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ABSTRACT

HCV-positive serum (HCVser, genotypes 1a-3a) or JFH1/HCVcc infection of primary normal human hepatocytes was assessed by measuring intracellular HCV RNA strands. Anti-CD81 antibodies and siRNA-CD81 silencing markedly inhibited (>90%) HCVser infection irrespective of HCV genotype, viral load, or liver donor, while hCD81-LEL had no effect. However, JFH1/HCVcc infection of hepatocytes was modestly inhibited (40-60%) by both hCD81-LEL and anti-CD81 antibodies. In conclusion, CD81 is involved in HCVser infection of human hepatocytes, and comparative studies on HCVser *versus* JFH1/HCVcc infection of human hepatocytes and Huh-7.5 cells revealed that the couple cell-virion is determinant of the entry process.

We recently reported that the low density lipoprotein receptor plays a critical role in serum-derived hepatitis C virus (HCVser) infection of human hepatocytes (26). However, several other receptor candidates have been proposed (4, 12, 24, 36, 37), including tetraspanin CD81 (32). HCV glycoprotein E2 binds with high affinity to the large extracellular loop (LEL) of human and chimpanzee CD81 in a genotype-dependent manner (29, 35, 38). In contrast, it does not bind mouse or rat CD81 and binds only weakly to african green monkey CD81 (14). A number of reports confirmed the role of CD81 in the entry of viral pseudoparticles (HCVpp) and cell culture particles (JFH1/HCVcc) in liver cell lines (6, 7, 10, 18, 22, 23, 43, 46). However, these experimental models do not mimic the natural infection process, and several lines of evidence indicate that the infectious properties of HCV may be modulated by host factors that are present *in vivo* (5, 27, 42).

The current study was undertaken to evaluate the role of CD81 in HCVser infection of highly differentiated normal primary human hepatocytes. Hepatocyte isolation, culture conditions, infection with serum from HCV chronically infected patients and treatments were performed as described elsewhere (9, 15, 26, 30). These cultures retain liver phenotypic markers (3, 8, 11, 13, 16, 28, 31) and are sensitive to HCV or hepatitis delta virus (HDV) infection (1, 9, 15, 26, 39). Intracellular accumulation of replicative and genomic HCV RNA strands, assessed by rTth-RT-PCR and quantitative RT-PCR respectively, were used as experimental end-points (9, 15, 20, 21, 26). Statistical analysis of data was performed using the non-parametric signed-rank Wilcoxon test (34). We verified that hepatocytes cultured under our standard conditions express both CD81 and CD9, a closely related tetraspanin (not shown). On average, after HCVser infection of $CD81^+/CD9^+$ hepatocytes at a genome equivalent per cell (Geq/cell) ratio of 0.025, the number of HCV genome copies per hepatocyte is 0.18±0.10 (n=13, range : 0.04 to 0.37).

Anti-CD81 monoclonal antibodies (mAbs) JS81 and JS64 inhibited HCVser (genotypes 1-3) infection of hepatocytes in a concentration-dependent manner (**Figure 1A-C**). On average, mAbsJS81 produced 92±9% inhibition. Anti-CD9 mAbsM-L13 or mouse IgG1 isotype control produced no inhibition. When cells were treated with mAbsJS81 at the time of inoculation (T0) or shortly after (30 min), the accumulation of both negative and positive HCV RNA strands was clearly reduced (>90% on positive strand) (**Figure 1D-E**). However, as the interval between HCVser inoculation and anti-CD81 treatment increased, the inhibitory effect gradually decreased. When antibodies were added 8 hours after inoculation, the inhibition was markedly reduced. Neither anti-CD81 nor anti-CD9 mAbs prevented the HDV infection of hepatocytes (not shown). The inhibitory effect of anti-CD81 mAbs is therefore interfering with an early step of HCVser cell entry, and specific to HCV infection.

To reduce CD81 expression, hepatocytes were transduced with FG12 lentivirus (33) expressing siRNAs directed against hCD81 mRNA, CD81/siRNA412 (5'-GCAGTTCTATGACCAGGCC-3'), CD81/siRNA619 (5'-GATCGATGACCTCTTCTCC-3') or CD81/siRNA1262 (5'-GAAGGAACATCAGGCATGC-3'). Cells transduced with lentivirus expressing human Constitutive Androstane Receptor (CAR) siRNA (5'-GCATGAGGAAAGACATGA, CAR/siRNAc382) (45) or GFP only were used as controls. More than 90% of cells were transduced as assessed by GFP expression and western blot analysis revealed that CD81 siRNA produced a >75% inhibition of CD81 expression (not shown). Levels of membrane-bound CD81 assessed by FACS analysis were strongly decreased in CD81 siRNA-transduced cells as compared to CAR siRNA- or FG12-transduced cells (**Figure 1F**). hCD81 siRNAs produced a consistent and significant inhibition (91±6% at MOI 1, $p<0.01$) of HCVser genome replication, while CAR siRNA or empty lentivirus did not (**Figure 1F**). These results confirm the role of CD81 in the HCVser infection of human hepatocytes.

Recombinant CD81-LEL GST-fusion proteins from man, african green monkey and mouse were synthesized as described (17). We first verified that hCD81-LEL binds glycoprotein E2 and that LEL and anti-CD81 mAbs JS81 and JS64 markedly inhibit (>90%) JFH1/HCVcc infection of Huh-7.5 cells (not shown), as observed by others (23, 43). Interestingly, hCD81-LEL did not inhibit HCVser infection of hepatocytes (**Figure 2A-B**). As this was rather unexpected, we compared HCVser and JFH1/HCVcc infection of human hepatocytes. We first established that under our standard conditions of infection (9, 15, 26), JFH1/HCVcc infect hepatocytes at Geq/cell ratio 0.03 and 0.1, as assessed by both genomic and replicative HCVcc RNA strand accumulation in cells and production of infectious virions in cell supernatant (not shown). JFH1/HCVcc genomic strand level was 3.3±1 (n=3; range: 2.5-4.1) copies per hepatocyte after infection at Geq/cell ratio 0.03 (and 9.7 at Geq/cell ratio 0.1). This is an order of magnitude greater than that observed (0.18±0.10) after HCVser infection at the same Geq/cell ratio. Unexpectedly, however, JFH1/HCVcc infection of human hepatocytes was inhibited significantly but only partly (40-60%) by both hCD81-LEL (**Figure 2C-D**) and anti-CD81 mAbs (compare **Figures 3A-B and 1B**), in sharp contrast with the strong inhibition (>90%) observed in JFH1/HCVcc infection of Huh-7.5 cells (not shown) and in HCVser infection of hepatocytes (**Figure 1, 92%**).

The current observation that hCD81-LEL does not inhibit HCVser infection of hepatocytes, and only partly inhibits JFH1/HCVcc infection of hepatocytes is puzzling on the basis that HCVser does infect human hepatocyte in a CD81-dependent manner that can be specifically inhibited with anti-CD81 mAbs or CD81 specific siRNA. Indeed, previous (and current) studies have shown that hCD81-LEL strongly reduces HCVpp or JFH1/HVCcc infection of Huh-7 or Huh-7.5 cells (6, 7, 10, 18, 23, 43, 46). Several hypothesis may be proposed for this observation. First, it is possible that within the infectious HCVser lipoviroparticles (2, 19, 25, 41), lipoproteins prevent the interaction between glycoprotein E2

and hCD81-LEL. This is supported by the findings that viral particles from human plasma do not bind hCD81-LEL (44) and that some human sera have to be treated with a detergent (deoxycholate) to release HCV particles able to interact with CD81 in Huh-7 cells (40). Second, it is possible that the affinity of hCD81-LEL for E2 is much lower than that of membrane-bound CD81 in normal hepatocyte. Third, a primary binding of HCVser to a non-CD81 membrane molecule as for example LDLR and SR-BI (14, 26), may trigger the virus binding to CD81 on the hepatocyte membrane so that hCD81-LEL cannot compete in the natural membrane environment.

In contrast to recombinant CD81-LEL proteins which are expected to compete with the natural membrane receptor for binding virus particles, antibodies directly target the cellular receptor. Their efficiency is therefore expected to be greater. This is consistent with current results revealing that the efficacy of mAbJS81 is approximately one to two orders of magnitude greater than that of recombinant hCD81-LEL, on a molar concentration basis (not shown). Anti-CD81 mAbs inhibited JFH1/HCVcc infection of primary hepatocytes to a weaker extent (60%) as compared with HCVser infection of primary hepatocytes (92%), and JFH1/HCVcc infection of Huh-7.5 cells (>90%, not shown). It is possible that the higher input of infectious virus in JFH1/HCVcc *versus* HCVser infection of hepatocytes, the difference in affinity or kinetics of HCV interaction with Huh-7.5 *versus* hepatocyte membrane receptors, and the difference in membrane protein population in Huh-7.5 cells *versus* hepatocytes account for these differences in inhibition efficiency. In sum, the data obtained on recombinant CD81-LEL and anti-CD81 antibodies suggest that CD81 has a much greater impact on the entry of JFH1/HCVcc in Huh-7.5 cells than it does on the entry of HCVcc particles in primary hepatocytes. Nevertheless, other data provided here show that CD81 indeed mediates the entry of HCVser in hepatocytes. It is therefore possible that the nature of the couple cell-virion is an important determinant of the virus entry process.

It is interesting that JFH1/HCVcc particles are able to infect primary human hepatocytes as revealed here by the intracellular accumulation of both genomic and replicative (not shown) strands. Indeed, although HCVser are infectious for primary hepatocytes, it has to be noted that less than 15% of the tested sera are actually infectious (15). The reason for this observation is unclear but could be related to the presence of anti-HCV antibodies in the serum of chronically infected patients. In addition, the Geq/cell ratio with HCVser can hardly be greater than 0.1 because of the limited amounts of infectious serum. Hence, the possibility to infect primary hepatocytes with unlimited amounts of JFH1/HCVcc at much greater Geq/cell ratio will strongly facilitate future studies. The rate of genome replication of JFH1/HCVcc in human hepatocytes appears to be one order of magnitude greater than that observed with HCVser, whereas it is one order of magnitude smaller than that observed in Huh-7.5 cells at the same Geq/cell ratio. Thus, although JFH1/HCVcc particles are adapted to Huh-7.5 cells, they can replicate their genome and proliferate in primary human hepatocytes, albeit at a lower rate. This could result from the greater antiviral response of human hepatocytes as compared to Huh-7.5 cells (L. Pichard-Garcia, S. Molina, P. Maurel, unpublished results). On the other hand, the fact that the rate of genome replication of JFH1/HCVcc in hepatocytes is greater than that of HCVser suggests that either only a small portion of HCVser virions are infectious, or antibodies present in the serum strongly affect their infectivity.

In conclusion, CD81 plays a critical role in an early step of HCVser infection of primary human hepatocytes. Comparative studies on HCVser *versus* JFH1/HCVcc infection of human hepatocytes and on JFH1/HCVcc infection of human hepatocytes *versus* Huh-7.5 cells suggest that the nature of the couple cell-virion is a determinant factor for virus entry. This would be consistent with the fact that several receptor candidates have been identified

for HCV and that their interactions with one another or with other membrane components might modulate the way they associate with the various virion populations.

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LEGENDS

FIGURE 1. Effect of anti-CD81 mAbs and of siRNA-mediated silencing of CD81 on HCVser infection of primary human hepatocyte (PHH)

Three days post-plating, hepatocytes FTBP from a 52y-old male with metastasis of colic tumor were exposed or not (UT) to increasing concentrations (0.5 to 6 μg/ml) of anti-CD81 mAbs JS81 or JS64, or anti-CD9 mAbs M-L13, or mouse IgG1 isotype MOPC-21 for 30 min before inoculation with HCV(+) serum S298 (genotype 3a, 1.5 10^6 IU/mL). Following overnight exposure, cells were washed three times and the culture was continued. Five days post-inoculation, cells were washed three times and total cellular RNA was extracted.

A. One μg of total RNA was analysed by rTth-RT-PCR for the replicative RNA strand.

B. One μg of the same RNA sample was analysed by real-time PCR for the genomic strand. RNA quality control was evaluated by performing a quantification of GAPDH mRNA in each sample. Amounts of HCV RNA were normalized to GAPDH mRNA. Results are expressed as percent of intracellular viral RNA relative to the amount obtained in *in vitro* infected hepatocytes in the absence of treatment (0). Data are mean of three independent assays. These results are representative of observations made with *all other hepatocyte cultures* from different donors used in this work.

C. Three days post-plating, hepatocytes FT168 (75y-old female with metastasis of colic tumor) were exposed to HCV(+) serum S42 (genotype 1b, 6 10^6 IU/mL), S31 (genotype 2, 13 10^6 IU/mL) or S34 (genotype 1b, 60 10^6 IU/mL), hepatocytes FT196 (35y-old female with metastasis of colic tumor) were exposed to serum S155 (genotype 1a, 1.1 10^6 IU/mL), hepatocytes FT 212 (52y-old male with metastasis of colic tumor) were exposed to serum S183 (genotype 3a, 2.6 10^6 IU/mL), and hepatocytes FT249 (52y-old male with metastasis of colic tumor) were exposed to serum S192 (genotype 3a, 2.5 10^6 IU/mL), in the absence (UT) or presence of anti-CD81 mAbs JS81 at 4 μg/ml (mAbs), and further analysis was carried out

as indicated above. Amounts of HCV RNA were normalized to GAPDH mRNA. Results are expressed as percent of intracellular viral RNA relative to the amount obtained in *in vitro* infected hepatocytes in the absence of treatment (UT). Data are mean of three independent assays. These results are representative of observations made with other hepatocytes cultures from different donors FT161 (35y-old male organ donor), 167 (57y-old male with metastasis of colic tumor), 176 (69y-old female with metastasis of colic tumor), 195 (17y-old male organ donor) and FTBP.

D and E. Three days post-plating, hepatocytes (FT196) were exposed to HCV(+) human serum S155. Cells were left untreated (UT) or treated with anti-CD81 mAbs JS81 (4 µg/ml) added either at the time of inoculation with HCV (+) serum (0), or 30 min, 1h, 2h, 4h, 8h later, and further analysis was carried out as indicated above.

D. One µg of total RNA was analysed by rTth-RT-PCR for the replicative RNA strand.

E. One µg of the same RNA sample was analysed by real-time PCR for the genomic strand. Amounts of HCV RNA were normalized to GAPDH mRNA. Results are expressed as percent of intracellular viral RNA relative to the amount obtained in *in vitro* infected hepatocytes in the absence of treatment (UT). Data are mean of three independent assays. These results are representative of observations made with another hepatocyte culture from a different donor (FT195).

F. Hepatocytes FT259 (76y-old male with hepatocellular carcinoma) were transduced overnight with suspensions of lentivirus expressing hCD81 siRNA 412, 619 or 1262, or expressing human Constitutive Androstane Receptor (hCAR) siRNA 382, at a multiplicity of infection (MOI) 0.5 or 1. Non-transduced cells were used as control (NT). Seven days later, GFP-positive hepatocytes were stained with anti-CD81 mAb JS-81 conjugated with Zenon, and analysed by flow cytometry. The results are expressed in % of GFP-positive cells expressing (+) or not expressing (-) CD81 protein. Then, hepatocytes were infected at day 8

with HCV positive serum sample S268 (genotype 1a, 4.9 10^6 IU/mL) and at day 3 post-inoculation the intracellular HCV RNA was analyzed by quantitative real-time RT-PCR as indicated in the legend of Figure 1. All experiments were carried out in duplicate. These results are representative of various cell cultures and infectious serum samples S297 (genotype 3a, 1.5 10^6 IU/mL) for culture FT256 (43y-old female with adenoma), and S268 and S301 (genotype 3a, 3.3 10^6 IU/mL) for culture FT259. Statistical analysis and p values were obtained using the Wilcoxon test.

FIGURE 2. **Effect of recombinant mouse, green monkey and human CD81-LEL on HCVser and JFH1/HCVcc infection of primary human hepatocyte (PHH)**

A and B. HCV positive serum S297 or S298 was pre-incubated for 30 minutes at 4°C with 10, 20 or 40µg/ml green monkey (G), mouse (M) or human (H) CD81-LEL fusion proteins and, three days post-plating, hepatocytes FT257 (30y-old female with hydatid cyst) or FT259 were inoculated with these mixtures under our standard conditions. After exposure, cells were washed three times to remove excess of inoculum and the culture was continued for 3 days. Total cellular RNA was extracted and one µg was analysed by rTth-RT-PCR for the replicative RNA strand (FT257/S297) or by real-time PCR for the genomic strand. Amounts of HCV RNA were normalized to GAPDH mRNA. All experiments were carried out in duplicate. These results represent average data obtained from these four series of experiments (FT257/S297 or S298; and FT259/S297 or S298). These experiments were repeated with two different lots of CD81-LEL fusion proteins with same results.

C and D. JFH1/HCVcc was pre-incubated or not (UT) with 10, 20 or 40 µg/ml of green monkey (G), human (H), or mouse (M, only 40 µg/ml) CD81-LEL during 30 min and used to inoculate hepatocytes FT273 (64y-old male with metastasis of colic tumor), FT276 (62y-old female with metastasis of colic tumor) and FT281 (56y-old male with metastasis of colic

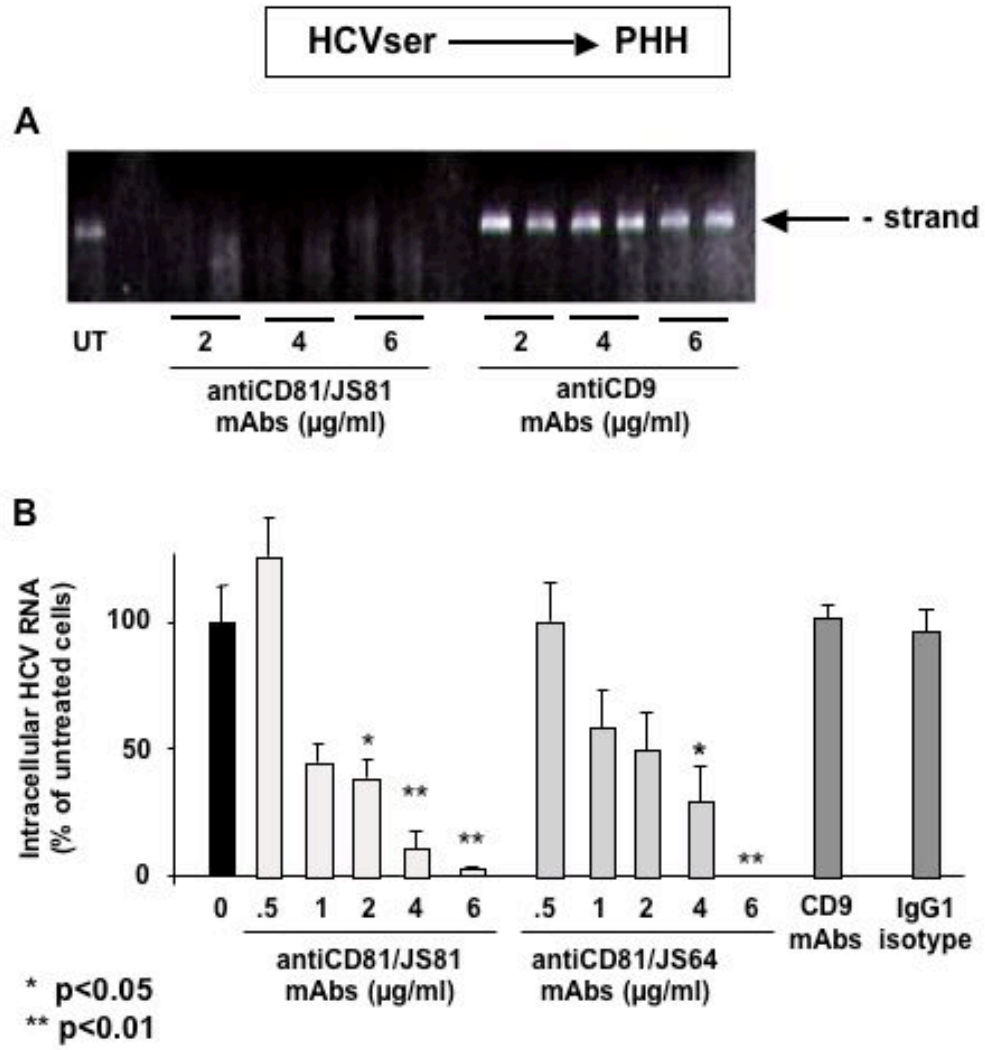
tumor) at different genome equivalent per cell ratios (0.03 and 0.1), three days post-plating. Three days later, cells were washed three times and total cellular RNA was extracted. One µg of the same RNA sample was analysed by real-time PCR for the genomic strand. RNA quality control was evaluated by performing a quantification of GAPDH mRNA in each sample. Amounts of HCV RNA were normalized to GAPDH mRNA. Results are expressed as genome equivalent copies per cell. Data are mean of three independent assays. Statistical analysis and p values were obtained using the Wilcoxon signed-rank test.

FIGURE 3. **Effect of anti-CD81 mAbs on JFH1/HCVcc infection of primary human hepatocyte (PHH)**

Human hepatocytes (FT273, FT276 and FT281) were inoculated with JFH1/HCVcc at different genome equivalent per cell ratios (0.03 and 0.1) in the absence (UT) or presence of 0.5 to 6 µg/ml of anti-CD81 mAbs JS81, or JS64 added for 30 min before inoculation. Control experiments were performed under the same conditions with anti-CD9 mAbs M-L13 (not shown). Following overnight exposure, cells were washed three times and the culture was continued. Three days later, cells were washed three times and total cellular RNA was extracted. One µg of total RNA was analysed by real-time PCR for the genomic strand. RNA quality control was evaluated by performing a quantification of GAPDH mRNA in each sample. Amounts of HCV RNA were normalized to GAPDH mRNA. Results are expressed as percent of control (UT). Data are mean ± SD of three independent assays. Statistical analysis and p values were obtained using the Wilcoxon signed-rank test.

1

Fig. 1A-B S Molina et al.



2
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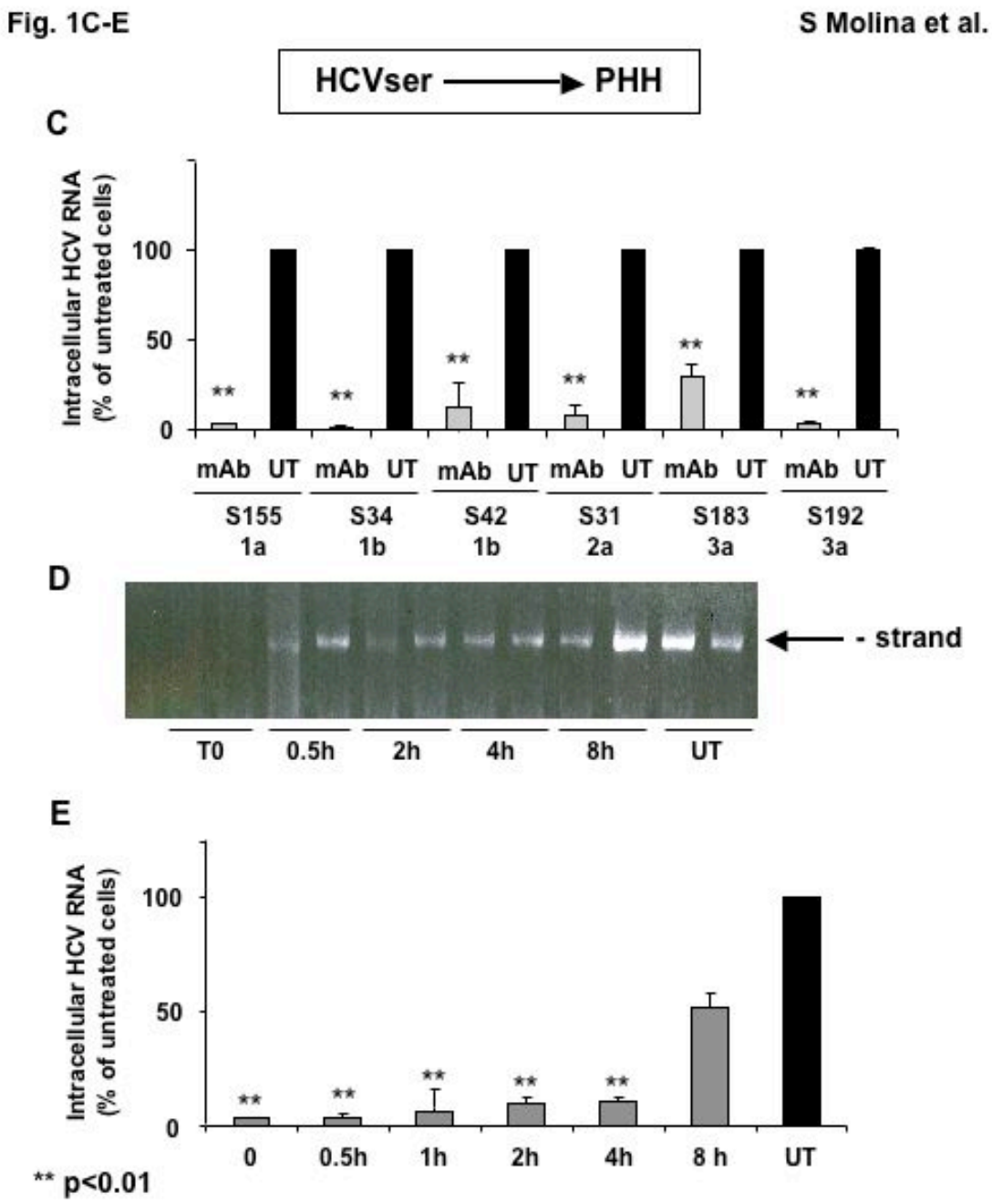
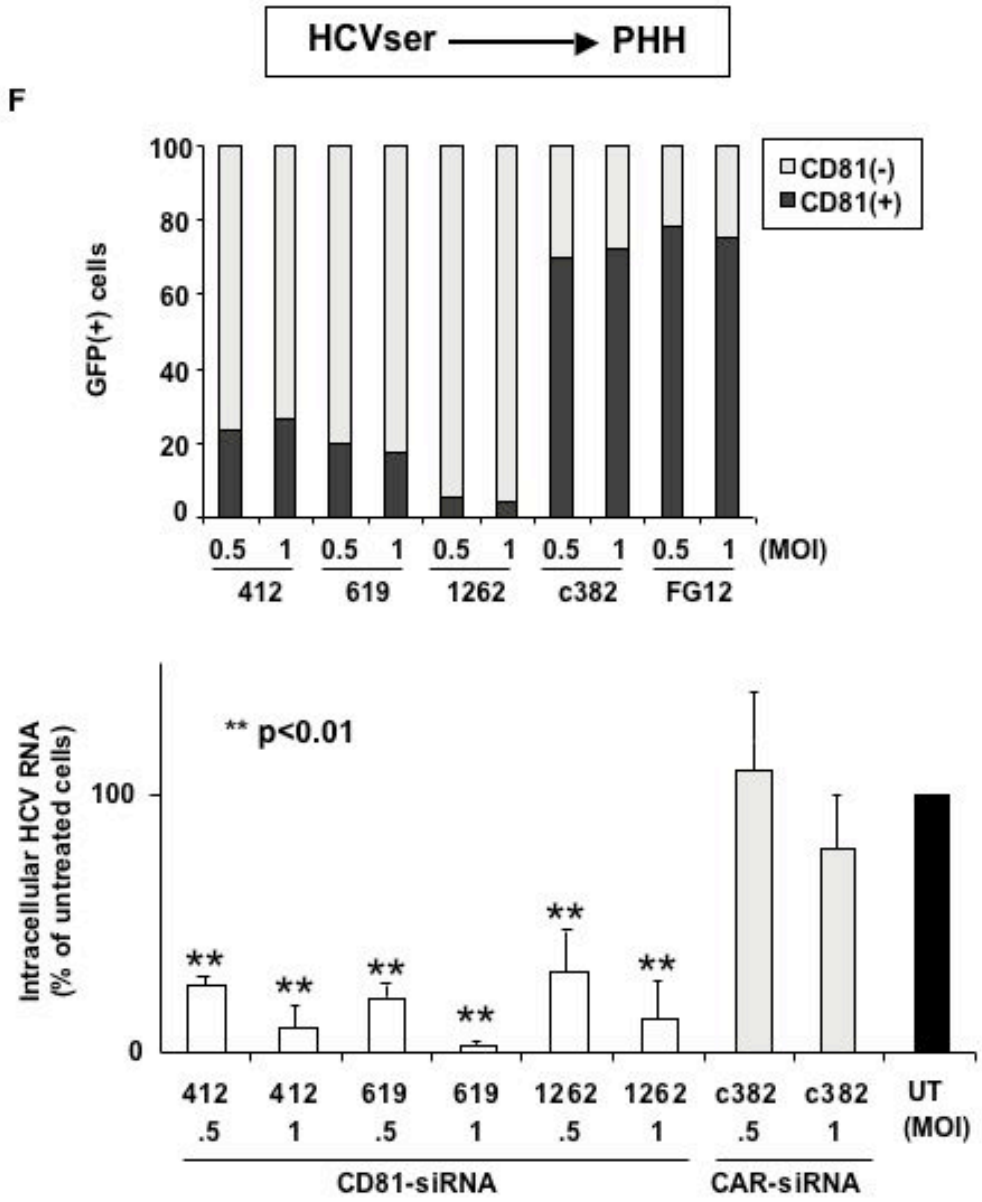


Fig. 1C-E
S Molina et al.
HCVser ⟶ PHH
C
Intracellular HCV RNA (% of untreated cells)
100
50
0
**
mAb UT mAb UT mAb UT mAb UT mAb UT mAb UT
S155 1a
S34 1b
S42 1b
S31 2a
S183 3a
S192 3a
D
- strand
T0 0.5h 2h 4h 8h UT
E
Intracellular HCV RNA (% of untreated cells)
100
50
0
0 0.5h 1h 2h 4h 8 h UT
** p<0.01

1
2

1

Fig. 1 F **S Molina et al.**



2
3

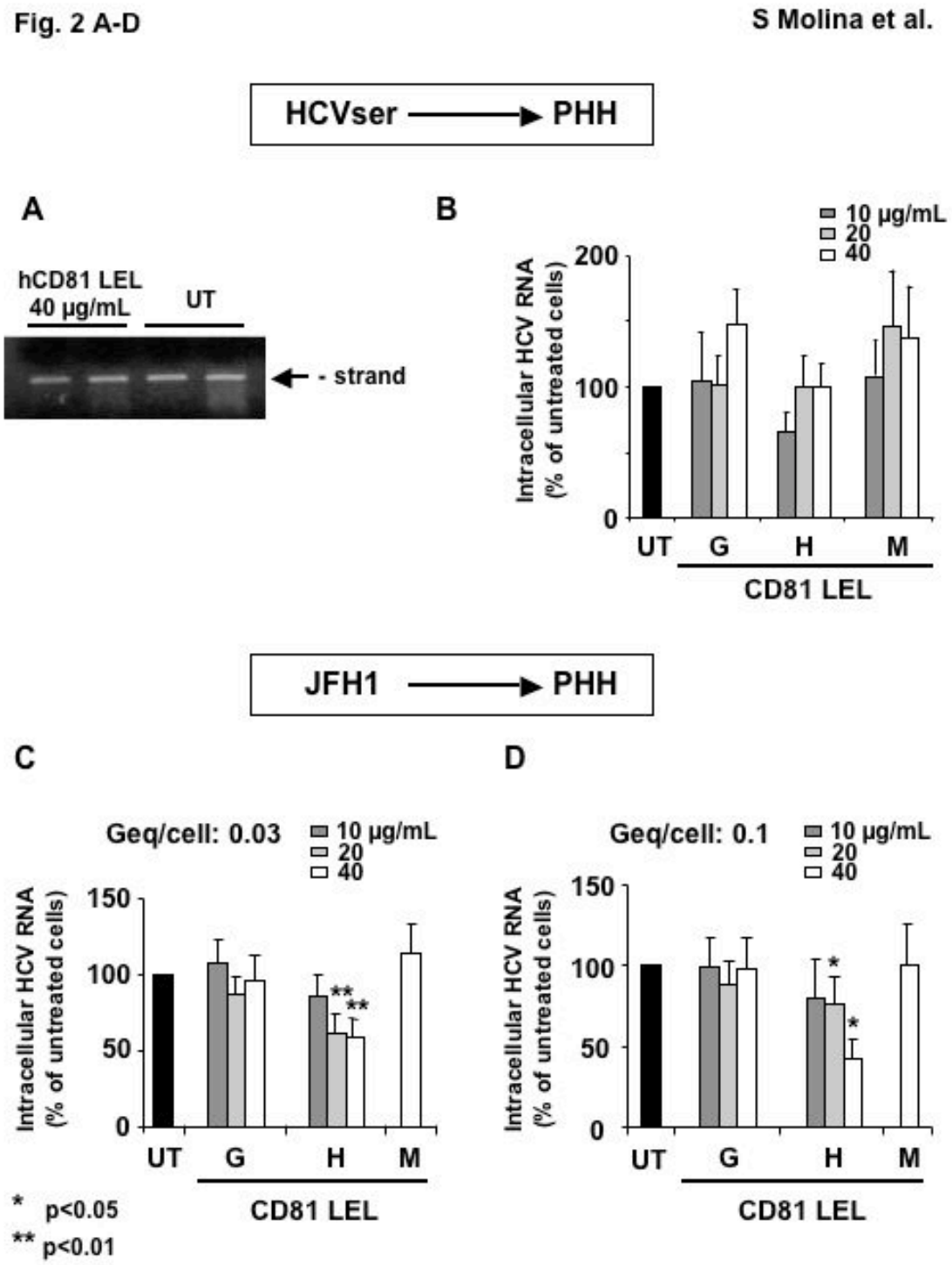


Fig. 2 A-D
S Molina et al.
HCVser → PHH
A
hCD81 LEL
40 µg/mL
UT
- strand
B
10 µg/mL
20
40
Intracellular HCV RNA
(% of untreated cells)
200
100
0
UT
G
H
M
CD81 LEL
JFH1 → PHH
C
Geq/cell: 0.03
10 µg/mL
20
40
Intracellular HCV RNA
(% of untreated cells)
150
100
50
0
**
**
UT
G
H
M
CD81 LEL
* p<0.05
** p<0.01
D
Geq/cell: 0.1
10 µg/mL
20
40
Intracellular HCV RNA
(% of untreated cells)
150
100
50
0
*
*
UT
G
H
M
CD81 LEL

1
2

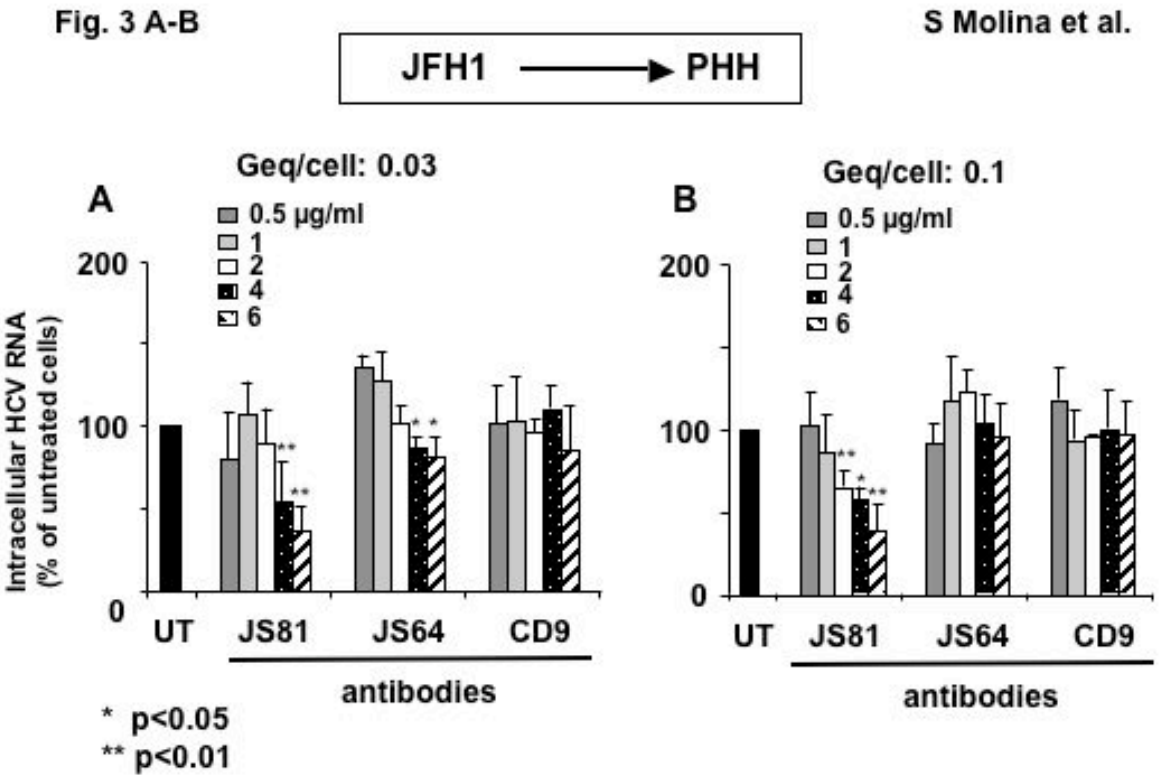


Fig. 3 A-B
S Molina et al.
JFH1 ⟶ PHH
Geq/cell: 0.03
A
0.5 µg/ml
1
2
4
6
Intracellular HCV RNA (% of untreated cells)
200
100
0
UT
JS81
JS64
CD9
antibodies
Geq/cell: 0.1
B
* p<0.05
** p<0.01

1