CD81 large extracellular loop-containing fusion proteins with a dominant negative effect on HCV cell spread and replication

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Abstract

The roles of CD81 in the hepatitis C virus (HCV) life cycle are multiple but remain ill characterized. CD81 is known to interact with the HCV glycoproteins as an attachment factor. It also has an important role in the post-attachment entry process. Its interaction with claudin-1, for example, is vital for viral uptake and trafficking. Furthermore, CD81 and its role in membrane organization and trafficking are thought to play a pivotal role in HCV replication. Some of these functions are particularly limited to human CD81; others can be substituted with CD81 molecules from other species. However, with the exception of the large extracellular loop sequence, the structure-function analysis of CD81 in the HCV infectious cycle remains ill characterized. We describe here the fusion molecules between the large extracellular loops of human or mouse CD81 and lipid-raft-associated or unassociated GPI anchors. These fusion molecules have strong antiviral activity in a dominant negative fashion, independent of membrane raft association. Their expression in the hepatoma cell line Huh7.5 blocks HCV uptake, transmission and replication. These molecules will be useful to decipher the various roles of CD81 in the HCV life cycle and transmission in more detail.

INTRODUCTION

CD81 is a surface molecule known to play a role in the life cycle of a number of different viruses including hepatitis C virus (HCV). It is a member of the tetraspanin family, characterized by four transmembrane segments linked by one short extracellular loop (SEL) and one large extracellular loop (LEL). CD81 is ubiquitously expressed and located in the liver on the basolateral membrane of hepatocytes and on the sinusoidal endothelium [1]. In the context of infection with HCV, CD81 is thought to play roles in several steps of the viral life cycle [2]. Knock down of CD81 and anti-CD81 antibodies block HCV entry and replication [3, 4]. Ectopic CD81 expression in CD81-negative hepatoma cell lines renders these cells susceptible to HCV [5]. The LEL of CD81 has been shown to bind to the ectodomain of the HCV glycoprotein E2 and CD81 is therefore considered to be an attachment factor [6]. However, mutant forms of CD81 that cannot interact with E2 still permit cell entry, suggesting that CD81 is more than a simple attachment factor and may play a role(s) in additional steps of the viral life cycle. Indeed, murine CD81, which fails to interact with HCV glycoproteins or to inhibit HCV infection in its soluble form [2, 6, 7], can support HCVpp and HCVcc infection [7–9]. Thus, CD81 is clearly required for canonical HCV cell entry, but its roles in cell–cell transmission and additional steps of the viral life cycle are much less well defined.

Viral spread by direct cell–cell contact involves complex inter-cellular adhesion, cellular polarity and intra-cellular trafficking, which allows the virus to pass through or accumulate in zones of tight cell–cell contact that are resistant to neutralizing antibodies and to reach a high local particle concentration. Cell–cell transmission may play an important role in the pathology associated with chronic hepatitis C. HCV cell–cell transmission was first suggested when infected cell foci were seen in infected human livers by RNA imaging analysis [10] and recently confirmed using the same approach [11]. Further evidence for in vitro cell–cell transmission also implied the cell entry factors claudin 1, occludin and particularly the scavenger receptor B1 [12–14], however the involvement of CD81 is still controversial. A number of publications reported cell–cell transmission to be CD81-dependent based on different experimental approaches. Indeed, cell–cell transmission in refractory
CD81-deficient cells could be rescued by ectopic CD81 expression [12, 15, 16]. However, other lines of evidence pointed out a CD81-independence for cell–cell transmission. HCVcc strains with E2 mutations that block CD81 binding remained infectious and infectivity was not altered by the presence of neutralizing anti-E2 antibodies [17]. The CD81 expression level of recipient hepatoma cells was shown not to influence the frequency of HCV cell–cell transmission [17]. However, other studies have reported the coexistence of CD81-dependent and -independent cell–cell transmission. Soluble CD81-LEL and anti-CD81 antibody abrogated not only cell-free infection of Huh-7.5 cells but also partially inhibited cell–cell transmission [18]. In vivo anti-CD81 antibodies prevented HCV infection when added before infection, but did not block HCV spread when administered right after infection [19]. Finally, CD81 expression levels in hepatoma cells have been shown to impact the HCV replication process [20], but the underlying mechanisms remain ill defined.

In order to analyse the role of CD81 in the HCV life cycle in more detail, we have developed recombinant fusion proteins displaying the LEL of human or murine CD81 fused to glycosylphosphatidylinositol (GPI) membrane anchors. The GPI anchors were derived either from the membrane-raft targeted decay-accelerating factor (DAF) receptor or the non-raft resident tumour necrosis factor-related apoptosis-inducing ligand 3 (TRAIL3) receptor [21]. The resulting LEL-GPI fusion proteins exhibited strong antiviral activity that was dominant over the proviral activity of wild-type CD81. They efficiently blocked cell-free infection, cell–cell transmission and replication of HCV. This effect was specific for HCV; no modulation of HIV-1 transmission, in which CD81 has been shown to play a role [22, 23], was detected.

RESULTS

Dominant negative CD81 LEL-GPI molecules block HCV infection

To investigate the role of CD81 in HCV cell–cell transmission and cell-free infection, the LELs of mouse or human CD81 were fused to ectopic membrane anchors. Sequences were chosen in analogy to soluble LEL constructs, in order to ensure efficient folding of the LELs [2]. As ectopic membrane anchors, GPI sequences derived from the lipid raft partitioned decay-accelerating factor (DAF) or from the non-raft associated TRAIL3 were chosen [21]. Outlines of the resulting chimeric expression constructs are depicted in Fig. 1(a). Stable Huh7.5 cell lines, expressing the ectopic LEL-GPI proteins from a resistance-carrying vector were selected with antibiotics and expression verified by immunofluorescence (IF) and fluorescence-activated cell sorting (FACS) using human and mouse reactive anti-CD81 antibodies recognizing epitopes in the LEL (Fig. 1b, c). A human-specific anti-CD81 antibody recognized both endogenous CD81 as well as ectopically expressed hsLEL-DAF and hsLEL-TRAIL, an anti-mouse CD81 antibody recognized only ectopically expressed mmLEL-DAF and –TRAIL by IF (Fig. 1b). These data were confirmed by FACS (Fig. 1c). Importantly, ectopic LEL-GPI fusion proteins did not modify the expression levels of other key HCV cell entry factors such as scavenger receptor B1 and claudin-1 (Fig. 1c). Neither did stable expression of the LEL-GPI fusion proteins result in any detectable cell toxicity compared to untransduced Huh7.5 cells (Fig. 1d). Infection of naïve Huh7.5 cells with JFH1 strain HCVcc at two different multiplicities of infection (m.o.i. of 0.02 and m.o.i. of 0.1) resulted in appearance of infection foci as soon as 3 days post-infection (days p.i.) with a marked spread by 6 days p.i. (Fig. 1e). In mmLEL-DAF or mmLEL-TRAIL cell lines, HCV foci were also detected at 3 days p.i., but by 6 days p.i., infection had spread more moderately compared to naïve Huh7.5 cells. In the hsLEL-DAF and hsLEL-TRAIL cell lines, only a very small number of cells were HCV-positive at 3 and 6 days p.i. suggesting efficient inhibition of HCV spread. These results were confirmed by quantitative reverse transcription PCR (RT-qPCR) (Fig. 1f). Upon inoculation at an m.o.i. of 0.02, HCV replication was reduced 19-fold in the hsLEL-DAF and 42-fold in the hsLEL-TRAIL cell lines at 3 days p.i. compared to infection of naïve Huh7.5 cells. At 6 days p.i., replication was inhibited by 155- to 530-fold.

In mmLEL-DAF or –TRAIL expressing cells, the inhibition of HCV replication was less pronounced at 3 days p.i. with a decline of about fourfold, which became more pronounced at 6 days p.i. (30-fold) (Fig. 1f). In conclusion, expression of the LEL of human but also mouse CD81 has a dominant negative effect on the ability of endogenous human CD81 to support HCV infection and transmission in hepatoma cells. To analyse the role of this dominant negative effect in more detail, we focused on LEL-GPI constructs displaying the human form of CD81 LEL in subsequent experiments.

HCV cell spread in the presence of human LEL-GPI fusion proteins

In order to assess the dominant negative effect of LEL-GPI molecules on HCV cell–cell transmission and cell-free spread, a co-culture system was set up to visualize and quantitate viral spread based on fluorescently labelled infected donor and naïve acceptor cells. As donor cells, naïve Huh7.5 cells were transduced with a red fluorescent marker (RFP) and persistently infected with HCVcc. To generate acceptor cells, uninfected hsLEL-TRAIL or hsLEL-DAF expressing Huh7.5 cells were transduced with a vector encoding the fusion protein eGFP-Interferon Beta Promoter Stimulator Protein 1 (IPS). IPS contains a mitochondrial localization sequence and an HCV-protease specific cleavage site in its C-terminus. In uninfected cells, the eGFP-IPS fusion protein thus displays a perinuclear mitochondrial, punctate green fluorescent pattern. When eGFP-IPS-expressing acceptor cells became infected, the viral protease cleaves the IPS-eGFP moiety, which then diffuses into the cytoplasm and nucleus [24] (Fig. 2a). Thus eGFP relocalization from mitochondria into the cytoplasm can be used as a read-out for infection in live cell cultures. Infected donor cells (RFP) and uninfected acceptor cells (eGFP-IPS) were mixed at a ratio of 1:30. The cell cultures...
were then incubated in the absence or presence of saturating concentrations of the neutralizing anti-E2 glycoprotein antibody CBH5, which blocks infection of cell-free virus. Infection events were quantified by counting green cells with diffuse, cytoplasmic eGFP distribution. At 3 days post co-culture, most of the naïve Huh7.5-eGFP acceptor cells were infected (Fig. 2b). In contrast, in hsLEL-DAF and -TRAIL-expressing acceptor cells, the total number of HCV-infected cells was

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**Fig. 1.** HCVcc replication in Huh7.5 cells transduced with CD81 LEL constructs. (a) Schema of the CD81 LEL-TRAIL and -DAF fusion proteins used in the study. (b) Confocal microscopy: the indicated naïve and transduced Huh7.5 cell lines were permeabilized or not upon fixation and stained with human or mouse reactive anti-CD81 antibodies as indicated. Representative images are shown. (c) FACS analysis of the indicated Huh7.5 cell lines using antibodies against human and murine CD81, claudin-1, and SR-BI. Histograms depicting staining with secondary antibody only are shown in white, histograms depicting staining of the indicated primary plus a secondary antibody are in grey. Graphs depicting the mean fluorescence intensity (MFI) of the histograms are shown on the right. (d) Cell toxicity in the stable cell lines expressing the indicated LEL-GPI fusion proteins and parental Huh7.5 cells was assessed by propidium iodide (PI) staining. As control, Huh7.5 cells were fixed in 2% PFA for 5 min prior to PI staining. (e, f) The indicated naïve and transduced Huh7.5 cell lines were infected with HCVcc JFH1 strain at an m.o.i. of 0.02 or 0.1. (e) Conventional fluorescent microscopy: to assess HCV infection, cells were fixed and stained for HCV (anti HCV core in green) and for nuclei (DAPI) at 3 and 6 days p.i. A representative scale bar is indicated. (f) In parallel, at the same time points and conditions, cells were harvested and HCV replication quantified by RT-qPCR using GUS as housekeeping gene. 2^{-ΔΔCt} values are shown. Differences in HCV replication are indicated as fold change on the graph. All differences between parental and LEL-GPI transduced cell lines were significant (**).
Fig. 1. (cont.)

(c) Huh7.5 hsLEL-DAF hsLEL-TRAIL mmLEL-DAF mmLEL-TRAIL

(d) 

(e) Huh 7.5 cells expressing:

(f)
Fig. 2. Virus spread in co-cultures between infected Huh7.5 cells and CD81 LEL cells. In order to estimate the impact of the CD81 LEL constructs on cell–cell and cell-free virus transmission, co-cultures were set up. Persistently infected donor cells expressed a red fluorescent tag within the nucleus. Wild-type or hsLEL-DAF or hsLEL-TRAIL-expressing Huh 7.5 cells were stably transduced with pTrip-
three- and eightfold lower, respectively (Fig. 2b, c). When cell–cell transmission was assessed in the presence of the neutralizing CBH5 antibody in naïve Huh7.5 cells, a >threefold drop of infection events was observed suggesting that at least one-third of the overall HCV spread in Huh7.5 cells is due to cell–cell transmission, which is consistent with previously reported data [12]. Compared to cell–cell transmission rates in wild-type Huh7.5 cells, cell–cell transmission rates in hsLEL-DAF and -TRAIL-expressing Huh7.5 cells was reduced over the 3 day infection period by two and fourfold, respectively (Fig. 2b, c). Due to its more potent inhibitory effect on cell-free and cell–cell transmission, further experiments were performed with the hsLEL-TRAIL-expressing cell line.

**CD81 LEL-expressing cells are refractory to HCV infection**

Discrimination between the expression of endogenous human CD81 and the ectopic human CD81-LEL-GPI fusion constructs is important for assessing the correlation between hsLEL-TRAIL expression and susceptibility of cells to HCV cell entry and replication. Therefore an N-terminal GFP was fused to hsLEL-TRAIL (Fig. 1a). GFP-hsLEL-TRAIL expression was detected in Huh7.5 cells by fluorescence microscopy on the cell surface and in a perinuclear pattern (Fig. 3a). As expected, GFP-hsLEL-TRAIL did not localize to lipid rafts and was not targeted to lysosomal degradation since no co-localization was detected with CD55 (known as DAF) or Lamp1, respectively (Fig. 3b). When co-stained with an anti-human CD81 antibody, the GFP-hsLEL-TRAIL-transfected cells displayed in comparison to untransfected cells in the same culture much stronger CD81 expression levels (Fig. 3b). To further investigate the correlation between GFP-hsLEL-TRAIL expression and susceptibility to HCV infection, Huh7.5 cells were transfected with a GFP-hsLEL-TRAIL expression construct. Twenty-four hours later, GFP-hsLEL-TRAIL-transfected as well as untransfected Huh7.5 cells as control were infected with HCVcc at an m.o.i. of 1. Two days post-infection, cells were fixed and stained for HCV core protein and co-localization of core and GFP-hsLEL-TRAIL was assessed. In non-transfected cell cultures, HCV core was detected in over 80 % of cells. Similarly, within the GFP-hsLEL-TRAIL-transfected cell culture, close to 80 % of non-transduced, GFP-hsLEL-TRAIL-negative Huh7.5 cells were HCV core-positive. In contrast, almost none of the CD81-LEL-GFP TRAIL expressing cells were positive for HCV core (Fig. 3c, d). This suggests that the hsLEL-TRAIL fusion protein efficiently blocks HCV spread and possibly also replication when expressed in cells prior to infection (Fig. 3c, d).

**CD81 LEL fusion proteins block HCV replication**

To assess a potential role of the dominant negative human and mouse CD81 LEL constructs in the post-entry steps of the HCV life cycle, infection conditions were set up where viral replication can be assessed independently of viral infection and spread. For that purpose, Huh7.5 cells were infected with HCV at an m.o.i. of 0.2 for 24 h to ensure low level uptake of virus into all cells. After the 24 h, infected cells were cultured in the presence or absence of the neutralizing antibody CBH5 for three more days and then harvested for RT-qPCR analysis. In parallel, infections were performed where CBH5 Nab was added to cells together with virus (m.o.i. of 0.2) in order to control its neutralizing capacity. These cultures were also harvested 3 days p.i. and processed for RT-qPCR analysis (Fig. 4a). While simultaneous addition of HCV and CBH5 Nab resulted in a significant neutralization of HCV infection, delayed addition of CBH5 Nab 24 h after the virus did not significantly inhibit HCV infection and replication. This shows that 24 h after addition of virus, virus has been taken up and replication can be assessed independently of viral spread in the presence of CBH5 Nab.

To assess the impact of the CD81 LEL-GPI fusion proteins on viral replication, Huh7.5 cells were infected with HCV at an m.o.i. of 0.2. Twenty-four hours later, the cells were treated or not with CBH5 Nab and concomitantly transduced with constructs expressing the CD81 LEL-GPI fusion proteins, wild-type CD81 or the GPI anchors fused to GFP as control. Infections were harvested 3 days later for IF and RTqPCR analyses. Successful transduction and expression of the CD81 LEL-GPI constructs and controls was verified by immunostaining or IF (Fig. 4b). Replication levels in wild-type Huh7.5 cells infected for 24 h and then treated with CBH5 Nab or not were comparable, as expected (Fig. 4c). In comparison, transduction with hsLEL-DAF or hsLEL-TRAIL reduced HCV replication by 50 % independently of the presence of CBH5 Nab (Fig. 4c). No inhibition of infection was observed in cells transduced with mouse LEL-DAF or an expression construct encoding wild-type hsCD81, nor in cells transduced with expression constructs for DAF and TRAIL GPI anchors only (GFP-DAF, GFP-TRAIL). Thus the inhibition observed in hsLEL-DAF and –TRAIL cells seemed to be specifically mediated by the hsLEL moieties in the hsLEL-DAF and –TRAIL fusion proteins.
These findings strongly suggest a post-entry role for human, but not murine CD81 in HCV replication.

HIV-1 replication is not affected by the CD81 LEL constructs

To check whether the observed dominant negative effect of the hsCD81 LEL constructs is specific for HCV, we tested their effect on human immunodeficiency virus type 1 (HIV-1), another RNA virus capable of cell-free and cell–cell propagation [25]. HeLaP4 cells were transduced with the hsLEL-DAF or hsLEL-TRAIL constructs. Expression levels were verified by flow cytometry (Fig. 5a) and confocal microscopy (Fig. 5(b)). CD81 expression was stronger in transduced cell lines compared to wild-type HeLaP4 cells.
Expression of the LEL-GPI fusion proteins did not affect HeLaP4 cell viability (Fig. 5c). Consequently, wild-type and hsLEL-DAF/-TRAIL HeLaP4 cell lines were infected with HIV-1 at an m.o.i. of 0.2. Two days later, cells were recovered to assess HIV-1 replication by IF and RT-qPCR. HIV infection was not sensitive to the presence of the hsLEL-GPI fusion proteins but could be fully inhibited with the HIV-specific T20 fusion peptide inhibitor (Fig. 5d, e). Thus, neither HIV-1 entry nor replication seem to be sensitive to the LEL-GPI fusion proteins. In addition, cell entry and infection of various HIV-1 based pseudoparticles were tested using the Huh7.5 cell lines stably expressing the CD81 LEL constructs (Fig. 5f). Infection rates of lentiviral pseudoparticles displaying the HCV glycoproteins, but not the vesicular stomatitis G protein VSV-G, were strongly inhibited by hsLEL-TRAIL and -DAF, suggesting that the observed dominant negative effects of the hsCD81-GPI fusion proteins are specific to HCV.

**DISCUSSION**

We have shown that the ectopic expression of the LEL of human and also mouse CD81 fused to different GPI anchors has a dominant negative effect on HCV infection. The effects observed with mouse LEL fusion proteins was somewhat slower and weaker than those observed with human LEL fusion constructs, even though final inhibitory efficacies were similar. The localization of the LEL-GPI fusion proteins did not seem to play a predominant role in the inhibition process, as raft associated and non-raft associated GPI anchors exhibited the same dominant negative effect. The observation that the hsLEL-GPI constructs exhibited a stronger and quicker effect on HCV spread and amplification than the mmLEL versions may be due to the fact that human but not murine LEL is known to bind and to sequester HCV virions. Indeed, human but not mouse CD81 is known to bind the glycoprotein complex (reviewed in [2]). This finding suggests indirectly that the conformation of the hsLEL within the fusion constructs has been conserved and is still capable of interacting with HCV. Binding and infection studies on cells not endogenously expressing CD81 and engineered to express the LEL-GPI constructs may shed further light on this issue.

The observation that the mouse LEL-GPI construct blocks HCV infection even though mouse CD81 is known to not
Fig. 5. hsLEL-DAF and -TRAIL have no effect on HIV-1 replication. HeLaP4 cells (expressing CD4) were transduced with hsLEL-DAF and -TRAIL constructs. (a) Expression of hsLEL-DAF and -TRAIL in HeLaP4 cells was assessed by FACS using an anti-hs CD81 antibody. Histograms depicting staining with secondary antibody only are shown in white, those depicting staining of anti-CD81 with a secondary antibody are in grey. MFI values of the histograms are shown in the right-hand graph. (b) Confocal microscopy: immunostaining of the indicated HeLaP4 cell lines with an anti-hsCD81 antibody. (c) Cell toxicity of the indicated HeLaP4 cell lines was
bind HCV suggests that CD81 may play additional roles in HCV infection apart from HCV binding, and that this function is conserved between human and murine CD81. In particular, it would be interesting to investigate the impact of LEL-GPI constructs on the oligomerization and membrane mobility of wild-type CD81, the confirmation of the tetraspanin web [26, 27] and the interaction of CD81 with particular partners such as claudin 1 in the tetraspanin web [28]. Interestingly, the effects of LEL-GPI fusion proteins are specific to HCV, since HIV-1 entry and amplification are not affected.

In contrast to entry and uptake, little is known of the role of CD81 in HCV replication. Zhang et al. have previously shown that CD81, possibly due to its physical proximity to the HCV replication site, helps the virus to direct the HCV RNA template towards the replication site [20]. Our data support a role for CD81 in replication. Indeed, introduction of the LEL of human but not murine CD81 into Huh7.5 cells that have already taken up but not yet efficiently amplified the virus, efficiently blocks viral genome replication. This may suggest that the LEL has – besides E2 binding – additional roles in HCV replication, and as already evident in the literature, not all of these roles can be substituted by murine LEls. Furthermore, these findings may suggest that, besides the LEL, additional domains in human CD81 are required to support the viral replication process. The absence of these domains in the hsLEL-GPI constructs is likely to explain the observed dominant negative effects of the hsLEL-GPI fusion proteins. While the molecular mechanisms of this antiviral effect remain to be defined, it is clear that it does not depend on raft- or non-raft-association of the fusion proteins. In conclusion, the LEL-GPI constructs described here will – due to their dominant negative effect – be useful to further explore the functions of CD81 in HCV replication, spread and other steps of the viral life cycle.

**METHODS**

**Cells and viruses**

Human hepatocellular carcinoma cells (Huh7.5), cervical carcinoma cells expressing CD4 molecule (HeLaP4) and embryo kidney cells (HEK293T) were cultivated in Dulbecco’s modified Eagle medium (Life Sciences) in the presence of 10% foetal calf serum, pyruvate (1 mM), L-glutamine (2 mM), streptomycin (100 µg ml⁻¹) and penicillin (100 U ml⁻¹) at 37°C in a 5% CO₂ incubator.

Huh7.5 cells were infected with HCVcc JFH-1 strain, and HeLaP4 cells with HIV-1 laboratory strain NL4-3. The neutralizing antibody used for cell–cell transmission assays was anti-E2 CBH-5 used at 2 µg ml⁻¹ final concentration. The Trimeris/Roche T-20 fusion inhibitor was obtained through the NIH AIDS Reagent Program (Division of AIDS, NIAID) and added at 2 µg ml⁻¹ prior to infection. Virus titres for HCV and HIV were obtained using the TCID₅₀ method [29].

**VLP production and transduction**

Vesicular stomatitis virus G protein (VSV-G) or HCV pseudotyped lentiviruses were generated by transient co-transfection of HEK293T cells using a calcium phosphate kit with a three-plasmid combination: one 10 cm culture plate containing HEK293T cells at about 40% confluence was transfected with 8 µg lentiviral vector pBFG encoding a puromycin resistance or GFP, 8 µg GagPol plasmid and 2 µg VSV-G or HCV E1/E2 expression plasmids [30]. Supernatants containing virus-like particles (VLPs) were collected 48 h post transfection, passed through a 0.45 µm filter and stored at −80°C until further use. To establish cell lines, Huh7.5 or HeLaP4 cells were seeded in a six-well plate (400 000 cells well⁻¹) in 1.5 ml of complete DMEM medium and 500 µl of VLPs added for 48 h. Cells were then passaged in DMEM medium containing puromycin in order to select transduced cells.

CD81 constructs pCR3-EGFP-GPI-(DAF) and pCR3-EGFP-GPI-(TRAIL-R3) were a kind gift from D. F. Legler (University of Konstanz, Germany). The EGFP moiety in these constructs was replaced by PCR with the LEL of human (aa94-182) and of mouse (aa94-182) CD81 (oligo sequences available upon request). A version of hsLEL-GPI-(TRAIL-R3) displaying an N-terminal GFP sequence was established by PCR. The ORFs of the resulting fusion proteins hsLEL-GPI-(DAF), mmLEL-GPI-(DAF) and hsLEL-GPI-(TRAIL-R3), mmLEL-GPI-(TRAIL-R3) alongside EGFP-GPI-(DAF) and EGFP-GPI-(TRAIL-R3) were then subcloned into the lentivirus vector pBFG containing a puromycin resistance.

**Virus replication**

To assess the impact of CD81LEL on HCV infection, Huh 7.5 cells were seeded in 12-well plates at 80 000 cells well⁻¹ in 1 ml DMEM. One day later, cells were infected with HCVcc at an m.o.i. of 0.02 or 0.1. Virus replication was quantified by real-time PCR. Cells were lysed in Extract All solution (Eurobio) according to manufacturer’s protocol. RNA (1 µg) was treated with DNase (Roche) and reverse transcribed using a Superscript II Reverse Transcriptase kit (Life Technologies). cDNA samples were diluted 1:5, mixed with SYBR Green PCR Master Mix (Roche) in a final
volume of 10 µl. Amplification of HCV cDNA was performed using RC1 and RC21 primers on a LightCycler 480 System (Roche) as already described [31]. For HIV-1 replication, HeLaP4 cell lines were seeded at 100 000 cells well−1 in 12-well plates and infected with HIV-1 NL4-3 at an m.o.i. of 0.2 and experiments performed 2 days later. For HIV-1 cDNA amplification, the following primers were used: Gag (MA) FW 5’-GGA-GCT-AGG-ACG-ATT-GGC-AGT-TA and Gag (MA)REV5’-GGT-TGT-AGC-TGT-CCC-AGT-ATT-TGTC. Beta-glucuronidase (GUS) was used as a reference gene [31].

**Flow cytometry**

For cell-surface FACS staining of CD81, cells were detached by trypsin treatment and incubated for 30 min at 4 °C with anti-CD81 (mouse monoclonal, JS-81, BD Bioscience) or rat anti-mouse CD81 [32] diluted 1/100 in 1 % BSA-PBS. Afterwards, cells were stained for 30 min at 4 °C with anti-mouse Alexa Fluor488 or anti-rat Alexa Fluor594 diluted 1/1000 in 1% BSA-PBS and analysed on a BD FACS Calibur. For HCV receptor analysis, cells were fixed in 1 % paraformaldehyde for 15 min, washed in PBS and incubated for 30 min at 4 °C with anti-CD81 (mouse monoclonal, JS-81, BD Biosciences; rat anti-mouse CD81 [32]), anti-Claudin-1 (goat polyclonal, sc-22932, SCBT) or anti-SR-B1 (mouse monoclonal, Cell Signaling; rat anti-mouse CD81 [32], goat Alexa Fluor488 conjugated antibody was used at 1:200). Pictures were taken using a Nikon Eclipse TE200-E.

For confocal microscopy studies, cells were seeded on glass coverslips (100 000 cells per well) in 24-well plates in 0.5 ml of culture medium. Forty-eight hours later, cells were fixed in 4 % paraformaldehyde (PFA) for 15 min followed by a quenching step of 5 min in 50 mM NH4Cl (in PBS). Cells were then permeabilized or not in 0.2 % Triton X-100 (in PBS) for 5 min and blocked in 3 % BSA-PBS solution. Primary and secondary antibodies were diluted in 1 % BSA-PBS and incubated for 1 h at room temperature. For transfection experiments cells were transfected 1 day after seeding using Jetprime reagent (Polyplus) according to manufacturer’s instructions. The next day, medium was replaced and HCVcc added at an m.o.i. of 0.2. One day post-infection, cells were fixed in 4 % PFA and treated as previously described. Primary antibodies: HCV Core C750, SCBT, 1:200; human-specific anti CD81 (TAPA-1) Abcam, 1:800; rat anti-mouse CD81 [32]; anti Lamp1, SCBT, 1:500, anti CD55 (gift from Denis Gerlier), 1:50. Secondary antibodies: Alexa Fluor555 conjugation and Alexa Fluor488 diluted 1/1000 and analysed on a BD FACS Calibur. For assessment of cell viability, cells were stained for 30 min at 4 °C with anti-mouse/anti-goat Alexa Fluor488 diluted 1/1000 and analysed on a BD FACS Calibur. For cell-surface FACS staining of CD81, cells were detached by trypsin treatment and incubated for 1 h at room temperature. For transfection experiments cells were transfected 1 day after seeding using Jetprime reagent (Polyplus) according to manufacturer’s instructions. The next day, medium was replaced and HCVcc added at an m.o.i. of 0.2. One day post-infection, cells were fixed in 4 % PFA and treated as previously described. Primary antibodies: HCV Core C750, SCBT, 1:200; human-specific anti CD81 (TAPA-1) Abcam, 1:800; rat anti-mouse CD81 [32]; anti Lamp1, SCBT, 1:500, anti CD55 (gift from Denis Gerlier), 1:50. Secondary antibodies: Alexa Fluor555 conjugation 1:1000. Cover slips were then mounted on glass slides using fluorescence mounting medium (Dako, Denmark) and analysed on a Leica Confocal Spectral TCS SP5X microscope.

**Microscopy**

For conventional fluorescence microscopy Huh7.5 cells were seeded in 48-well plates (20 000 cells well−1) in 0.25 ml of complete DMEM medium. The next day, they were infected with HCVcc (m.o.i. of 0.02 or m.o.i. of 0.1) and at 3 and 6 days p.i. fixed in ice cold methanol:acetone (1:1) solution. After three washes in PBS, cells were blocked for 30 min in 3 % BSA-PBS, and incubated for 1 h with an anti-HCV human serum (1:500) in blocking buffer. For HIV-1 imaging, HeLaP4 cell lines were seeded at 100 000 cells well−1 in 12-well plates, infected with HIV-1 NL4-3 at an m.o.i. of 0.1 and fixed 2 or 3 days p.i. just like the Huh7.5 cells. HIV-1 infection was evaluated by p24 Gag staining using HIV-1 p24 Gag Monoclonal diluted at 1:1000 (#24-4; from Dr Michael H. Malim obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID) [33]. Alexa Fluor488 conjugated antibody was used at 1:2000 and incubated together with DAPI (1 µg ml−1) for 1 h in blocking buffer. Pictures were taken using a Nikon Eclipse TE200-E.

For confocal microscopy studies, cells were seeded on glass coverslips (100 000 cells per well) in 24-well plates in 0.5 ml of culture medium. Forty-eight hours later, cells were fixed


