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Research Article **Journal of Virological Methods**

Enhancing Hepatitis C Virus pseudoparticles infectivity through p7NS2 cellular expression

Running Title: Enhanced infectivity of $HCVpp_{p7NS2}$

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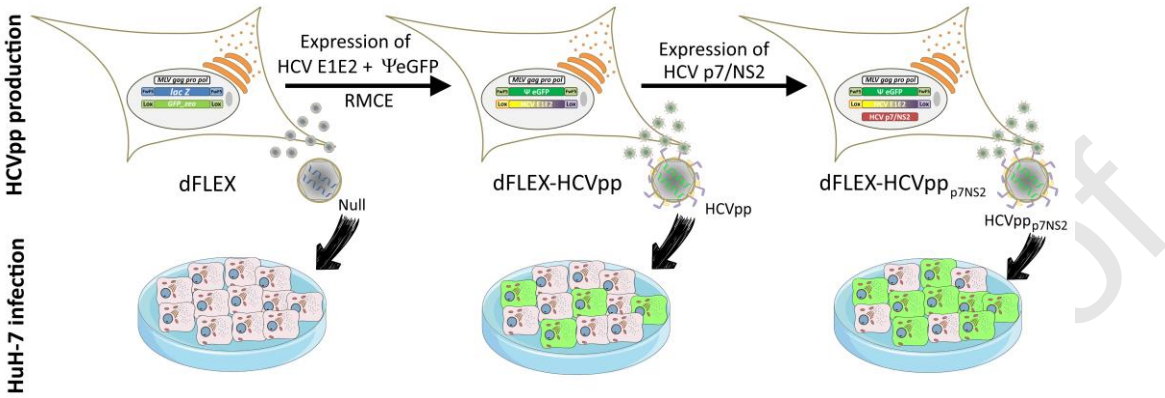
Graphical abstract



Highlights

- Establishment of a versatile cell line producing high titers of Hepatitis C Virus pseudoparticles;
- Cellular co-expression of HCV p7NS2 ORF enhances HCVpp infectivity;
- The inhibitory effect of Rimantadine, a HCV p7 inhibitor, reveals a major contribution of p7 for the enhanced infectivity of HCVpp.

ABSTRACT

Hepatitis C pseudoparticles (HCVpp) are used to evaluate HCV cell entry while screening for neutralizing antibodies induced upon vaccination or while screening for new antiviral drugs. In this work we explore the stable production of HCVpp aiming to reduce the variability associated

with transient productions. The performance of stably produced HCVpp was assessed by evaluating the influence of Human Serum and the impact of CD81 cellular expression on the infectivity of HCVpp. After evaluating the performance of stably produced HCVpp we studied the effect of co-expressing p7NS2 open-reading frame on HCVpp infectivity. Our data clearly shows an enhanced infectivity of $HCVpp_{p7NS2}$. Even though the exact mechanism was not completely elucidated, the enhanced infectivity of $HCVpp_{p7NS2}$ is neither a result of an increase production of virus particles nor a result from increased envelope density. The inhibitory effect of p7 inhibitory molecules such as rimantadine suggests a direct contribution of p7 ion channel for the enhanced infectivity of $HCVpp_{p7NS2}$ which is coherent with a pH-dependent cell entry mechanism. In conclusion, we report the establishment of a stable production system of HCVpp with enhanced infectivity through the overexpression of p7NS2 ORF contributing to improve HCV entry assessment assays widely used in antiviral drug discovery and vaccine development.

Keywords:

Hepatitis C Virus, p7, NS2, Rimantadine, dFLEX

1. Introduction

Recently approved direct-acting antiviral drugs (DAAs) revolutionized the treatment of Hepatitis C virus infections (Cheung et al., 2016; Li and De Clercq, 2017). Nonetheless a wider administration of DAAs is hindered by the almost prohibitive costs, the emergence of resistant viral strains and other limitations, such as the poor diagnosis of acute HCV infection and social stigmas thus urging the need for a prophylactic vaccine (Baumert et al., 2014). Common to the discovery process of both antivirals and vaccines is the need to have efficient reporter assays which monitor the capacity of HCV to infect and replicate. There are two major *in vitro* models

to assess HCV infection, (i) HCV pseudoparticles (HCVpp) and (ii) cell culture adapted HCV (HCVcc). HCVpp are spherical chimeric particles with a diameter of 90-140 nm, containing a retroviral core and both HCV envelope proteins E1 and E2 (Bartosch et al., 2003; Dreux and Cosset, 2009). HCVcc are full length replicative Hepatitis C viruses, this particles have a diameter of 50-80 nm (Steinmann et al., 2008; Wakita et al., 2005). While the latter is a more physiological model, it strongly depends from JFH1 clone and JFH1 chimeras (Catanese and Dorner, 2015), has challenging production protocols (Paulson, 2010), and some gain-of-function mutations introduced to improve HCVcc system that are not present in wild-type strains (Grobler et al., 2003). On the other hand, HCVpp enable assessing the infectivity of unrestricted HCV envelope sequences (Urbanowicz et al., 2016, 2015) using simple production protocols (Bartosch and Cosset, 2009). The major disadvantage of HCVpp system is their limited application to infection studies, being not suitable to access HCV replication. A recent study by Wasilewski and colleagues (2016) has established a positive correlation between HCVpp and HCVcc systems to evaluate the inhibitory potential of monoclonal antibodies thus strengthening the use of HCVpp as model particles.

The protocol for the production of HCVpp is well established and has undergone minor changes over the last 10 years. It involves co-transfecting HEK 293 derived cell lines with 3 or 4 expression plasmids for the production of pseudotyped gammaretroviral (Murine Leukemia Virus, MLV) or lentiviral (Human Immunodeficiency Virus, HIV) vectors, respectively (Bartosch and Cosset, 2009). The use of HCVpp containing luciferase reporter gene increased significantly due to higher assay sensitivity when compared to GFP reporter HCVpp. Disadvantages of transient transfection systems are (i) batch-to-batch variability (Carrondo et al.,

2011; Urbanowicz et al., 2016), and (ii) loss of infectious titers due to cell death and limited time of production.

The work described herein uses recombinase mediated-cassette exchange (RMCE) to develop a cell line stably producing HCVpp from genotype 1a (strain H77) with an eGFP reporter gene. The stable production of HCVpp was attained after exchanging the original GFP-zeo and *lacz* reporter genes present in dual-FLEX cells (dFLEX) (Coroadinha et al., 2006; Soares et al., 2018) by two ORFs encoding HCV envelope proteins E1E2 and an eGFP proceeded by a MLV encapsidation signal, respectively. The successful production of HCVpp in stable enabled studying the effect of HCV nonstructural protein p7 and NS2 expression in HCVpp infectivity. Our results show a direct contribution of p7 ion channel functions to enhance the infectivity of $HCVpp_{p7NS2}$ which was reverted by the addition of the p7 inhibitory molecules.

2. Materials and Methods

2.1 Cell Lines and Culture Media

dFLEX#11 (Soares et al., 2018) cell line derived from human embryonic kidney (HEK 293, ATCC CRL-1573) stably producing non-pseudotyped murine leukemia virus (MLV) based vectors. dFLEX-HCVpp cells derive from dFLEX#11 by site-specific recombination (supplementary information) for expression of HCV envelope proteins and continuous secretion of HCVpp. HuH-7 (JCRB 0403) were purchased from the Japanese Collection of Research Bioresources Cell Bank, HuH7-Lunet cells (Koutsoudakis et al., 2007) were kindly provided by Prof. Ralf Bartenschlager from the University of Heidelberg, Lunet-N cells and Lunet-N hCD81 (Bitzegeio et al., 2010) were kindly provided by Dr. Thomas Pietschmann, TWINCORE Hannover. All cells were maintained in Dulbecco's modified Eagle's medium, DMEM, (Gibco,

Paisley, UK) supplemented with 10 % (v/v) Fetal Bovine Serum (FBS) (Gibco) at 37 ºC inside an incubator with an humidified atmosphere of 5 % CO_2 in air.

2.2 HuH-7 infection assay

Infection of HuH-7 cells with HCVpp was performed as following: HuH-7, HuH7-Lunet, Lunet N or Lunet N hCD81 cells were seeded at $2.5x10^4$ cells cm^{-2} in 24 well plates one day before infection. Transduction was performed in triplicates by transferring the supernatant of HCVpp and $HCVpp_{p7NS2}$ producing cells to target cells in culture. Infection was performed with 0.5 mL of filtered viral supernatant supplemented with 8 mg mL^{-1} of polybrene (Sigma-Aldrich, St. Louis, U.S.A.). Cells were centrifuged at 1500 x g for 1 h at room temperature and placed at 37 ºC for additional 4 h after which culture medium was replaced with DMEM supplemented with 10 % (v/v) FBS. 72 h post-infection, cells were harvested and analyzed by flow cytometry (CyFlow Space, Sysmex, Horgen, Switzerland) to determine the percentage of GFP positive cells. Infectious particles titers as determined as described before (Bartosch et al., 2003a; Tomás et al., 2018). Unless indicated otherwise in the results section, figure legends or discussion, HCVpp production was performed in 2 % (v/v) Fetal Bovine Serum supplemented with 5 % (v/v) Human Serum.

2.3 Antibodies and Reagents

The following antibodies were used in this study: rat anti-MLV p30 monoclonal antibody produced by the hybridoma R187 (ATCC CRL-1912), mouse anti-GFP monoclonal antibody clone 4B10 (Cell Signaling Technology, Danvers, U.S.A.), rabbit anti-β-Galactosidase (Thermo Fisher Scientific Inc, Waltham, U.S.A.), mouse anti-β-Tubulin antibody (Santa Cruz, Dallas,

U.S.A.), mouse monoclonal antibodies anti-HCV E1 (Acris Antibodies, Herford, Germany), mouse anti-HCV E2 (Austral Biologicals, CA, U.S.A.), sheep anti-mouse IgG HRP conjugated GE Healthcare (GE Healthcare Life Sciences, Little Chalfont, UK), Donkey anti-rabbit IgG HRP conjugate (GE Healthcare). Rimantadine and Telaprevir were acquired from Selleck Chemicals (Houston, TX U.S.A.), Chlorcyclizine, *N*N-DNJ, Polybrene, Human Serum, Tannic Acid and Galanthus nivalis agglutinin (GNA) are all from to Sigma–Aldrich and Polyethylenimine (PEI) used for cell transfection was acquired from Polysciences (Warrington, PA).

3. Results

3.1 Production of eGFP-reporter HCVpp in dFLEX cells

To generate HCVpp in dFLEX cells, pZeoCre was co-transfected with pTarLoxHCV thus targetting Cre/Lox RMCE locus to drive the expression of HCV envelope glycoproteins E1 and E2 (Fig. 1A). After antibiotic selection, dFLEX cells were co-transfected with pSVFlpe and pEmMFG-WPRE to drive the incorporation of GFP reporter gene into nascent HCVpp (Fig. 1A). The presence of β-Gal and GFP-Zeo proteins in parental dFLEX but not in dFLEX-HCVpp in contrast with eGFP and HCV E1 and E2 which are present in the opposite samples confirmed the success of double RMCE (Fig. 1B). The incorporation of HCV envelope proteins in purified HCVpp (Fig. 1C), confirmed successful gene exchange which resulted in the production of HCV pseudoparticles (HCVpp) carrying eGFP reporter gene. The presence of high-mannose as well as complex and hybrid glycans in both HCV E1 and E2 envelope proteins (Fig. 1D) is consistent with the glycosylation profiles reported for HCV E1 and E2 incorporated in transiently produced HCVpp (Op De Beeck et al., 2004; Vieyres et al., 2010). Additionally, the capacity of HCVpp to infect HuH-7 cells (Fig. 2A) validates the functionality of HCVpp produced in dFLEX-HCVpp

cells. The contrasting number of HuH-7 cells transduced by HCVpp and HCVpp co-pseudotyped with VSV-G (Fig. 2A), which preferentially enter cells via the latter envelope receptors, suggests that full functional HCVpp particles are produced by dFLEX-HCVpp cells but unable to efficiently infect HuH-7 cells thus stressing the possibility to increase HCVpp infectivity by improving its entry efficiency.

3.2 Optimization of HCVpp production and infectivity

The possibility to improve HCVpp infectivity was initially assessed by Human Serum (HS) supplementation. HCVpp produced in decreasing concentrations of FBS were divided in two test groups, to one test group 2 % (v/v) HS was added immediately before HuH-7 infection while the other was maintained without HS. As shown in Fig. 2B, the titers of infectious HCVpp decreased with the reduction of FBS supplementation, suggesting impaired HCVpp production. The addition of 2 % (v/v) of Human Serum immediately before HuH-7 cells infection reverted this effect associated with lower FBS supplementations. Hence different medium formulations were tested to optimize HCVpp production conditions. As observed in Fig. 2C highest titers of infectious particles (1.5 $x10^6$ IP / mL) were generated in the condition with minimal FBS (2 % (v/v)) and maximal HS concentrations (10 % (v/v)) were used. To discriminate whether HS enhanced the infectivity of HCVpp or increased the permissiveness of target cells, HuH-7 cells were pre-conditioned with increasing concentrations of HS for 48h prior to infection. As visible in Fig. 2D, pre-conditioned HuH-7 cells were similarly transduced by HCVpp as determined by equal infectious titers obtained for the different test conditions. Altogether these data suggests improved cell entry capacity of HCVpp when in the presence of HS.

3.3 HCVpp infection is depend on HCV co-receptor CD81

Engineered target cell lines and HCV entry inhibitors were used to discriminate between specific CD81-mediated HCVpp infection from unspecific HCVpp internalization. To evaluate the dependence of CD81 expression, HuH-7, HuH7-Lunet (Koutsoudakis et al., 2007) and two derived cell lines, (i) Lunet-N which are negative for CD81, and (ii) Lunet-N-hCD81 in which CD81 expression was restored (Bitzegeio et al., 2010) were used as target cells. As shown in Fig. 3A the titers of HCVpp determined in all CD81 positive cells are equivalent. In contrast, only residual infection of Lunet-N, near lower limit of detection, was observed. HCVpp infectivity was further challenged with the addition of HCV-entry inhibitors concomitant to HuH-7 cells infection. All tested inhibitors – an anti-CD81 antibody, a recombinant CD81 protein, the lectin *Galanthus Nivalis* Agglutinin (GNA) and the polyphenol tannic acid – reduced the ability of HCVpp to infect HuH-7 cells while no impact in HCVpp infection was observed when an irrelevant antibody (anti-CXCR5) was used (Fig. 3B). Overall these data attests the dependency of HCVpp infection from CD81 expression.

3.4 Overexpression of p7NS2 ORF enhances HCVpp infectivity

The putative contribution of viral proteins p7 and NS2 to enhance HCVpp infectivity was hypothesized previously due to their role in HCV assembly. Therefore, p7NS2 ORF was delivered to dFLEX-HCVpp cells through lentiviral transduction enabling stable expression and the generation of dFLEX- $HCVpp_{p7NS2}$ cells (Fig.4A). As shown in Fig.4B and Fig. 4C, particles produced by dFLEX- $HCVpp_{p7NS2}$ are significantly more infectious than parental HCVpp. The productivity of engineered dFLEX-$HCVpp_{p7NS2}$ cells is maintained, as determined by similar MLV p30 protein levels in cell culture medium (Fig. 5A) and by similar numbers of total

physical particles secreted to the cell culture medium (Fig. 5B), as is the ultrastructural organization of HCVpp, as indicated by similar incorporation levels of CD81, HCV E2 and HCV E1 determined by ELISA (Fig.5C and 5D) or western blotting (Fig.5E). CD81 protein was used here to control coating efficiency since it is commonly incorporated by retrovirus and retrovirus-based vectors (Segura et al., 2008; Soares et al., 2016). In addition, the effect of p7NS2 expression in the infectivity of non-HCV pseudotypes was evaluated (Fig. S1). GaLV and 4070A pseudotyped particles produced in the presence of p7NS2 expression plasmid showed similar infectivity to particles produced without p7NS2. Altogether, these data indicate that p7NS2 expression specifically affect the performance of retrovirus particles pseudotyped with HCV envelope proteins but not with other viral envelope proteins.

3.5 Rimantadine reverts the infectivity of HCVpp$_{p7NS2}$

Specific inhibitors of HCV p7 were used to evaluate the contribution of HCV p7 to the enhanced infectivity of HCVpp$_{p7NS2}$. As shown in Fig. 6A, the presence of rimantadine during production period reverted the infectivity of HCVpp$_{p7NS2}$ to levels compared to HCVpp. Consistently, the addition of Telaprevir, an inhibitor of HCV NS3/4A protease used as control for DMSO solubilizer, did not affect HCVpp$_{p7NS2}$ infectivity. Additionally, Rimantadine addition did not affect the infectivity of GaLV and 4070A pseudotyped particles (Fig. S1). Noteworthy, the productivity and viability of dFLEX-HCVpp$_{p7NS2}$ cells was maintained in the presence of p7 inhibitor, rimantadine (Fig. S2). To evaluate the potential role of p7 in post-production i.e. in cell entry steps, increasing concentrations of rimantadine, chlorcyclizine and *N*N-DNJ were added to HCVpp and HCVpp$_{p7NS2}$ concomitant to HuH-7 infection. As shown in Fig. 6B, C and D HCVpp$_{p7NS2}$ infectivity is inhibited by the addition of p7 inhibitory molecules in a dose-response

manner. These results suggest that functional p7 protein contribute to the entry process of $HCVpp_{p7NS2}$, either directly or as co-factor for HCV envelope proteins.

4. Discussion

Hepatitis C pseudoparticles are important tools to evaluate the efficacy of vaccine candidates (Huret et al., 2013) and to screen new antiviral compounds (Izquierdo et al., 2016; Liu et al., 2015). Nonetheless, the production of HCVpp in transient depends on a fine balance of plasmid stoichiometry which influences assay performance (Urbanowicz et al., 2016) thus challenging direct comparisons between independent experiments and laboratories. Packaging cell-lines have been extensively used in the past to produce retrovirus with different genomes and envelopes (Coroadinha et al., 2006). Here, the stable production of HCVpp was performed in FLEX cells (Soares et al., 2018); stable production of HCVpp is associated with immediate advantages, such as: (i) the elimination of periodical cell transfections and of the variability associated with different transfection reagents, protocols and batch to batch, (ii) the reduction of reagents and materials (e.g. transfection reagents, plasmids), (iii) reduction of materials preparation and bioreaction time, (iii) the elimination of DNA and other transfection contaminants present in HCVpp-containing cell culture medium; hence enhancing reproducibility of HCVpp production (Carrondo et al., 2011; Schucht et al., 2006). In this work, eGFP reporter gene was used to enable monitoring absolute viral titers as well as the use of non-invasive detection methods such as microscopy and flow cytometry.

Biologically functional HCVpp were produced in dFLEX-HCVpp cells after efficient site specific recombination on dFLEX cells with a targeting plasmid encoding HCV envelope proteins. The characterization of stably produced HCVpp demonstrated the incorporation of

correctly glycosylated forms of HCV E1 and E2 (Fig.1) which promoted the transduction of HuH-7 cells (Fig.2) in a CD81-dependent manner (Fig. 3). Moreover, the infectious titers obtained with the stable production system are similar to those reported for transient productions (Bartosch et al., 2005; Bartosch et al., 2003a) thus validating these cells as an alternative production method. The manipulation of Fetal Bovine Serum (FBS) and Human Serum (HS) concentration in culture medium enabled the optimization of HCVpp production and infection. In contrast to Fetal Bovine Serum which is a known inhibitor of HCV cell attachment (Qin et al., 2013), Human Serum is described as a facilitator of HCV infection (Bartosch et al., 2005; Lavillette et al., 2005; Meunier et al., 2005; Steenbergen et al., 2013). As expected, the simple reduction of FBS during HCVpp production reduced the number of infectious particles (Fig. 2B) which is in agreement with an impaired production of viral particles (Rodrigues et al. 2012). The supplementation of low-FBS cell culture medium with increasing concentrations of HS enhanced the number of infectious particles up to 52-fold (Fig. 2C). These numbers are in agreement with Bartosch et al. (2005) which reported a 20x improvement in the transient production HCVpp when 0.1 % (v/v) FBS cell culture medium was supplemented with 2.5 % (v/v) HS. Likewise, the 48 hours pre-conditioning of HuH-7 cells with HS did not alter HuH-7 cells permissiveness to HCVpp infection (Fig. 2D) as reported by Bartosch et al. (2005) for 1h pre-conditioning study. Aiming to further increase the infectivity of stably produced HCVpp, dFLEX-HCVpp cells were forced to express HCV non-structural proteins p7 and NS2 through lentiviral transduction. HCV p7 and NS2 proteins are described as adjuvants of HCV assembly and maturation (Boson et al., 2011; Jones et al., 2007; Mankouri et al., 2016; Steinmann et al., 2007; Wozniak et al., 2010). NS2 is involved in membrane rearrangement (Mankouri et al., 2016) while p7 oligomers work as a viral-encoded ion-channel regulating pH of newly formed virions (Griffin, 2009). It is known

that natural HCV replication produces different variants of E2p7NS2 cleavages due to incomplete processing of HCV polyprotein. Some of these variants were shown to impact the production of infectious HCV particles. While the inefficient separation of E2 from p7 completely inhibits virus production, the separation of p7 from NS2 has no impact in the infectivity of HCV particles (Shanmugam and Yi, 2013). Moreover the production of infectious HCV particles is enhanced when *in trans* delivered p7 protein is fused to its natural signal peptide sequence (Vieyres et al., 2013). Consequently a (sp)p7NS2 ORF was delivered to dFLEX-HCVpp through lentiviral transduction and the infectivity of produced HCVpp $_{p7NS2}$ was evaluated.

The expression of HCV genes p7 and NS2 in dFLEX-HCVpp$_{p7NS2}$ cells resulted in the production of 4x more infectious particles than parental dFLEX-HCVpp cells (Fig. 4C). A detailed characterization of production supernatants indicated similar productivities and similar pseudotyping of HCVpp (Fig. 5). This is in agreement with the different exit mechanism employed by MLV and HCV which predicted no impact of p7NS2 ORF expression in plasma membrane associated events such as MLV particles assembly, enveloping and release.

HCV p7 has multiple roles during HCV infection such as protecting E2 from premature degradation, or to control the pH of nascent virions (Atoom et al., 2013; Sharma et al., 2011; Steinmann et al., 2013). Moreover the participation of p7 in post-production HCV cell entry events is supported by several evidences such as the facilitation of membrane fusions (Lee et al. 2016), evidences of pH-dependent HCV infection (Bartosch et al., 2003; Fénéant et al., 2015; Sharma et al., 2011) and accumulated evidences that impaired p7 activity reduces infectivity of HCV particles (Bentham et al., 2013; Denolly et al., 2017; Wozniak et al., 2010). Here, we show that neither p7NS2 expression nor rimantadine addition influence the infectivity of MLV

particles pseudotyped with GaLV or 4070A. In contrast, the addition of rimantadine, chlorcyclizine and *N*N-DNJ inhibitors (Breitinger et al., 2016; Griffin et al., 2008) did revert the infectivity of $HCVpp_{p7NS2}$ to levels comparable to HCVpp (Fig.6), supporting the hypothesis that functional p7 molecules participate in HCV fusion events, either directly or by improving HCV E1/E2 envelope infectivity. In summary this work validates the functionality of HCVpp produced in stable by dFLEX-HCVpp and dFLEX-$HCVpp_{p7NS2}$. Moreover it shows that HCV p7 contributes directly to enhance HCVpp infectivity thus grating the possibility to develop improved screening assays based in HCVpp with applicability across different research areas.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. Generation of eGFP-reporter HCVpp using dFLEX cells. **(A)** Schematic representation of pTarLoxHCV and pEmMFG-WPRE plasmids used to target envelope and transgene expression cassettes, respectively. **(B)** Western Blotting of total cell extracts prepared from HEK 293, dFLEX and dFLEX-HCVpp cell lines showing the expression of β-gal, GFP-Zeo, GFP, HCV E1 and E2, MLV p30 and β-Tubulin proteins; **(C)** Western Blotting of purified retroviral particles produced before and after RMCE indicating the incorporation of HCV envelope glycoproteins E1 and E2 in MLV-derived particles. **(D)** Analysis of HCV E1 and E2 glycosylation profile of HCVpp non-treated (Lane 1) or digested with Endo H (Lane 2) or PNGase (Lane 3).

Figure 2. Functional validation of HCVpp. **(A)** Microscopy phase contrast and green-fluorescence images of HuH-7 cells infected with non-pseudotyped retrovirus (top panel), eGFP reporter HCVpp (middle panel) and HCVpp co-pseudotyped with VSV-G envelope glycoprotein (bottom panel). **(B)** Determination of infectious particles titers of dFLEX-HCVpp supernatants produced in decreasing concentrations of FBS with or without the concomitant addition of 2% (v/v) Human Serum at infection. A 1x, 7x, 4x and 2x fold-increase, respectively, is observed when 2% (v/v) human serum is added at infection. **(C)** Infectious titers of eGFP-reporter HCVpp produced with 5% or 2% (v/v) FBS in the presence of increasing concentration of human serum. Significant fold-increases in infectious particles are: for 5% (v/v) FBS, 3x and 6x increase with 5% (v/v) and 10% (v/v) HS, respectively; for 2% (v/v) FBS, 15x and 52x increase with 5% (v/v) or 10% (v/v) HS, respectively. **(D)** pre-conditioning of HuH-7 cells with increasing concentrations of Human Serum resulted in similar HCVpp infectious titers; standard deviation were calculated based on at least three independent experiments; Student t-test was used to analyze statistical significance (** $p<0.001$, * $p<0.05$).

Figure 3. Validation of HCVpp entry through CD81 interaction. **(A)** Titers of infectious HCVpp evaluated in CD81 positive and negative cells. **(B)** Titers of infectious HCVpp evaluated in HuH-7 cells when entry inhibitors are added to the infection mixture; concentration of inhibitors used: 10 μg mL^{-1} of recombinant CD81; 5 μg mL^{-1} of anti-CD81 and anti-CXCR5; 100 μg mL^{-1} of GNA and 20 μM of Tannic acid; standard deviation were calculated based on at least three independent experiments; Student t-test comparing tested conditions with control, non-infected condition was used to analyze statistical significance (*** $p<0.0001$, * $p<0.05$).

Figure 4. p7NS2 ORF enhances infectivity of HCV pseudoparticles (HCVpp). **(A)** Schematic representation of the expression plasmids containing Lentivirus LTR, packaging signal and regulatory elements, the composite promoter human elongation factor1 and HTLV, p7 and NS2 open reading frame, and internal ribosome entry site and the hygromycin resistance gene. Underline sequence represents p7 ion-channel, black triangles represent signal peptide cleavage sites, the sequence upstream of p7 represent the last 17 amino acids of E2 which function as a signal peptide for p7 and the sequence downstream p7 is NS2 amino acid sequence. **(B)** Microscopy images of HuH-7 cells transduced by HCVpp and $HCVpp_{p7NS2}$ and **(C)** Infectious titers of HCVpp and $HCVpp_{p7NS2}$ determined by flow cytometry; standard deviation were calculated based on at least three independent experiments; Student t-test was used to analyze statistical significance (*** $p<0.0001$).

Figure 5. Production of HCVpp and HCVpp$_{p7NS2}$ **(A)** analysis of p30 titer determined by ELISA; **(B)** fully assembled particles determined by Nanosight; evaluation of envelope proteins incorporated on HCVpp and HCVpp$_{p7NS2}$ determined by ELISA for **(C)** CD81 and **(D)** E2 and by **(E)** Western Blot; standard deviation were calculated based on at least three independent experiments. Null VLPs are non-pseudotyped particles used as control; student t-test was used to analyze statistical significance (* $p<0.05$).

Figure 6. Susceptibility of HCVpp and HCVpp$_{p7NS2}$ to p7 inhibitory molecules; **(A)** Addition of rimantadine during particle production reduces their infectivity; Addition of **(B)** Rimantadine **(C)** NN-DNJ or **(D)** chlorcyclizine concomitant with HuH7 infection reduces particles infectivity in a dose dependent manner; standard deviation were calculated based on at least three independent experiments; Student t-test was used to analyze statistical significance (***$p<0.0001$).

Fig. 1.

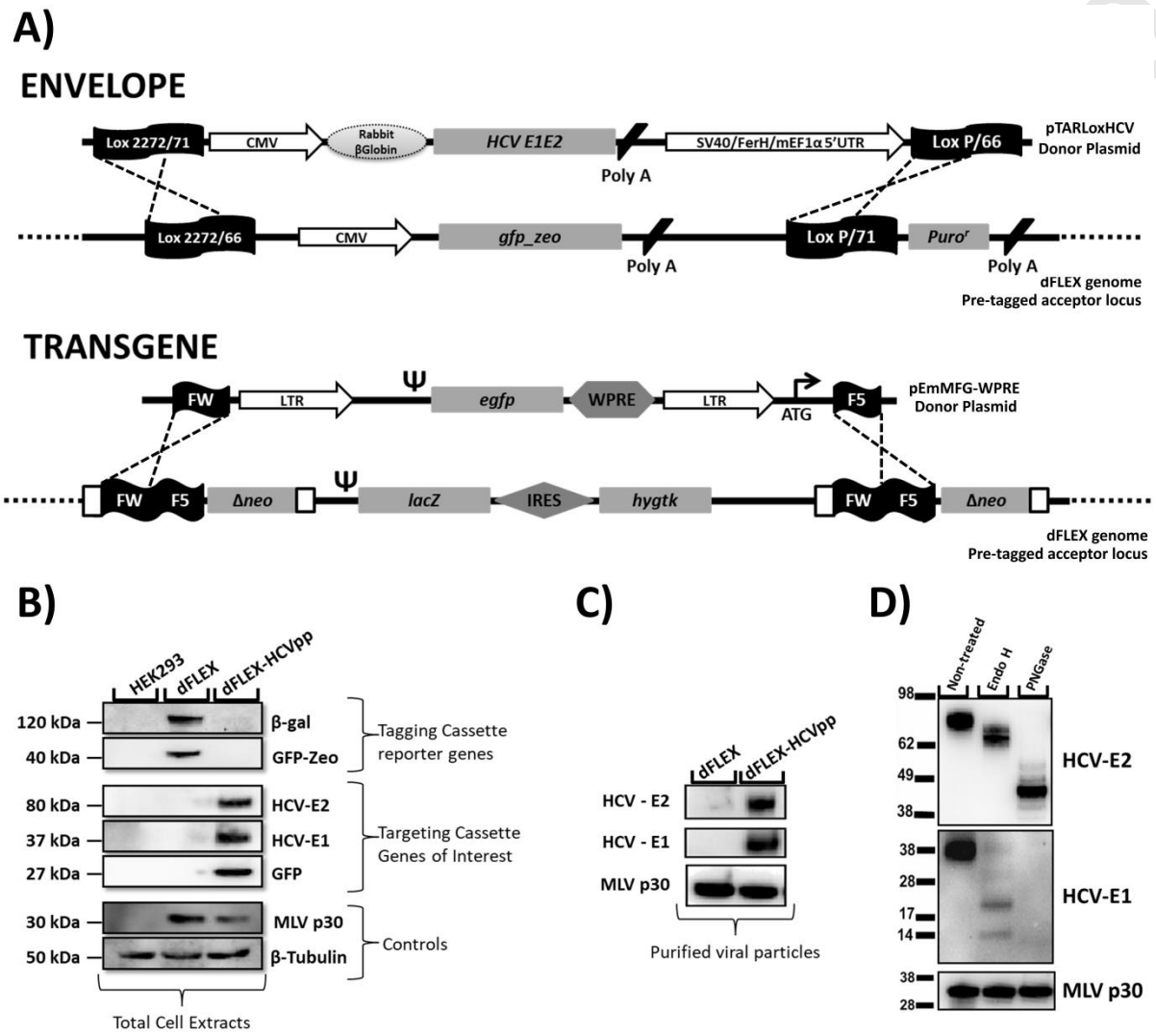

A)
ENVELOPE
Lox 2272/71
CMV
Rabbit βGlobin
HCV E1E2
Poly A
SV40/FerH/mEF1α 5'UTR
Lox P/66
pTARLoxHCV Donor Plasmid
Lox 2272/66
CMV
gfp_zeo
Poly A
Lox P/71
Puro^r
Poly A
dFLEX genome Pre-tagged acceptor locus
TRANSGENE
FW
LTR
Ψ
egfp
WPRE
LTR
ATG
F5
pEmMFG-WPRE Donor Plasmid
FW
F5
Δneo
Ψ
lacZ
IRES
hygtk
FW
F5
Δneo
dFLEX genome Pre-tagged acceptor locus
B)
HEK293
dFLEX
dFLEX-HCVpp
120 kDa
β-gal
40 kDa
GFP-Zeo
Tagging Cassette reporter genes
80 kDa
HCV-E2
37 kDa
HCV-E1
27 kDa
GFP
Targeting Cassette Genes of Interest
30 kDa
MLV p30
50 kDa
β-Tubulin
Controls
Total Cell Extracts
C)
dFLEX
dFLEX-HCVpp
HCV - E2
HCV - E1
MLV p30
Purified viral particles
D)
Non-treated
Endo H
PNGase
98
62
49
38
HCV-E2
38
28
17
14
HCV-E1
38
28
MLV p30

Fig. 2.

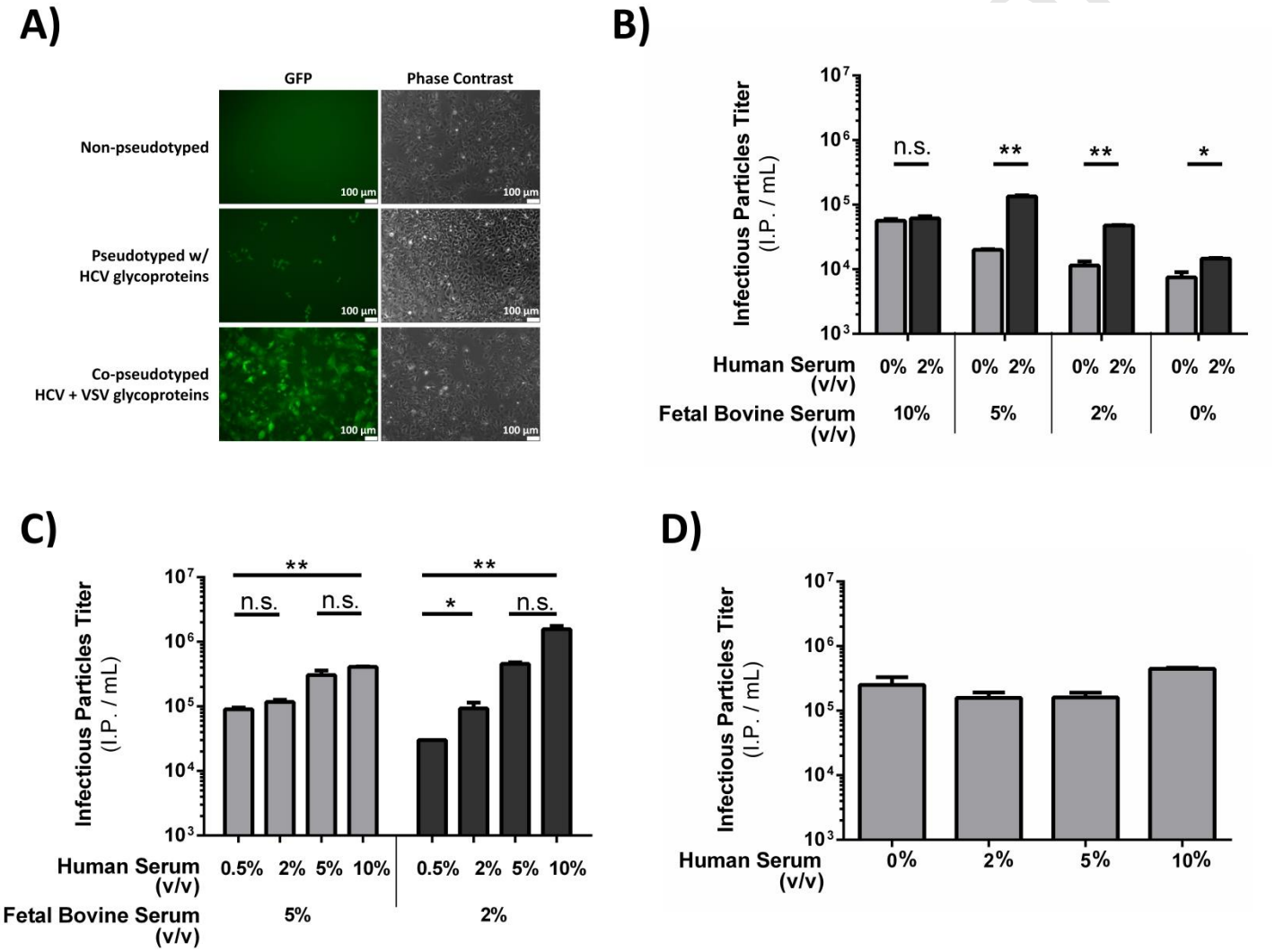

A)
GFP
Phase Contrast
Non-pseudotyped
Pseudotyped w/ HCV glycoproteins
Co-pseudotyped HCV + VSV glycoproteins
100 µm
B)
Infectious Particles Titer (I.P. / mL)
n.s.
**
**
*
Human Serum (v/v)
0% 2%
Fetal Bovine Serum (v/v)
10%
5%
2%
0%
C)
Infectious Particles Titer (I.P. / mL)
**
**
n.s.
n.s.
*
n.s.
Human Serum (v/v)
0.5% 2% 5% 10% 0.5% 2% 5% 10%
Fetal Bovine Serum (v/v)
5%
2%
D)
Infectious Particles Titer (I.P. / mL)
Human Serum (v/v)
0%
2%
5%
10%

Fig. 3.

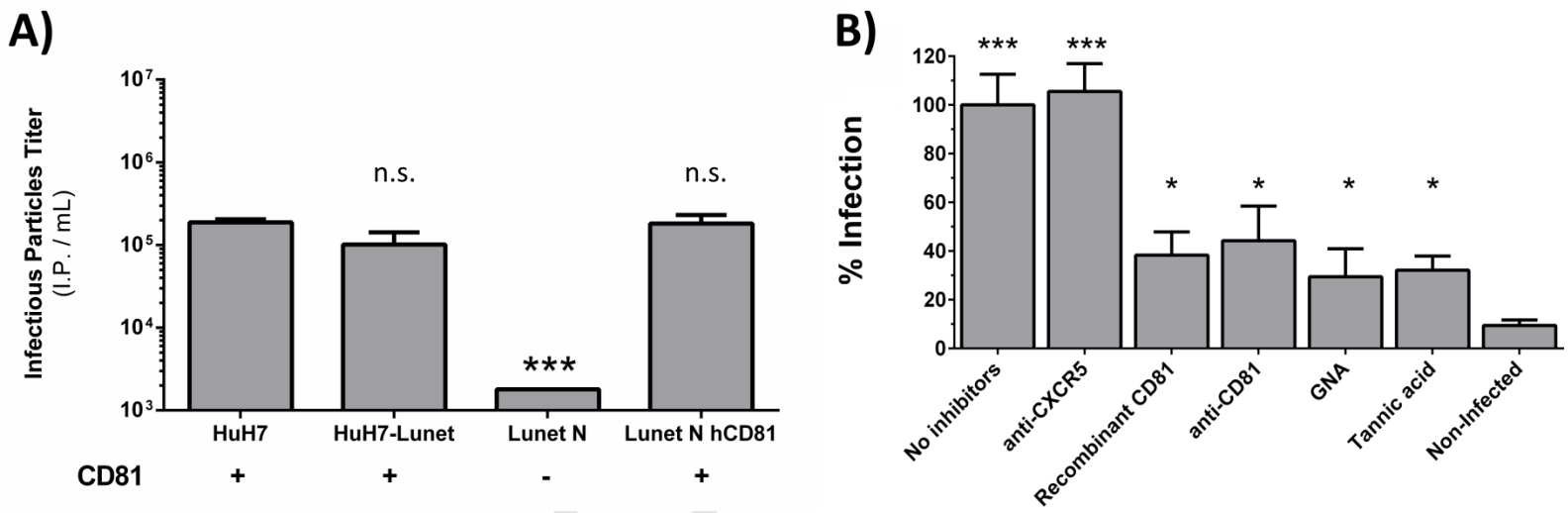


Fig. 4.

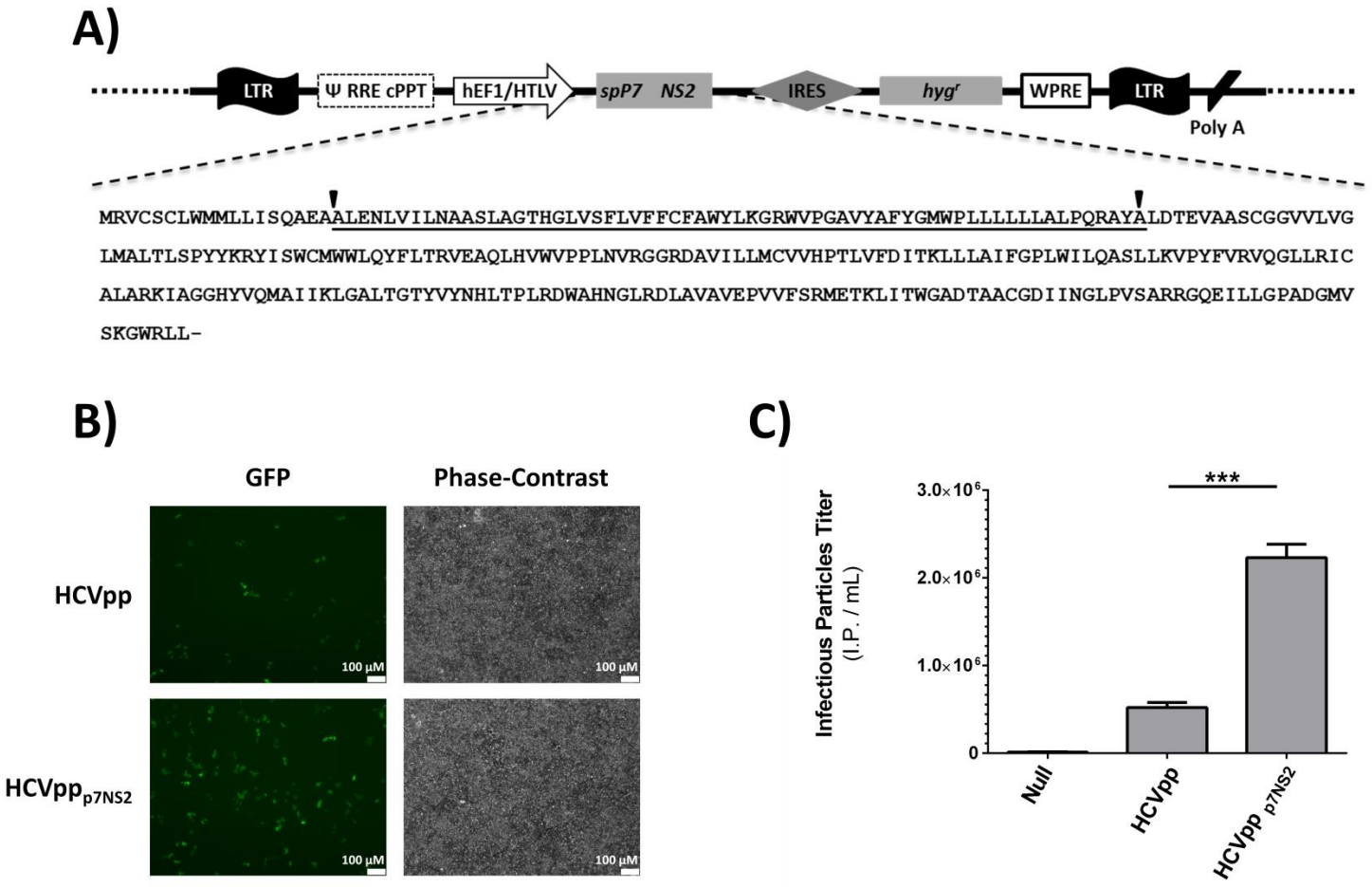

A)
LTR
Ψ RRE cPPT
hEF1/HTLV
spP7 NS2
IRES
hygr
WPRE
LTR
Poly A
MRVCSCLWMMLLISQAEAALENLVILNAASLAGTHGLVSFLVFFCFAWYLKGRWVPGAVYAFYGMWPLLLLLLALPQRAYALDTEVAASCGGVVLVG
LMALTLSPYYKRYISWCMWWLQYFLTRVEAQLHVWVPPLNVRGGRDAVILLMCVVHPTLVFDITKLLLAIFGPLWILQASLLKVPYFVRVQGLLRIC
ALARKIAGGHYVQMAIIKLGALTGTYVYNHLTPLRDWAHNGLRDLAVAVEPVVFSRMETKLITWGADTAACGDIINGLPVSARRGQEILLGPADGMV
SKGWRLL-
B)
GFP
Phase-Contrast
HCVpp
HCVpp p7NS2
100 µM
C)
Infectious Particles Titer
(I.P. / mL)
3.0×10⁶
2.0×10⁶
1.0×10⁶
0
***
Null
HCVpp
HCVpp p7NS2

Fig. 5.

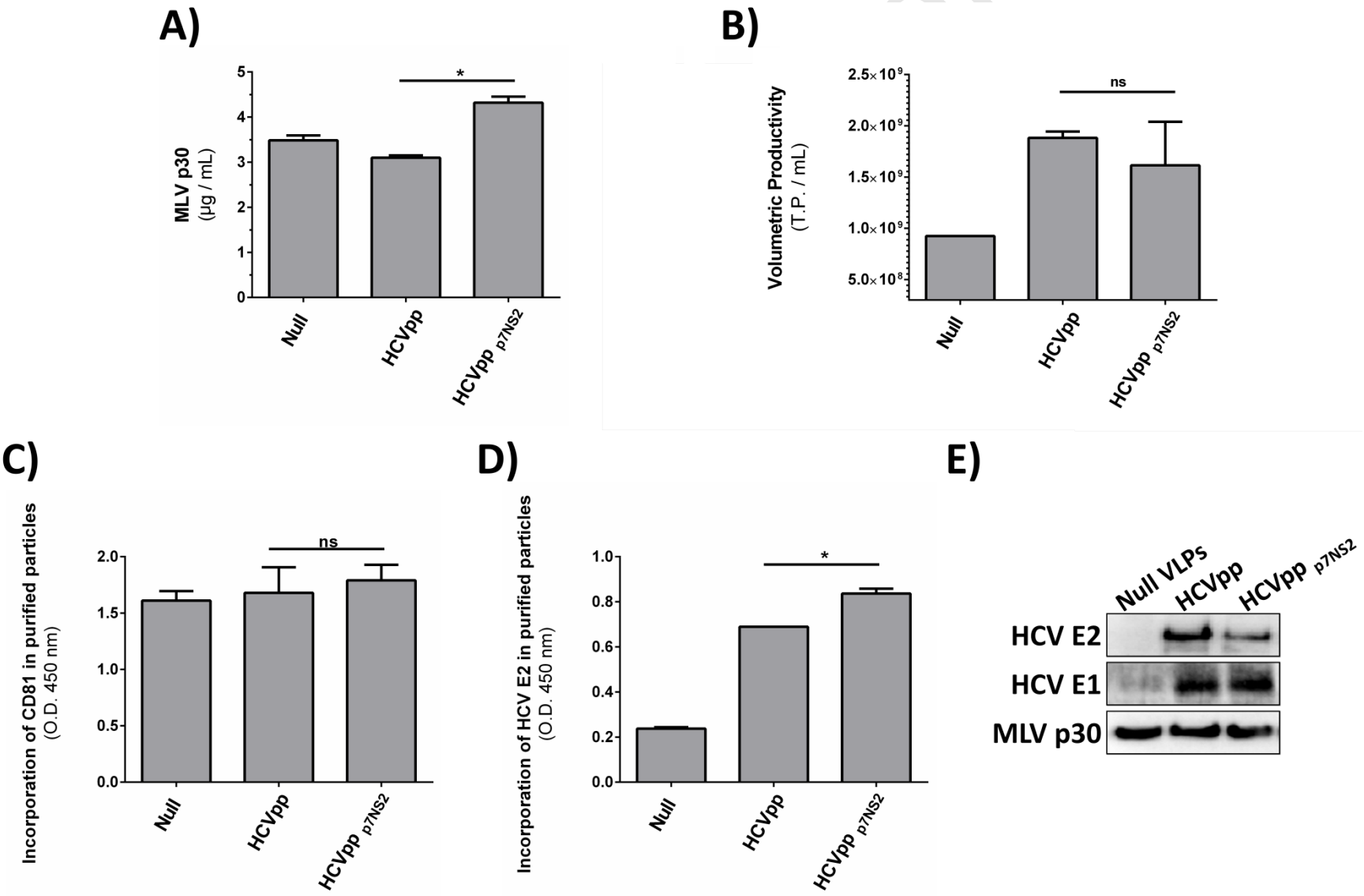
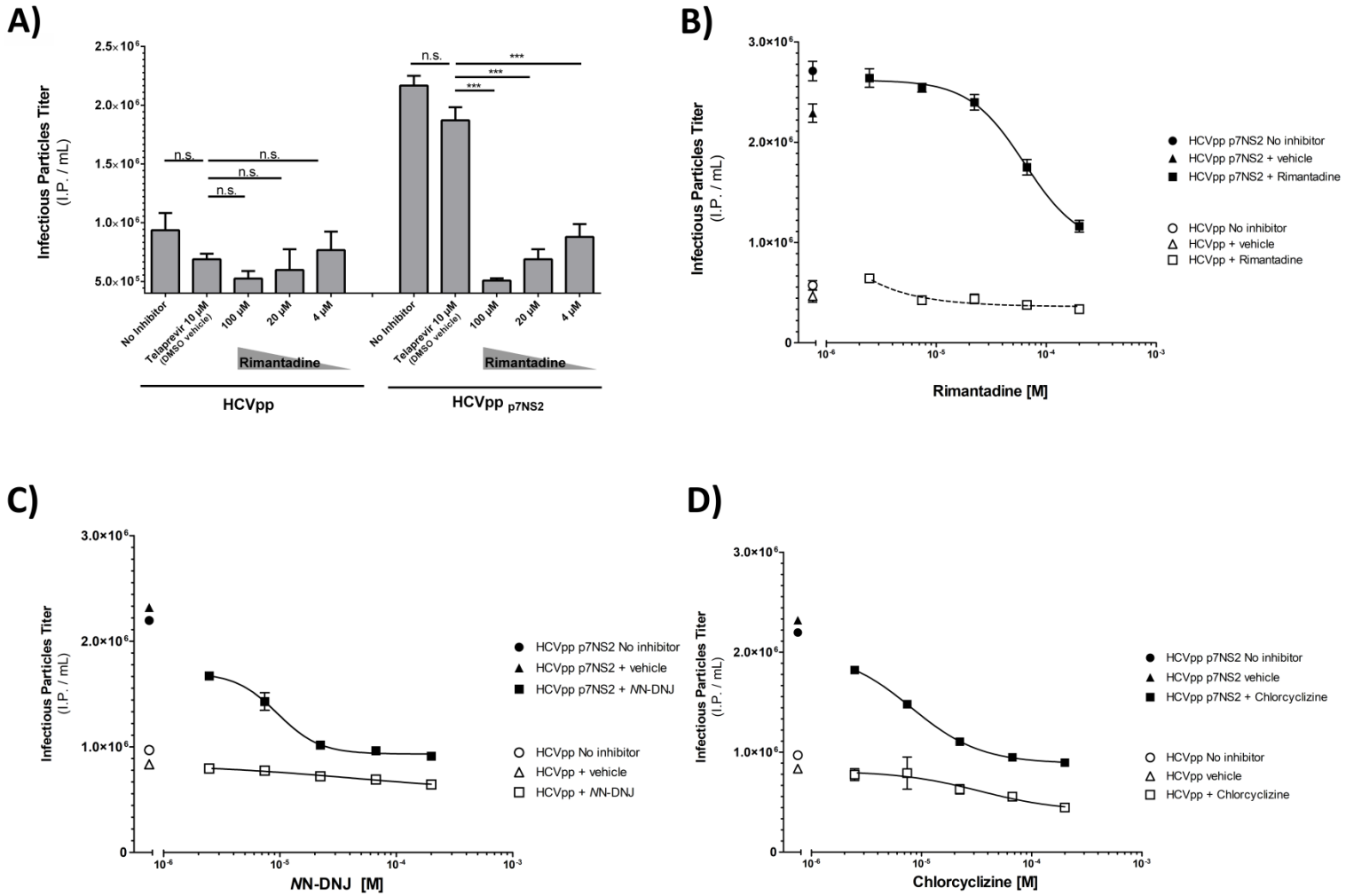

A)
MLV p30
(µg / mL)
*
Null
HCVpp
HCVpp p7NS2
B)
Volumetric Productivity
(T.P. / mL)
ns
Null
HCVpp
HCVpp p7NS2
C)
Incorporation of CD81 in purified particles
(O.D. 450 nm)
ns
Null
HCVpp
HCVpp p7NS2
D)
Incorporation of HCV E2 in purified particles
(O.D. 450 nm)
*
Null
HCVpp
HCVpp p7NS2
E)
Null VLPs
HCVpp
HCVpp p7NS2
HCV E2
HCV E1
MLV p30

Fig. 6.


A)
Infectious Particles Titer (I.P. / mL)
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
n.s.
n.s.
n.s.
n.s.
***
***
***
No Inhibitor
Telaprevir 10 µM (DMSO vehicle)
100 µM
20 µM
4 µM
Rimantadine
HCVpp
HCVpp p7NS2
B)
Infectious Particles Titer (I.P. / mL)
3.0×10^6
2.0×10^6
1.0×10^6
0
10^-6
10^-5
10^-4
10^-3
Rimantadine [M]
HCVpp p7NS2 No inhibitor
HCVpp p7NS2 + vehicle
HCVpp p7NS2 + Rimantadine
HCVpp No inhibitor
HCVpp + vehicle
HCVpp + Rimantadine
C)
Infectious Particles Titer (I.P. / mL)
3.0×10^6
2.0×10^6
1.0×10^6
0
10^-6
10^-5
10^-4
10^-3
NN-DNJ [M]
HCVpp p7NS2 No inhibitor
HCVpp p7NS2 + vehicle
HCVpp p7NS2 + NN-DNJ
HCVpp No inhibitor
HCVpp + vehicle
HCVpp + NN-DNJ
D)
Infectious Particles Titer (I.P. / mL)
3.0×10^6
2.0×10^6
1.0×10^6
0
10^-6
10^-5
10^-4
10^-3
Chlorcyclizine [M]
HCVpp p7NS2 No inhibitor
HCVpp p7NS2 vehicle
HCVpp p7NS2 + Chlorcyclizine
HCVpp No inhibitor
HCVpp vehicle
HCVpp + Chlorcyclizine

Supplementary Information

Plasmids

pTarLoxHCVE1E2 targeting plasmid resulted from cloning HCVE1E2 gene upstream the composite FerH promoter in pTarLoxmcs (Soares et al., 2018). HCV E1E2 envelope sequence from genotype 1a, strain H77, was amplified from pEPX145-71 plasmid (Garrone et al., 2011). The pEmMFG-WPRE plasmid used for expression of the gammaretroviral genome derives from pEmMFG (Coroadinha et al., 2006) into which a WPRE element was cloned downstream the eGFP reporter gene. Lentiviral transgene expression cassette is based in pRRLSin.hPGK.GFP.wPRE (Addgene plasmid 12252) kindly provided by D. Trono through the Addgene plasmid repository (Cambridge, MA U.S.A.). The original pRRLSin.hPGK.GFP.wPRE plasmid was digested with Sal I and Xho I restriction enzymes (New England Biolabs, Ipswich, MA EUA) to eliminate the internal promoter and the GFP reporter gene thus preserving the origin of replication, ampicillin resistance, long-terminal repeats (LTRs), packaging signal (Ψ), RRE element, the cPPT, the WPRE regulatory element and a SV40 polyadenylation site. HCV p7 and NS2 cDNA sequences (strain H77) were obtained from SinoBiological Inc. (Beijing, China) cloned in a pGEM-T vector; hEF1/HTLV hybrid promoter derives from pSELECT-puro (Invivogen San Diego, USA) and IRES and Hygromycin resistance gene derive from pIRESGALEO (Coroadinha et al., 2006). All inserts were amplified using PCR reactions and initially cloned into pGEM-T vector. Noteworthy, a signal peptide of 54 nucleotides, corresponding to the final 17 amino acids of HCV E2, was introduced by PCR assembly upstream the p7 sequence. Finally, hEF1/HTLV_spP7NS2_IRES_Hygromycin expression cassette was subcloned as a single unit into linearized pRRLSin-based vector and the final plasmid named pRRLSin-HCVp7NS2. All cloning reactions described were conducted using In-

Fusion HD Cloning system (Takara, Mountain View, USA). All plasmids were sanger-sequenced before use and all DNA sequences are available upon request. MLV4070A and GaLV derived from pENVAhis and pGaLV, respectively, as described in (Soares et al., 2018).

Recombinase Mediated Cassette Exchange

The Cre/Lox site-specific cassette exchange in dFLEX cells was performed as described in Soares et al. (2018). Briefly dFLEX cells were co-transfected with 2 μg of targeting plasmid pTarLoxHCVE1E2 and 6 μg of pZeoCre recombinase expressing plasmid (Fernandes et al., 2013) per million cells. After selecting recombinant cells with puromycin (Invivogen) eGFP negative cells were isolated by limiting dilution in 50 % conditioned media, 20 % (v/v) FBS and 15 μg ml-1 of puromycin. The surviving recombinant dFLEX cells population was submitted to a second round of RMCE for transgene cassette exchange using Flp/FRT system. Briefly, 4 μg of pEmMFG-WPRE plasmid and 12 μg of pSVFlpe were co-transfected as described before (Coroadinha et al., 2006). After selecting recombinant cells with 1 mg mL-1 of G418 (Invivogen) for 21 days, surviving cells were amplified and cryopreserved before further processing.

Virus production and purification

Retroviral particles purification was performed as described elsewhere (Soares et al., 2016), briefly cells were seeded in 225 cm2 tissue-culture flasks (Corning Life Sciences, Tewksbury, MA U.S.A.) and cultured until 80 % confluence, culture medium was then replaced with new culture medium. After 24 h virus production, culture containing viral particles was harvested, filtered with a 0.45 μm pore membrane and purified by two-step ultracentrifugation. The final

product was resuspended in D-PBS (Gibco). The quantification of MLV p30 capsid was performed using QuickTiter™ MuLV Core Antigen ELISA Kit (Cell Biolabs, San Diego, USA) kit according to manufacturer's instructions. The determination of total physical particles in solution was performed with NanoSight NS500 instrument (NanoSight, Amesbury, UK), following the manufacturer's instructions.

Western Blot

Cell lysates were prepared by adding 100 µL of M-PER extraction buffer (Thermo Fisher Scientific) to $1x10^6$ cells. 16 µg of cell lysates or $5x10^9$ purified retroVLPs were separated in a 4–12 % (w/v) acrylamide NuPAGE gradient pre-cast gel (Thermo Fisher Scientific). Samples were resolved for 45 minutes at a constant voltage of 180 V and transferred into a PVDF membrane using a Trans-Blot® Turbo™ Transfer System (BioRad, California, USA). Afterwards, membranes were blocked with 4 % (w/v) skimmed milk (Merck Millipore, Billerica, USA) and incubated with the respective primary antibody overnight. Detection was performed with the corresponding anti-mouse or anti-rabbit conjugated secondary antibody and developed with the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) before imaging with ChemiDoc XRS+ imaging system (Biorad, Hercules,USA).

Deglycosylation assay

HCVpp were produced and purified as described above. Purified HCVpp were treated with peptide N-glycosidase F (PNGaseF) (New England Biolabs, Ipswich, USA) or endoglycosidase H (Endo H) (New England Biolabs) according to the manufacturer's instructions. Non-digested controls were subjected to the exact same procedure except for the enzymes, which were

omitted. After digestion, proteins were precipitated with ethanol 80 % (v/v) overnight at -20 ºC. On the next day, precipitated proteins were resuspend in loading buffer, and analyzed by western blotting as described above.

HCVpp-ELISA

96-wells Maxisorp plates (NUNC, Waltham, MA USA) were coated with the equivalent to 10 µg of MLV p30 particles over night at 4ºC. In the following day, after plate washing and blocking with 4 % (w/v) skimmed milk (Merck Millipore), membrane proteins HCV E2 and CD81 were detected with anti-HCV E2 (Austral Biologicals) and anti-CD81 (Sigma-Aldrich). Bound antibodies were detected with an anti-mouse or an anti-rabbit HRP conjugated antibody.

Fig. S1.

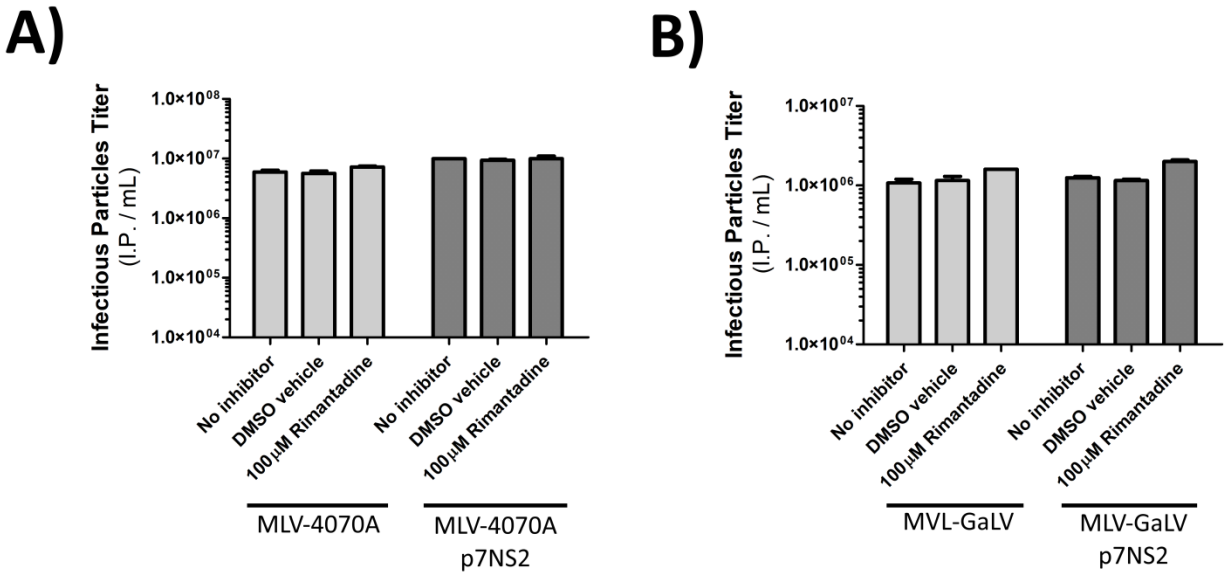


Figure S1. Neither the expression of p7NS2 nor the addition of Rimantadine influence the infectivity of non-HCV pseudotypes; **(A)** infectious MLV particles pseudotyped with 4070A or **(B)** GaLV envelope proteins were used to infect HuH-7 cells in the presence of rimantadine or control molecules.

Fig. S2.

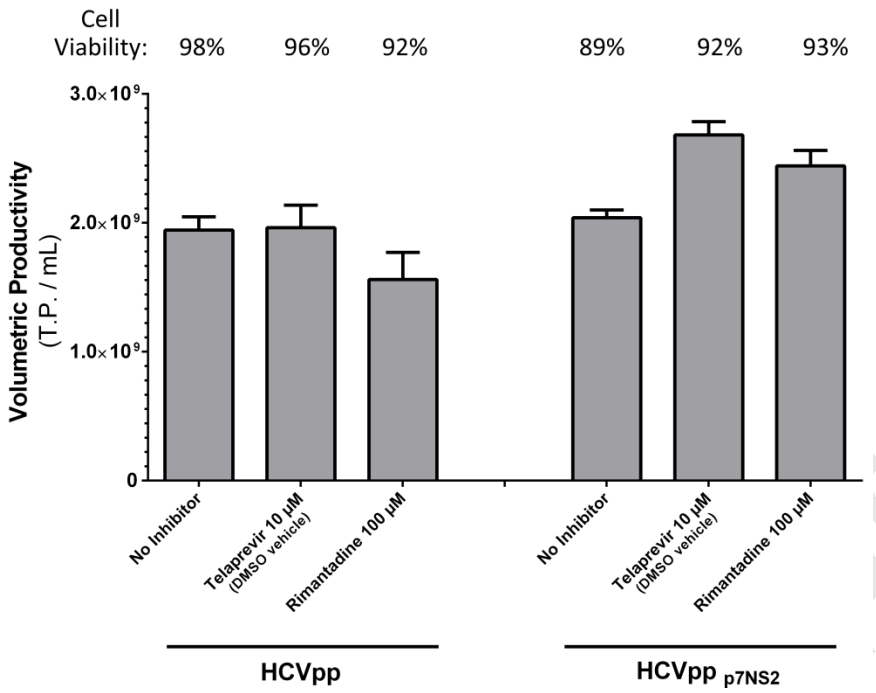


Figure S2. Effect of DMSO and rimantadine in cell viability during the production of HCVpp and HCVpp$_{p7NS2}$ physical particles.

References

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