studies demonstrate that CLDN6- and CLDN9-specific
145 mAbs had no effect on HCV infection of Huh7.5.1 cells or PHHs (Fig. 3-5).
146 Interestingly, silencing of CLDN6 in Huh7.5 cells also did not affect HCV infection
147 (17). Furthermore, these mAbs were unable to further decrease HCVpp entry into
148 CLDN1-silenced Huh7.5.1 cells or when added simultaneously with CLDN1-specific
149 mAb to naive Huh7.5.1 cells, suggesting that CLDN6 and CLDN9 are not able to
150 complement absence of CLDN1 expression or blockage by anti-CLDN1 mAb (Fig. 4).

Given their low expression (Fig. 5C-D), other CLDN members may not be able to compensate CLDN1-specific inhibition of HCV entry into PHHs. In contrast, a recent study showed that silencing CLDN6 expression in Huh7.5 cells further reduced CLDN1-specific antibody-mediated inhibition of HCV infection of genotype 1b and 3a (17). Differences of CLDN6 expression in Huh7-based cell lines or experimental design may account for the different findings. Given the very low levels of CLDN6 protein expression in the human liver *in vivo* (Fig. 5) and its absent or limited functional role in human hepatocytes (Fig. 5), the relevance of CLDN6 escape observed in Huh7.5 cells (17) remains to be determined in human hepatocytes or *in vivo*. If relevant, the CLDN6-specific mAbs (Fig. 1,2) could address this issue.

In summary, this study demonstrates that although CLDN6 and CLDN9 can serve as entry factors upon exogenous expression in certain cell culture models such as CLDN-deficient 293T cells, they do not play a major role for HCV entry into human hepatocytes. Yet, given their functional activity, the novel antibodies described in this study may represent interesting tools to investigate the role of CLDN6 and CLDN9 in physiology and disease.

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193 The authors declare no conflict of interest. Inserm and Aldevron have previously filed
194 a patent application on the use of anti-Claudin-1 antibodies for inhibition of HCV
195 infection (WO2010034812).
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FIGURE LEGENDS

Figure 1. CLDN6- and CLDN9-specific mAbs inhibit HCVpp infection of 293T cells engineered to express CLDNs. (A) Reactivity of anti-CLDN mAbs for 293T cells transfected with different human CLDNs. CLDN-deficient 293T cells were transfected to express Aequorea coerulescens green fluorescent protein (AcGFP) tagged human CLDN1, CLDN6 or CLDN9 as described (6) before detachment and staining with CLDN-specific mAbs or control isotype-matched irrelevant rat IgG at 20 µg/ml. Primary bound antibodies were detected with goat anti-rat PE conjugated $F(ab')_2$ fragment (PN IM 1623, Beckman Coulter). After washing, the cells were fixed with 2% PFA and analyzed by flow cytometry (FACscan). AcGFP tagged CLDN protein expression was confirmed by flow cytometric quantification of AcGFP expression (data not shown). Specific binding of CLDN1 (OM-7D3-B3) (6), CLDN6 (WU-8F5-E7, WU-9E1-G2, WU-5H6-D6, WU-10A4-B9, WU-3C9-B11) and CLDN9 (YD-4E9-A2, YD-6F9-H2, YD-1C4-A4) mAbs to AcGFP tagged CLDNs is shown as the difference of the mean fluorescence intensity of cells stained with CLDN-specific mAb and cells stained with control rat mAb isotype. (B) Expression of HCV entry factors in 293T, Hela, HepG2, Bcl-2 and Huh-7.5 cells. RNA was purified from these cell lysates using a Qiagen miniRNA kit following the manufacturer's instructions. Samples were amplified using a Cells Direct RT-PCR kit (Invitrogen). A primer-limited GAPDH referent primer set was included in all PCRs and used to compare expression. (C) Entry factor expression in CLDN1-, CLDN6- or CLDN9-transfected 293T cells. The relative expression of each entry factors was determined by flow cytometry and is indicated as fold expression compared to parental 293T cells. (D) HCVpp (strains H77 (1a), HCV-J (1b), JFH1 (2a), UKN3A1.28 (3a), UKN4.21.16 (4); produced as described in reference (16)) were allowed to infect CLDN1-, CLDN6- or

CLDN9-293T cells, and infection assessed by luciferase activity in cell lysates 72 h post-infection. Results are expressed in relative light units (RLU). The threshold for a detectable infection in this system is indicated by a dashed line, corresponding to the mean ± 3 SD of background levels, i.e., luciferase activity of naive non-infected cells or cells infected with pseudotypes without HCV envelopes. Means ± SD from three independent experiments performed in triplicate are shown. (E) 293T cells engineered to express CDLN1 (left panel), CLDN6 (middle panel) or CLDN9 (right panel) were pre-incubated for 1 h at 37°C with CD81-specific (10 µg/mL), CLDN-specific or control (50 µg/ml) mAbs before infecting with HCVpp (strains H77, genotype 1a; HCV-J, genotype 1b) for 4 h at 37°C. HCVpp entry was assessed as described in (D). Means ± SD from three independent experiments performed triplicate are shown. Statistical analysis relative to the control mAb was performed using the Student's *t* test, *p<0.05.

Figure 2. Anti-CLDN genotype independent inhibition of HCVpp infection of 293T cells engineered to express CLDN receptors. CLDN1-, CLDN6- and CLDN9-293T cells were pre-incubated for 1 h at 37°C with (A-B) serial dilutions of or (C) a fixed dose (100 µg/ml) of CLDN1-, CLDN6- (WU-9E1-G2), CLDN9- (YD-4E9-A2) specific or control mAbs before infecting with HCVpp (strains H77 (1a), HCV-J (1b), UKN3A1.28 (3a), UKN4.21.16 (4)) or MLVpp for 4 h at 37°C. Pseudoparticle entry was assessed as described in Fig. 1. Means ± SD from three independent experiments performed in triplicate are shown. Statistical analysis relative to the control mAb was performed using the Student's *t* test, *p<0.05

Figure 3. CLDN6- and CLDN9-specific mAbs do not inhibit HCV infection in Huh7.5.1 cells. (A) Binding of CLDN-specific mAbs to Huh7.5.1 cells. Huh7.5.1 cells were detached and incubated with CLDN-specific mAbs. MAb binding (4 µg/ml) was revealed by flow cytometry using CLDN1-specific mAb (OM-7D3-B3) as a positive control. Inhibition of (B) HCVpp entry or (C) HCVcc infection by CLDN-specific mAbs. Huh7.5.1 cells were pre-incubated for 1 h at 37°C with CLDN-specific or control mAbs (100 µg/ml) before infection with HCVpp (strains H77 (1a), HCV-J (1b), JFH1 (2a)) or HCVcc (strains Luc-Con1, genotype 1b/2a and Luc-Jc1, genotype 2a/2a) for 4 h at 37°C. HCV infection was assessed by luciferase activity in cell lysates 72 h post-infection. (D) Inhibition of HCVpp entry in CLDN1-silenced Huh7.5.1 cells. CLDN1-silenced Huh7.5.1 (4) were pre-incubated for 1 h at 37°C with CLDN-specific or control mAbs (100 µg/ml) before infection with HCVpp (strains H77 (1a), HCV-J (1b), JFH1 (2a), UKN3A1.28 (3a), UKN4.21.16 (4)). Western blot demonstrating CLDN1 silencing is indicated below. Means ± SD from three independent experiments performed in triplicate are shown. Statistical analysis relative to the control mAb was performed using the Student's *t* test, *p<0.05

Figure 4. Combining CLDN6- or CLDN9-specific mAbs with anti-CLDN1 mAb does not result in an additive neutralizing effect. Huh7.5.1 cells were pre-incubated with serial concentrations of CLDN1-specific or respective isotype control mAb for 1 h at 37°C and either (A) CLDN6- (WU-9E1-G2, upper panel) or (B) CLDN9- (YD-4E9-G2, lower panel) specific mAbs (10 or 100 µg/ml) before infection with HCVpp (strains H77 (1a), HCV-J (1b), JFH1 (2a), UKN3A1.28 (3a)) in the presence of both compounds. HCVpp infection was analyzed as described in Fig. 3. Means ± SD from one representative experiment performed in triplicate are shown.

Figure 5. CLDN6- and CLDN9-specific mAbs do not inhibit HCVpp infection of PHHs. (A) Binding of CLDN-specific mAbs to PHHs. Following PHH isolation, cells were incubated with CLDN-specific mAbs. MAb binding (20 µg/ml) was assessed as described in Fig. 3. (B) Inhibition of HCVpp entry into PHHs by CLDN-specific mAbs (100 µg/ml) was performed as described in Fig. 3. Means ± SD from three independent experiments performed in triplicate are shown. Statistical analysis relative to the control mAb was performed using the Student's *t* test, *p<0.05. (C) Expression of HCV entry factors in PHHs. Total RNA was extracted from hepatocyte samples (260-282), Hela, Huh7.5 and Caco-2 cells and mRNA copies determined by RTqPCR. Absolute quantities were normalized to GAPDH and data are displayed as % expression relative to CD81 and means ± SD of one experiment performed in quadruplicate are shown. Any signal <0.1 % of CD81 (1,000 fold lower) is negligible. (D) CLDN6 expression in normal liver. CLDN6 expression was analysed by immunohistochemistry on formalin fixed paraffin embedded normal donor and normal liver (right panel), distal to tumour resection tissues (21). A Seminoma case was used as a positive control (middle panel) and a control IgG performed as negative control (left panel). Positive staining is shown in red. Microscopic analysis of tissues showed strong membranous staining of the tumour cells for CLDN6 in the positive Seminoma control section. Normal donor and resection liver tissue demonstrated no detectable CLND6 expression and IgG controls on all sections were negative.