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► **To cite this version:**

Sonia Molina, Valerie Castet, Lydiane Pichard-Garcia, Czeslaw Wychowski, Eliane Meurs, et al.. Serum-derived hepatitis C virus infection of primary human hepatocytes is tetraspanin CD81 dependent.: CD81 and HCV infection of primary hepatocyte. *Journal of Virology, American Society for Microbiology*, 2008, 82 (1), pp.569-74. 10.1128/JVI.01443-07 . inserm-00374410

**HAL Id: inserm-00374410**

**<https://www.hal.inserm.fr/inserm-00374410>**

Submitted on 8 Apr 2009

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1                   **Serum derived HCV infection of primary human hepatocytes**  
2                                   **is tetraspanin CD81 dependent**

3  
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7 Running Title : CD81 and HCV infection of primary hepatocyte

8 **Keywords** : virus receptor, virus entry, genome replication

9 Abstract :98 words ; text : 1600 words

10

1 **ABSTRACT**

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

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12

1           We recently reported that the low density lipoprotein receptor plays a critical role in  
2 serum-derived hepatitis C virus (HCVser) infection of human hepatocytes (26). However,  
3 several other receptor candidates have been proposed (4, 12, 24, 36, 37), including tetraspanin  
4 CD81 (32). HCV glycoprotein E2 binds with high affinity to the large extracellular loop  
5 (LEL) of human and chimpanzee CD81 in a genotype-dependent manner (29, 35, 38). In  
6 contrast, it does not bind mouse or rat CD81 and binds only weakly to african green monkey  
7 CD81 (14). A number of reports confirmed the role of CD81 in the entry of viral  
8 pseudoparticles (HCVpp) and cell culture particles (JFH1/HCVcc) in liver cell lines (6, 7, 10,  
9 18, 22, 23, 43, 46). However, these experimental models do not mimic the natural infection  
10 process, and several lines of evidence indicate that the infectious properties of HCV may be  
11 modulated by host factors that are present *in vivo* (5, 27, 42).

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

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

12           To reduce CD81 expression, hepatocytes were transduced with FG12 lentivirus (33)  
13 expressing siRNAs directed against hCD81 mRNA, CD81/siRNA412 (5'-  
14 GCAGTTCTATGACCAGGCC-3'), CD81/siRNA619 (5'-GATCGATGACCTCTTCTCC-  
15 3') or CD81/siRNA1262 (5'-GAAGGAACATCAGGCATGC-3'). Cells transduced with  
16 lentivirus expressing human Constitutive Androstane Receptor (CAR) siRNA (5'-  
17 GCATGAGGAAAGACATGA, CAR/siRNAc382) (45) or GFP only were used as controls.  
18 More than 90% of cells were transduced as assessed by GFP expression and western blot  
19 analysis revealed that CD81 siRNA produced a >75% inhibition of CD81 expression (not  
20 shown). Levels of membrane-bound CD81 assessed by FACS analysis were strongly  
21 decreased in CD81 siRNA-transduced cells as compared to CAR siRNA- or FG12-transduced  
22 cells (**Figure 1F**). hCD81 siRNAs produced a consistent and significant inhibition (91±6% at  
23 MOI 1, p<0.01) of HCVser genome replication, while CAR siRNA or empty lentivirus did  
24 not (**Figure 1F**). These results confirm the role of CD81 in the HCVser infection of human  
25 hepatocytes.

1           Recombinant CD81-LEL GST-fusion proteins from man, african green monkey and  
2 mouse were synthesized as described (17). We first verified that hCD81-LEL binds  
3 glycoprotein E2 and that LEL and anti-CD81 mAbs JS81 and JS64 markedly inhibit (>90%)  
4 JFH1/HCVcc infection of Huh-7.5 cells (not shown), as observed by others (23, 43).  
5 Interestingly, hCD81-LEL did not inhibit HCVser infection of hepatocytes (**Figure 2A-B**). As  
6 this was rather unexpected, we compared HCVser and JFH1/HCVcc infection of human  
7 hepatocytes. We first established that under our standard conditions of infection (9, 15, 26),  
8 JFH1/HCVcc infect hepatocytes at Geq/cell ratio 0.03 and 0.1, as assessed by both genomic  
9 and replicative HCVcc RNA strand accumulation in cells and production of infectious virions  
10 in cell supernatant (not shown). JFH1/HCVcc genomic strand level was  $3.3 \pm 1$  (n=3; range:  
11 2.5-4.1) copies per hepatocyte after infection at Geq/cell ratio 0.03 (and 9.7 at Geq/cell ratio  
12 0.1). This is an order of magnitude greater than that observed ( $0.18 \pm 0.10$ ) after HCVser  
13 infection at the same Geq/cell ratio. Unexpectedly, however, JFH1/HCVcc infection of  
14 human hepatocytes was inhibited significantly but only partly (40-60%) by both hCD81-LEL  
15 (**Figure 2C-D**) and anti-CD81 mAbs (compare **Figures 3A-B and 1B**), in sharp contrast with  
16 the strong inhibition (>90%) observed in JFH1/HCVcc infection of Huh-7.5 cells (not shown)  
17 and in HCVser infection of hepatocytes (**Figure 1, 92%**).

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

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

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



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

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

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

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1 **ACKNOWLEDGMENTS**

2           We would like to express our thanks to: Dr Shoshana Levy (Stanford University  
3 Medical Center) for providing the transformed E. coli strains expressing recombinant CD81-  
4 LEL fusion proteins, Dr Takaji Wakita (Tokyo Metropolitan Institute of Neuroscience) for  
5 providing the pJFH1 plasmid, Dr Charles Rice (Rockefeller University New York) for  
6 providing the Huh-7.5 cell line, and Drs François-Loïc Cosset, Marlène Dreux and Dimitri  
7 Lavillette (Inserm U412, Lyon) for their help in the preparation of lentivirus and their  
8 experiments on the CD81-LEL protein effect on HCVpp entry in Huh-7 cells. This work was  
9 supported by grant 01031 from the “Agence Nationale de Recherches sur le SIDA et les  
10 hépatites virales” (ANRS) and grant 5869 C from “Association pour la Recherche sur le  
11 Cancer” (ARC) to C. F-W. Both V.C and S.M were supported by an ANRS fellowship. J.D.  
12 is an international scholar of the Howard Hughes Medical Institute.

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- 10  
11

1 **LEGENDS**

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

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

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

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

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



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

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

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

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

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

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

8

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

11 A and B. HCV positive serum S297 or S298 was pre-incubated for 30 minutes at 4°C with 10,  
12 20 or 40µg/ml green monkey (G), mouse (M) or human (H) CD81-LEL fusion proteins and,  
13 three days post-plating, hepatocytes FT257 (30y-old female with hydatid cyst) or FT259 were  
14 inoculated with these mixtures under our standard conditions. After exposure, cells were  
15 washed three times to remove excess of inoculum and the culture was continued for 3 days.  
16 Total cellular RNA was extracted and one µg was analysed by rTth-RT-PCR for the  
17 replicative RNA strand (FT257/S297) or by real-time PCR for the genomic strand. Amounts  
18 of HCV RNA were normalized to GAPDH mRNA. All experiments were carried out in  
19 duplicate. These results represent average data obtained from these four series of experiments  
20 (FT257/S297 or S298; and FT259/S297 or S298). These experiments were repeated with two  
21 different lots of CD81-LEL fusion proteins with same results.

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

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

8

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

11 Human hepatocytes (FT273, FT276 and FT281) were inoculated with JFH1/HCVcc at  
12 different genome equivalent per cell ratios (0.03 and 0.1) in the absence (UT) or presence of  
13 0.5 to 6  $\mu\text{g}/\text{ml}$  of anti-CD81 mAbs JS81, or JS64 added for 30 min before inoculation.  
14 Control experiments were performed under the same conditions with anti-CD9 mAbs M-L13  
15 (not shown). Following overnight exposure, cells were washed three times and the culture  
16 was continued. Three days later, cells were washed three times and total cellular RNA was  
17 extracted. One  $\mu\text{g}$  of total RNA was analysed by real-time PCR for the genomic strand. RNA  
18 quality control was evaluated by performing a quantification of GAPDH mRNA in each  
19 sample. Amounts of HCV RNA were normalized to GAPDH mRNA. Results are expressed  
20 as percent of control (UT). Data are mean  $\pm$  SD of three independent assays. Statistical  
21 analysis and p values were obtained using the Wilcoxon signed-rank test.

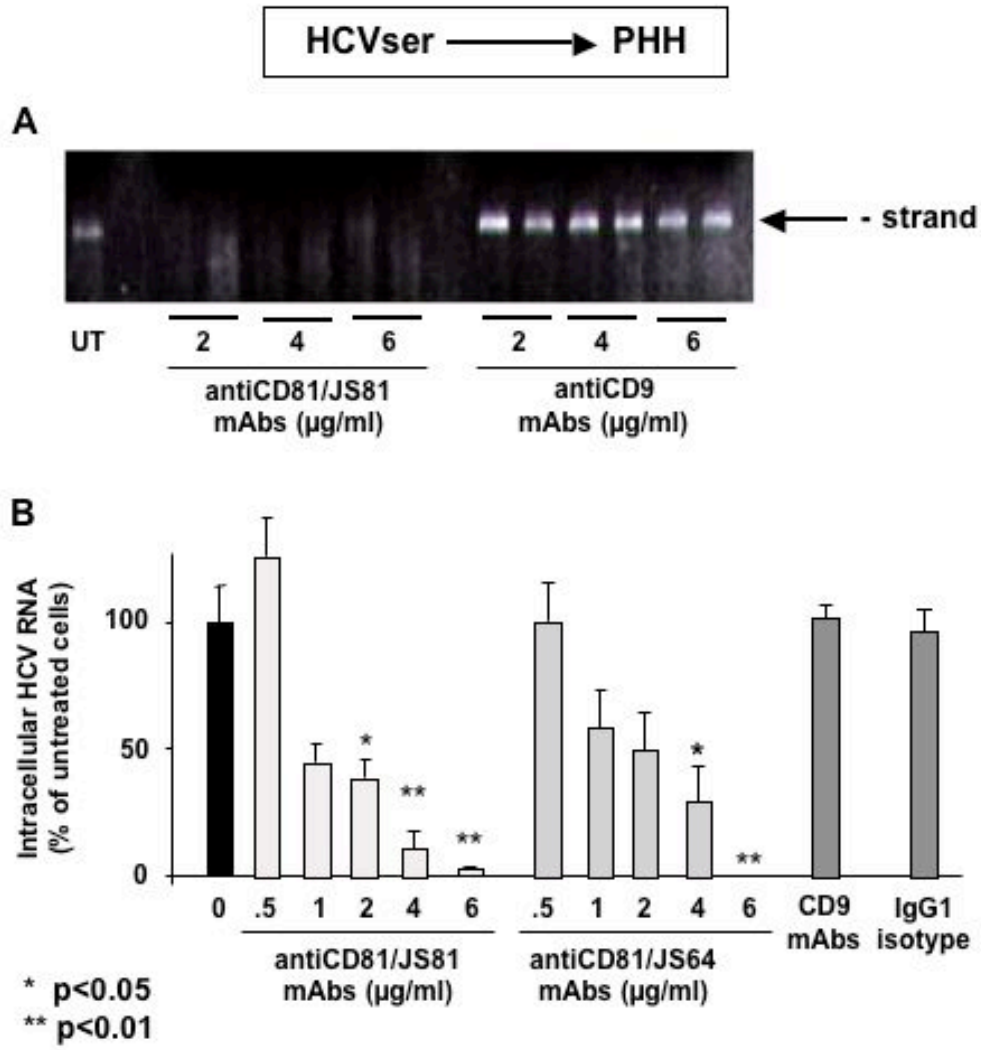
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Fig. 1A-B

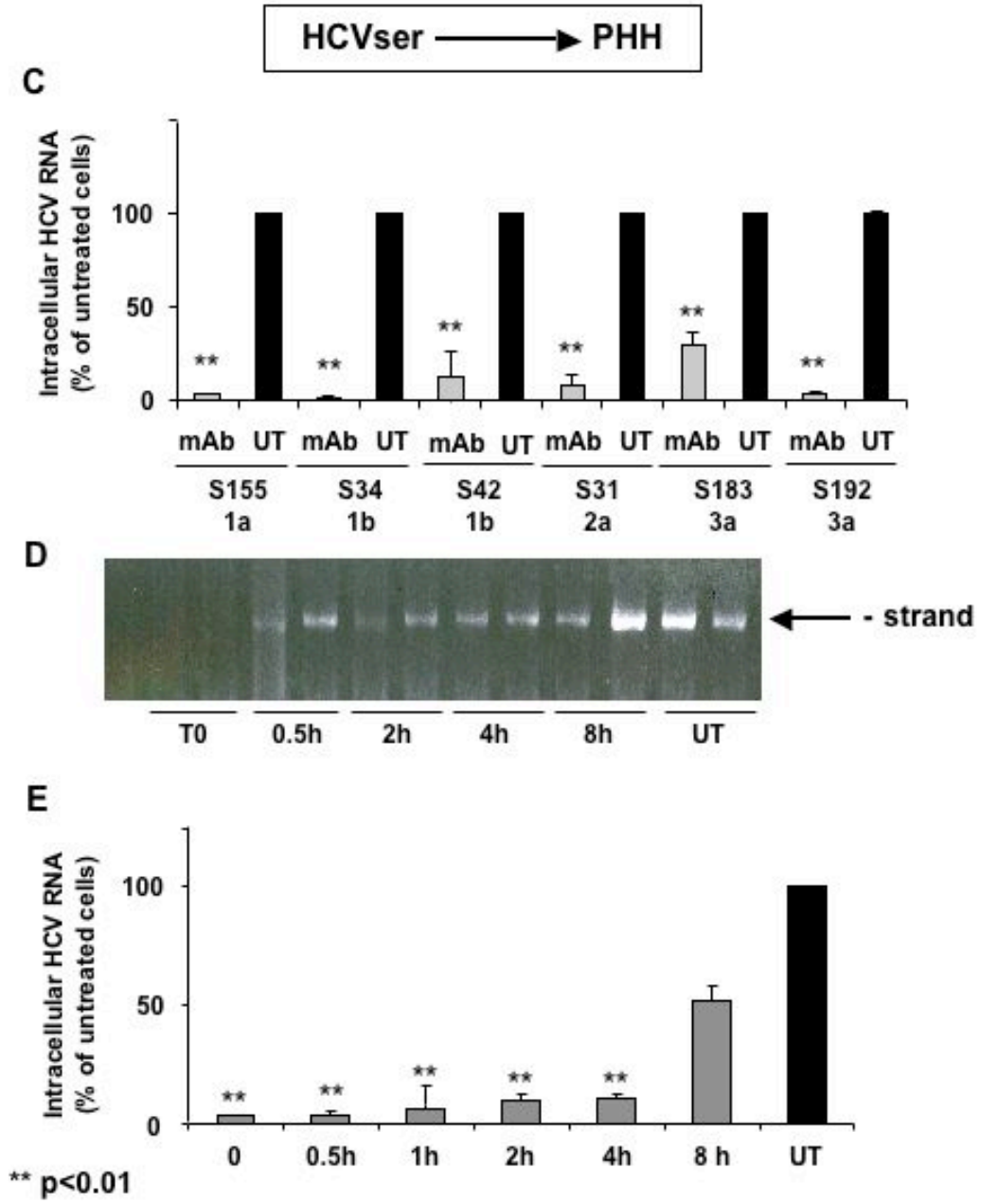
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Fig. 1C-E

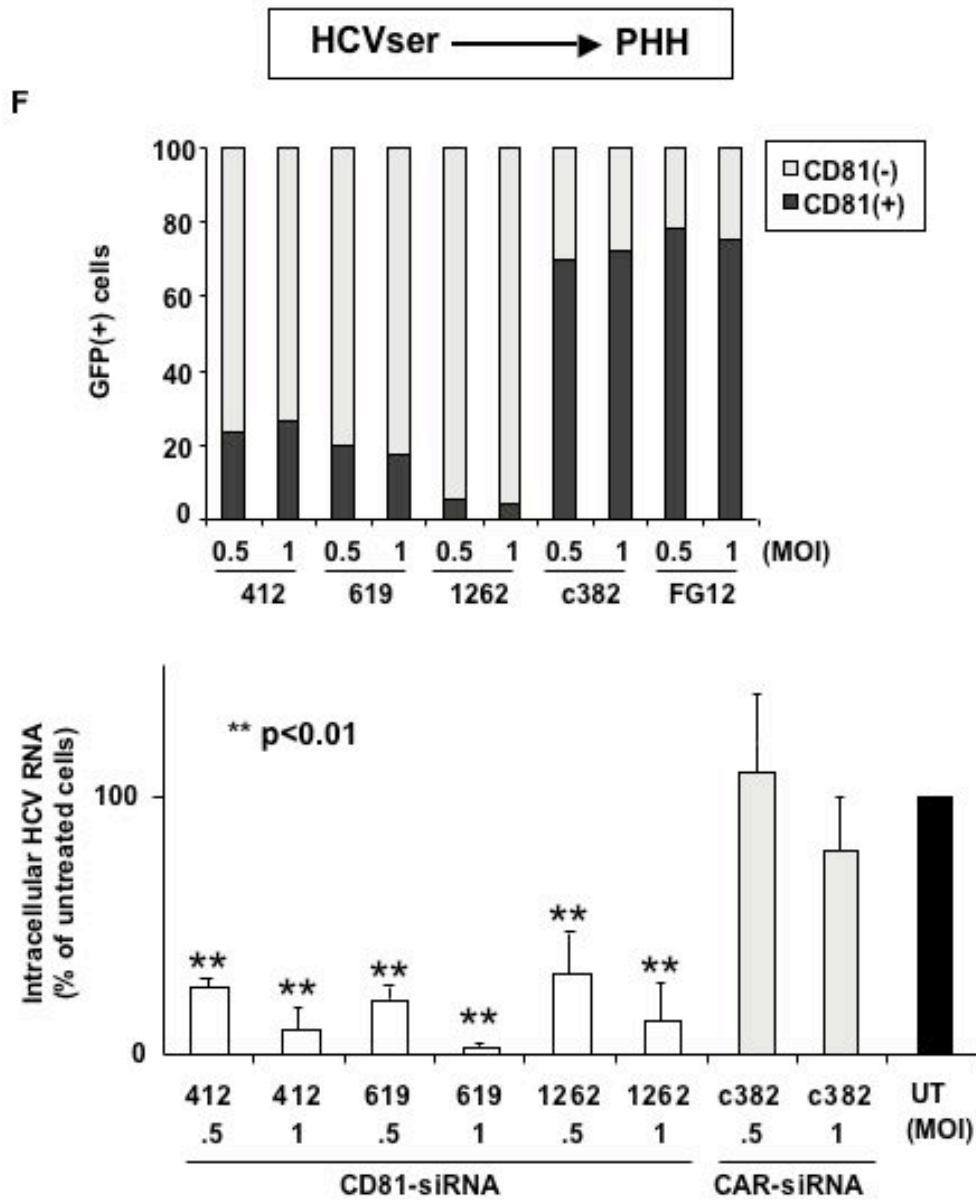
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Fig. 1 F

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Fig. 2 A-D

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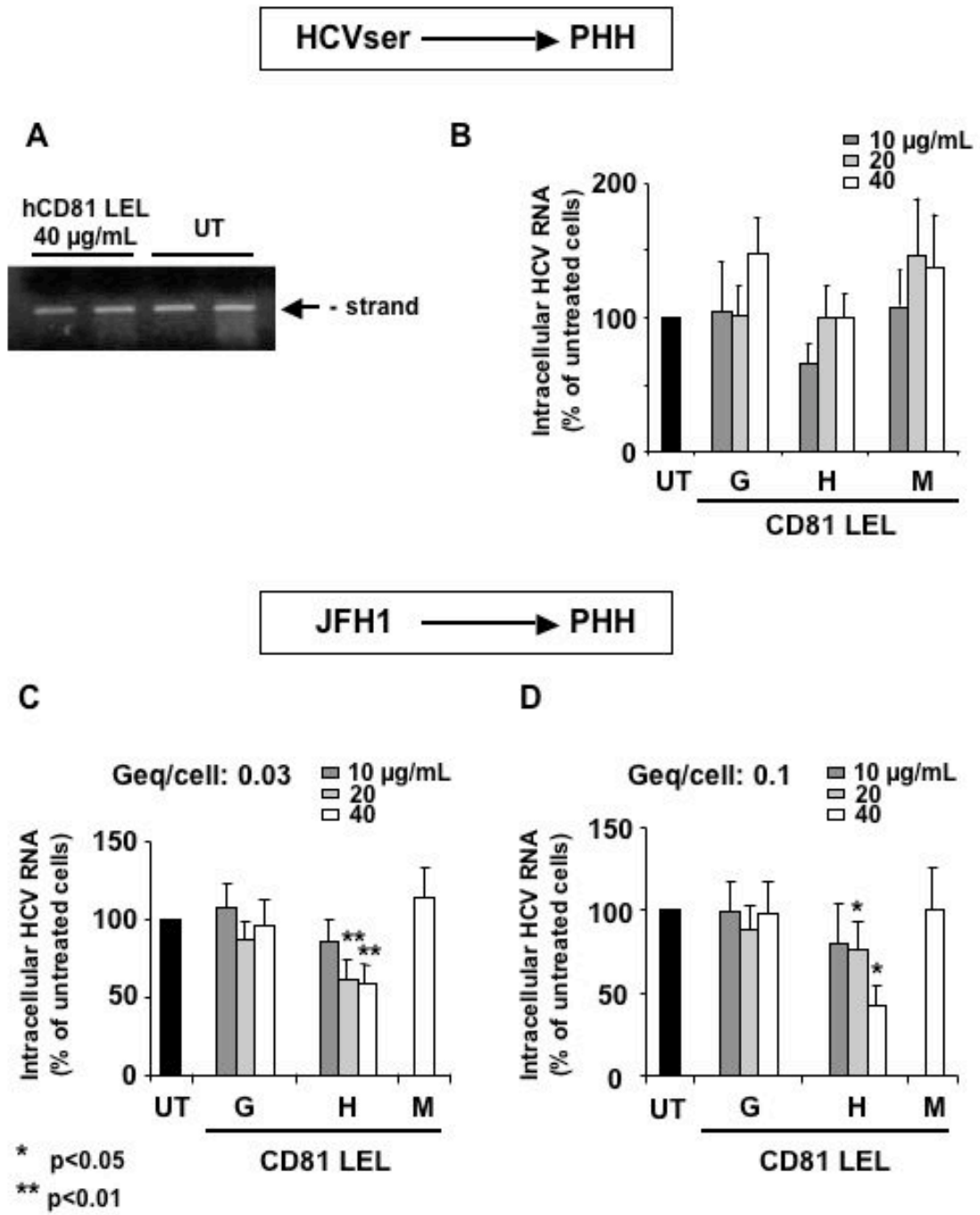


Fig. 3 A-B

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