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Abstract

The hepatitis E virus (HEV) is an underestimated RNA virus of which the viral life cycle and pathogenicity remain partially understood and for which specific antivirals are lacking. The virus exists in two forms: nonenveloped HEV that is shed in feces and transmits between hosts; and membrane-associated, quasi-enveloped HEV that circulates in the blood. It is suggested that both forms employ different mechanisms for cellular entry and internalization but little is known about the exact mechanisms. Interestingly, the membrane of enveloped HEV is enriched with phosphatidylserine, a natural ligand for the T-cell immunoglobulin and mucin domain-containing protein 1 (TIM1) during apoptosis and involved in 'apoptotic'mimicry, a process hijacked by viruses to promote infection. We here investigated the role of TIM1 in the entry process of HEV. We determined that HEV infection with particles derived from culture supernatant, which are cloaked by host-derived membranes (eHEV), was significantly impaired after knockout of TIM1, whereas infection with intracellular HEV particles (iHEV) was unaffected. eHEV infection was restored upon TIM1 expression; and enhanced after ectopic TIM1 expression. The significance of TIM1 during entry was further confirmed by viral binding assay, and point mutations of the PS-binding pocket diminished eHEV infection. Additionally, Annexin V, a PS-binding molecule also significantly reduced infection. Taken together, our findings support a role for TIM1 in eHEV-mediated cell entry, facilitated by the PS present on the viral membrane, a strategy HEV may use to promote viral spread throughout the infected body.

Keywords

Hepatitis E virus; viral hepatitis; T-Cell Immunoglobulin and Mucin Domain-Containing Protein 1; HAVCR1; virus-host interaction; viral entry; cell surface receptor; apoptotic mimicry

1. Introduction

The hepatitis E virus (HEV) is annually responsible for infection of at least 20 million people and is increasingly recognized as a medical problem in the Western world [1-3]. Most of the human cases are attributed to 4 viral genotypes (gts) that differ in geographical distribution, routes of transmission, disease course and pathogenicity. Gt-1 and gt-2 transmit fecal-orally and are sources of outbreaks in developing countries with high mortality seen in gt-1 infected pregnant women [4]. Gt-3 and gt-4 are zoonotic and predominant in industrialized countries and chronicity has been observed in gt-3 infected immunocompromised patients [5]. No HEV-specific therapy exists, necessitating the identification and development of novel and more effective therapies [1].

HEV is a positive-sense, single-stranded RNA virus with a 7.2 kb genome. Depending on viral gt, the viral genome comprises three or four open reading frames (ORF1-4). ORF1 encodes a nonstructural polyprotein, comprised of several domains essential for viral replication [6]. ORF2 and ORF3 are encoded from a bicistronic subgenomic RNA. ORF2 encodes the viral capsid protein, binds to the host cells and elicits an antibody response [7]. ORF3 encodes a small phosphoprotein important for viral release. The more recent discovered ORF4 is only expressed in gt-1 HEV and influences viral replication by interacting with both host and viral proteins [8].

HEV has long been neglected as it seemed a virus confined to developing countries, and the lack of appropriate cell culture systems as well as animal models made it difficult to study this virus in detail. Recent advances in this field have been made but parts of the viral life cycle remain poorly understood, such as viral entry. The virus is considered quasi-enveloped, but in fact different forms of the viral particle exist. Non-enveloped virus particles are retrieved from feces and are the major form present in (intra)cellular lysate (designated iHEV), whereas HEV particles circulating in the blood stream and cell culture supernatant are cloaked with membrane lipids, therefore considered enveloped (eHEV) [9]. Naked viral particles mediate HEV spread in the population, whereas enveloped viral particles are presumably more important for viral spread within the body. Experimental evidence suggests that both forms have distinct ways of entering a host cell and understanding both of these processes is necessary for insight into viral pathogenesis and developing highly needed antivirals [10]. Although several host factors have been shown to be involved in cell attachment and/or entry of iHEV, not one protein is generally accepted as the true HEV receptor. The cell entry of enveloped particles is even less studied, and it seems a more inefficient process compared to naked virus [9, 11]. It was previously described that eHEV particles contain phosphatidylserine (PS) [11]. This negatively charged phospholipid is a marker for apoptosis and normally present at the cytosolic side of the membrane. Upon apoptosis, PS is exposed and binds to PS-receptors, thereby modulating phagocytosis. This process is hijacked by a variety of viruses for their entry and is termed ‘apoptotic

mimicry' [12]. Not only enveloped viruses such as Ebola virus, Dengue virus or Marburg virus use this process but also hepatitis A virus (HAV) has been shown to bind the PS-receptor TIM1 (T cell immunoglobulin mucin domain 1) [13-16]. HEV is similar to HAV in that they both circulate as quasi-enveloped viruses [17]. The presence of PS in the eHEV particle might bind PS-receptors such as TIM1 and thereby mediate eHEV entry, but this has not previously been experimentally determined. In this study, we explored the role of TIM1 by using CRISPR-Cas9 technology, as well as TIM1 rescue and ectopic expression. We provide evidence that TIM1 mediates the infection of enveloped HEV but not intracellular HEV and facilitates eHEV internalization. Moreover, we show that PS present in viral membrane is crucial in this process. Our results suggest that TIM1 acts as an entry cofactor for eHEV.

2. Materials & Methods – (*A complete overview of the materials and methods can be found in the online supplement)*

2.1 Virus production and infection

The gt-3 HEV Kernow-C1 Passage 6 (p6) plasmid (GenBank accession number JQ679013) was linearized with *MluI* (New England Biolabs), the gt-3 HEV 83-2-27 plasmid (GenBank accession number AB740232) was linearized with *HindIII*. Capped RNA was produced either using T7 RiboMAX Express Large Scale RNA production (Promega), followed by ScriptCap m7G capping system (Cellscript) or using T7 mMESSAGE mMACHINE kit (Ambion).

Capped RNAs were electroporated into PLC3 using a Gene Pulser (Bio-Rad). Extracellular viral particles (eHEV) were harvested from centrifuged and filtered (0.22 µm) supernatant twice a week, and concentrated using Amicon Ultra Centrifugal Filters (Merck). Lysis of transfected cells was performed to release intracellular viral particles (iHEV) by 3 freeze-thaw cycles in distilled water, followed by addition of 10X phosphate-buffered saline (PBS) and centrifugation at 10.000 x g for 30 minutes to separate cell debris. A detergent/protease-treated viral preparation (tHEV) was made by treating culture supernatant with 0.1% trypsin and 0.1% sodium deoxycholic acid at 37°C for 1 hour.

To determine the HEV infectivity titer, Huh-7.5 cells were seeded in Poly-L-Lysine-coated black 96 well plates and exposed to serial dilutions of respectively eHEV, iHEV and tHEV preparations. Three (p6) or five (83-2-27) days after infection, indirect immunofluorescence staining was used to check for HEV ORF2 positive cells.

2.2 Neutralization assays

Huh-7.5 cells were pre-incubated for 30 minutes with a polyclonal anti-TIM1 (AF1750, R&D systems) or isotype control (R&D systems) antibody at concentrations of 1 µg/ml, 3.3 µg/mL and 10 µg/mL after which eHEV or iHEV viral particles were added to the cell culture. Culture media was refreshed and antibodies were replenished. After three days, infection was quantified as described. In order to monitor cell viability under antibody neutralization, a MTT assay (Roche) was performed according to manufacturer's instructions. Cells were incubated with the indicated amounts of anti-TIM1 or isotype control antibody or left untreated. After incubation time, cells were incubated with 10 µL MTT solution, solubilization solution was added after 4 hours and incubated

overnight. Absorbance was measured by ELISA and cell viability of anti-TIM1 or isotype control were normalized to the untreated wells.

2.3 TIM1 rescue and ectopic expression

The TIM1 expressing plasmid or empty vector control (cloning information in supplemental information) were transfected into Huh-7.5$^{TIM1\text{-}KO}$ or HEKT293T cells, seeded in 96 well-plates, using Lipofectamine 2000 (Thermo Fisher Scientific). Cells were exposed to eHEV or iHEV 24 hours post transfection. Three days after infection TIM1 and ORF2 staining was performed.

2.4 Generation of HEK293T cells stably expressing TIM1 WT or TIM1 ND mutant

pTRIP lentiviral vectors containing wild-type or mutated TIM1 were described previously [13]. In the mutated variant further referred to as TIM1 ND, both the Asparagine and Aspartate residues are substituted by Alanine (N114A and D115A) within the metal ion-dependent ligand-binding site (MILIBS) of TIM1. The pTRIP vectors were transfected together with pCMV-VSV-G envelope and pCMV-gag-pol packaging vectors (Sigma), in HEK293T using JetPEI (Polyplus) according to manufacturer's instructions. Viral supernatant was harvested 48 and 72h post transfection. HEK293T cells expressing these variants were generated by lentiviral transduction, followed by cell sorting of the populations expressing TIM1 by TIM1 antibody staining (P365D) and a FACS Aria II (BD Biosciences). After expansion, cells were exposed to eHEV or iHEV followed by determination of infection levels.

2.5 Annexin V blocking assay

Equal infectious units of eHEV or iHEV preparations were pre-incubated for 30 minutes at 4°C with 1 or 10 µL of Annexin V (ANX5) (A35108 – Invitrogen, concentration proprietary information) in the presence of Ca^{2+}. After incubation, eHEV or iHEV virus was added to plated Huh-7.5 WT cells and infection was determined three days later by ORF2 immunostaining.

2.6 Virus attachment and internalization assays

For virus attachment assays, 10^6 cells were incubated with eHEV or iHEV for 60 minutes at 4°C. Cells were washed thrice to remove unbound virus and lysed, followed by total RNA extraction using the RNeasy kit (Qiagen). For internalization assays, virus was allowed to adhere for 1 hour at 4°C and temperature was shifted to 37°C to allow endocytosis for 2 hours, then treated by trypsin to eliminate non-internalized virus and total RNA was extracted as stated above. Bound and internalized virus was then quantified by qPCR as stated in the online supplement.

2.7 Statistical Analyses

Statistical analysis was performed using GraphPad Prism 8.0.1 software. Statistical significance of differences between groups was tested with a t-test or two-way ANOVA. A probability value of $p < 0.05$ was considered significant.

3. Results

3.1 TIM1 is involved in the infectious cycle of eHEV

To get a general idea of the potential role(s) of TIM1 in the HEV life cycle, we used Huh-7.5 cells that are permissive for HEV and in which TIM1 expression was knocked-out by CRISPR/Cas9, as verified by TIM1 flow cytometry and western blot (**Fig. 1a**, left panels). Cells were exposed to virus preparations of different type (**Fig. 1a**). The first viral preparation consisted of extracellular viral particles (eHEV) isolated from supernatant of gt-3 HEV p6 electroporated cells, of which the majority are quasi-enveloped virions (**Supplemental Figure 1a**) [9]. The second viral preparation consisted of intracellular particles (iHEV) isolated from lysate of HEV p6-electroporated cells; most of the iHEV particles are considered non-enveloped [9].

Three days after virus inoculation, infection levels were determined by immunofluorescence staining against intracellular HEV ORF2 protein. Compared to wild-type (WT) Huh-7.5 cells, we observed a 97% reduction of eHEV infection in Huh-7.5$^{TIM1\text{-}KO}$ cells ($p<0.0001$) (**Fig. 1a**). Interestingly, infection rates were similar in both cells types infected with iHEV ($p=0.5126$) (**Fig. 1a**).

Given this intrinsic difference between eHEV and iHEV, we performed a treatment of eHEV (tHEV) with a solution comprising sodium deoxycholate and trypsin, thereby mimicking the process viral particles undergo in the bile duct and duodenum; i.e. the removal of lipid membranes and incorporated proteins. Upon treatment, a 50% reduction of infection in Huh-7.5$^{TIM1\text{-}KO}$ was seen compared to Huh-7.5^{WT} ($p=0.0006$), and is consequently positioned between the eHEV or iHEV infection levels, relating to presumably only a partial loss of lipid membranes **(Fig. 1a)**.

We corroborated our findings in A549^{WT} and A549$^{TIM1\text{-}KO}$, where TIM1 expression was verified by flow cytometry and western blot (**Supplemental Fig. 2**). In accordance with eHEV infections in Huh-7.5 cells, infection was inhibited by more than 95% in A549$^{TIM1\text{-}KO}$ cells compared to A549^{WT} ($p<0.009$) (**Supplemental Fig. 2**). iHEV or tHEV preparations failed to establish robust infection levels (**Supplemental Fig. 2**).

These findings were also further confirmed with the gt-3 viral isolate 83-2-27. General infection levels were much lower compared to p6 but upon eHEV inoculation, we could again demonstrate a decrease in infection of 99% in the Huh-7.5$^{TIM1\text{-}KO}$ cells, compared to Huh-7.5^{WT} ($p=0.004$) (**Supplemental Fig. 3**). iHEV infection was the same in the two cell lines ($p=0.9271$).

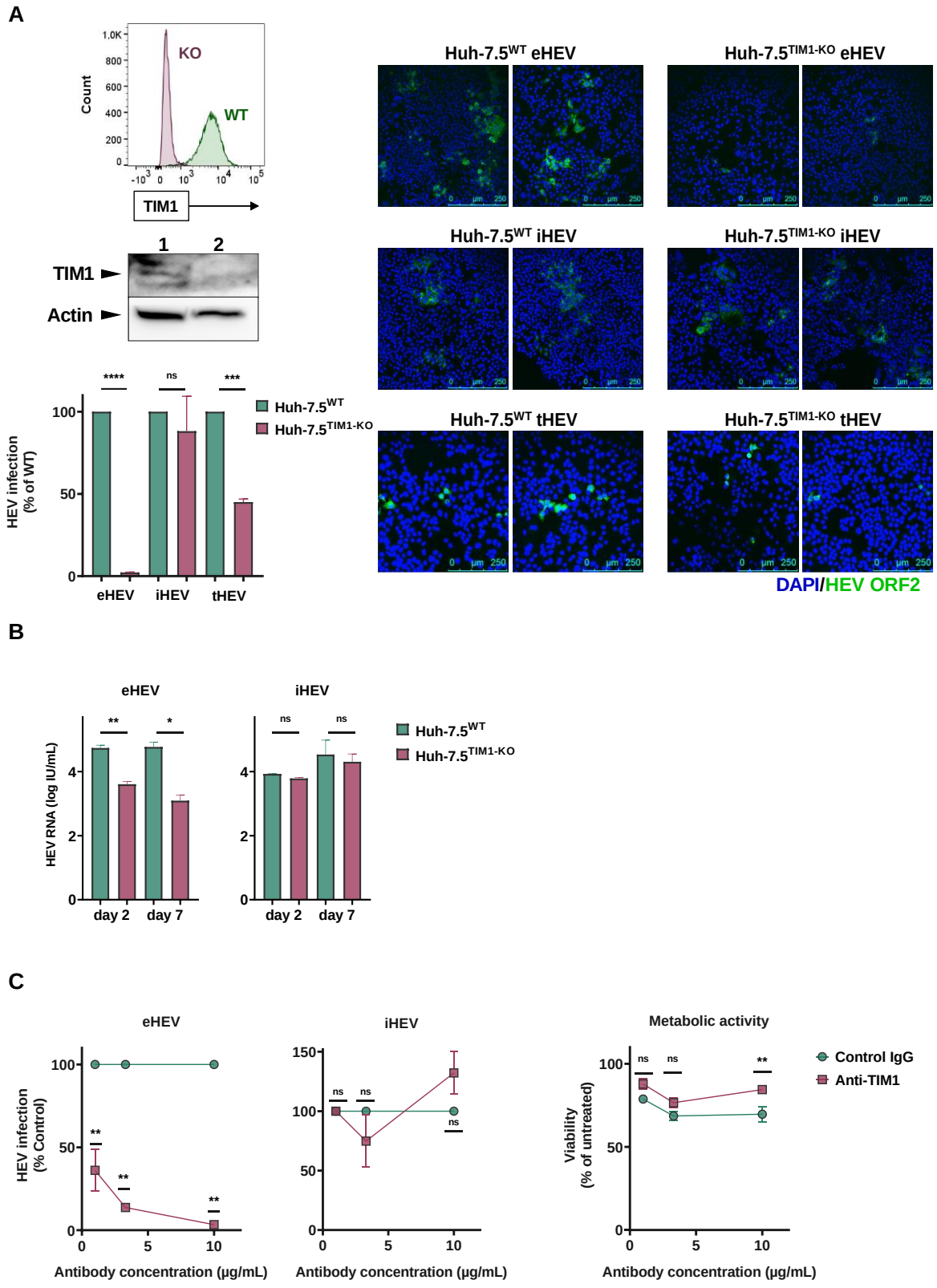


Fig. 1

Knockout or blocking of TIM1 decreases eHEV infection in Huh-7.5 cells.

(A) Upper left panel shows an overlay of TIM1 cell surface expression in Huh-7.5^{WT} (green) and Huh-7.5$^{TIM1\text{-}KO}$ (purple) cells. Expression evaluated by flow cytometry using a TIM1 recombinant rabbit monoclonal antibody. Middle left panel shows confirmation of TIM1 KO by western blot, 1 = Huh-7.5^{WT} 2 = Huh-7.5$^{TIM1\text{-}KO}$. Lower left panel shows infection in Huh-7.5^{WT} and Huh-7.5$^{TIM1\text{-}KO}$ exposed to different preparations of HEV inoculum: extracellular HEV (eHEV), intracellular HEV (iHEV) and extracellular HEV treated with deoxycholate/trypsin (tHEV). Infection was determined 3 days post infection by indirect immunostaining against the HEV ORF2 protein. Representative confocal images of the different HEV preparations are on the right with DAPI staining in blue and HEV ORF2 in green.

(B) Quantification of HEV RNA by qRT-PCR in supernatants of Huh-7.5^{WT} and Huh-7.5$^{TIM1\text{-}KO}$ at two different time-points after challenge with eHEV on the left or iHEV on the right.

(C) Huh-7.5^{WT} were pre-incubated with different concentrations (1 µg/mL, 3 µg/mL or 10 µg/mL) of a polyclonal anti-TIM1 antibody or normal IgG as control for 30 minutes. Pre-incubated cells were challenged with eHEV (left panel) or iHEV (middle panel) and infection was determined 3 days later by HEV ORF2 immunostaining. Right panel shows cell viability as measured by MTT assay and normalized to untreated control.

Data are represented as mean ± SEM of at least 2 independent experiments. Each experiment consisted of at least 3 technical replicates. Significance was calculated using a two-tailed t-test (A&B) or two-way ANOVA (C). ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 ns non-significant

Next we made different ratios of co-cultures of Huh-7.5^{WT} and Huh-7.5$^{TIM1\text{-}KO}$ cells and exposed them to either eHEV or iHEV to check the distribution of infection with regard to the TIM1 status. The ratio of WT cells ranged from 52% to 17% in the total cell population **(Supplemental Fig. 4a and 4c)**. Interestingly, eHEV preferentially infects the TIM1 positive population with roughly 90% of the infected cells being TIM1-positive, irrespectively of the percentage of TIM1-cells within the total population **(Supplemental Fig. 4b)**. On the other hand, iHEV infection does not preferentially infect the TIM1-positive cells as the percentage of infected TIM1 cells correlated more-or-less with the percentage of TIM1-positive cells within the total population (**Supplemental Fig. 4d)**. For example, when a population consisting of 15,4-17,7% TIM1-expressing cells was exposed to eHEV, 87% of the infected cells were of the TIM1-positive type, whereas if exposed to iHEV, only 25% of the infected cells belonged to the TIM1-positive subpopulation (**Supplemental Fig. 4a-d, fourth column**).

Additionally, we quantified the level of secreted HEV into culture supernatant of eHEV and iHEV infected cultures at 2- and 7-days post-infection. For eHEV infection, extracellular RNA titers were on average 1-$\log_{10}$ lower in supernatant of Huh-7.5$^{TIM1\text{-}KO}$ cell cultures compared to Huh-7.5^{WT} cultures at both time points (p=0.0098 and p=0.0378 for day 2 and day 7 respectively) **(Fig. 1b, left panel)**. For iHEV infection, viral load on day 2 and day 7 were comparable in both Huh-7.5$^{TIM1\text{-}KO}$ and Huh-7.5^{WT} cell cultures (p=0.0608 and p=0.4705 respectively)**(Fig. 1b, right panel)**.

The involvement of TIM1 in HEV infection was further explored by targeting the protein with increasing amounts of a neutralizing anti-TIM1 polyclonal antibody, followed by eHEV or iHEV inoculation. We found a concentration-dependent decrease in eHEV infection. The lowest concentration of antibody tested (1 µg/mL) already showed a 60% reduction in infection compared to control IgG (p=0.0097), while a reduction of more than

95% was observed at the highest antibody concentration (p=0.0029) **(Fig. 1c, left panel) (Supplemental Fig. 5)**. TIM1 neutralization followed by iHEV infection did not diminish the infection in any of the treated conditions (p values 0.3709, 0.5251. >0.9999 from high to low concentration) (**Fig. 1c, middle panel**) (**Supplemental Fig. 5)**. Cell viability under both anti-TIM1 and IgG antibody treatment was determined by an MTT assay. Viability only slightly reduced under treatment, but the TIM-1 antibody was not considered more cytotoxic than the IgG control in all tested conditions (**Fig. 1c, right panel**).

3.2 eHEV infection is rescued by TIM1 expression in KO-cells.

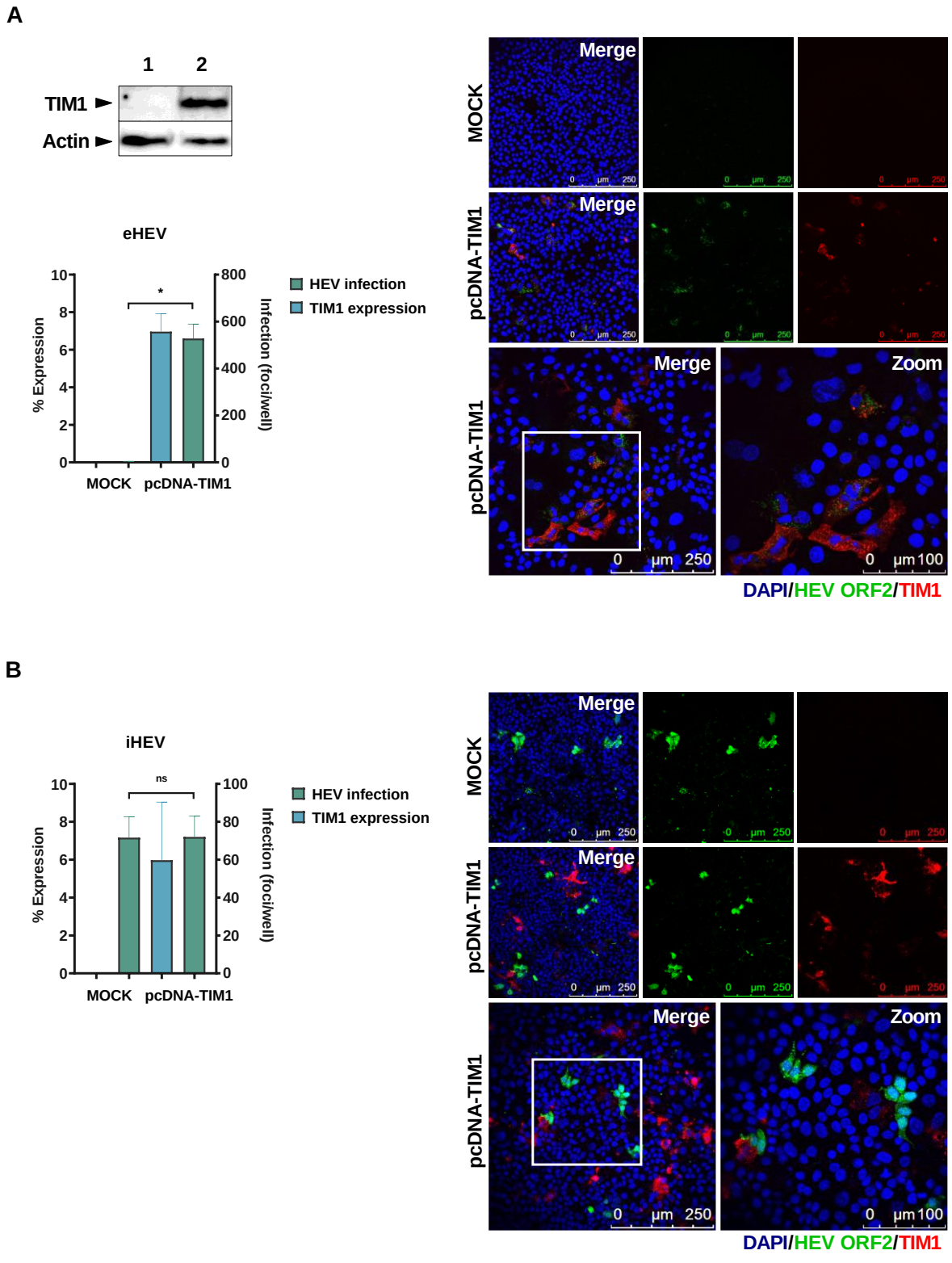
To prove that the absence of TIM1 is responsible for decreased eHEV infection in TIM1-KO cells, these cells were transiently transfected with the pcDNA3.1-TIM1 expression vector. Successful TIM1 transfection was verified via western blot (**Fig. 2a, upper panel**). To investigate the effect of HEV infection in transiently transfected Huh-7.5$^{TIM1\text{-}KO}$ cells, both HEV ORF2 and TIM1 were visualized by immunofluorescence staining in Huh-7.5$^{TIM1\text{-}KO}$ MOCK transfected or Huh-7.5$^{TIM1\text{-}KO}$ pcDNA-TIM1 transfected cells. At the time-point of HEV analysis on day three, an average of 6% of the cells showed TIM1 expression (**Fig. 2, blue bars**).

In accordance with our previous infection experiments in TIM1-KO cells, an average eHEV infection rate of only 4 foci/well could be observed in Huh-7.5$^{TIM1\text{-}KO}$ cells that were MOCK transfected. However, upon TIM1 rescue, we observed a nearly 150-fold increase in eHEV infection (p=0.0135) **(Fig. 2a, green bars)**. Moreover, it is clear that the HEV infection is observed in the cells expressing TIM1, as illustrated by the immunostaining images where HEV and TIM1 signals are predominantly observed in the same cells (**Fig. 2a, confocal images**). On the contrary, rescue of TIM1 had no effect on iHEV infection levels, as infection was similar in MOCK or pcDNA-TIM1 transfected cells (p=0.9849) (**Fig. 2b, green bars**) and infected cells are generally not the TIM1-positive cells (**Fig. 2b, confocal images**) This further confirms the role of TIM1 in eHEV infection.

3.1 Ectopic expression of TIM1 facilitates eHEV infection in HEK293T cells

We further evaluated the ability of ectopic TIM1 expression to mediate eHEV infection in HEK293T cells, which do not express TIM1. We first confirmed that HEK293T could support HEV genome replication (**Fig. 3a**). Next, we transfected HEK293T cells transiently with the pcDNA3.1-TIM1 expression vector and confirmed TIM1 expression first via western blot (**Fig. 3b**). To investigate the effect of HEV infection in transiently transfected HEK293T cells, both HEV ORF2 and TIM1 were visualized by immunofluorescence staining in HEK293T

MOCK transfected or HEK293T pcDNA-TIM1 transfected cells. At the time-point of HEV analysis on day three, almost 20% of the cells showed TIM1 expression (**Fig 3c and 3d, blue bars**). Parental MOCK transfected HEK293T were nearly completely resistant to eHEV infection, whereas TIM1 expression enhanced eHEV infection level to on average 15% (p=0.0063) (**Fig. 3c**). iHEV infection in parental MOCK transfected cells was generally low, but infected cell foci could be observed and transient TIM1 expression did not alter iHEV infection (p=0.7889) (**Fig. 3d**).



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278 **Fig. 2**

279 Rescue of TIM1 enhances HEV infection.

(A) Huh-7.5$^{TIM1\text{-}KO}$ cells were transfected with an empty plasmid (MOCK) or pcDNA3.1-TIM1 expression vector. Expression was verified via western blot, 1= Huh-7.5$^{TIM1\text{-}KO}$/MOCK and 2= Huh-7.5$^{TIM1\text{-}KO}$/pcDNA-TIM1. eHEV infection was performed 24h after transfection and analyzed three days later. eHEV infection levels determined by HEV ORF2 indirect immunofluorescence staining (right axis, green). TIM1 expression on the same day is depicted on the left y-axis (blue) and was measured by TIM1 immunofluorescence staining. On the right side, representative confocal microscopy images of MOCK and TIM1 transfected Huh-7.5$^{TIM1\text{-}KO}$ cells are depicted. Cells were fixed and permeabilized, followed by immunofluorescence staining against TIM1 (red) and HEV ORF2 (green), DAPI nuclei in blue. Lowest panel shows a merged image where a region of interest was acquired by 40x objective lens instead of 20x objective (white square) to demonstrate overlap between the cells expressing TIM1 and being infected with HEV.

(B) Huh-7.5$^{TIM1\text{-}KO}$ cells were transfected with an empty plasmid (MOCK) or pcDNA3.1-TIM1 expression vector and infected with iHEV. Experiment and graph same as for the upper panel. On the right side, representative confocal microscopy images are depicted, the same as described for the upper panel.

Data are represented as means ± SEM from two independent experiments. Each experiment included at least 3 technical replicates. Significance was calculated using a two-tailed t-test. *$p<0.05$ ns non-significant

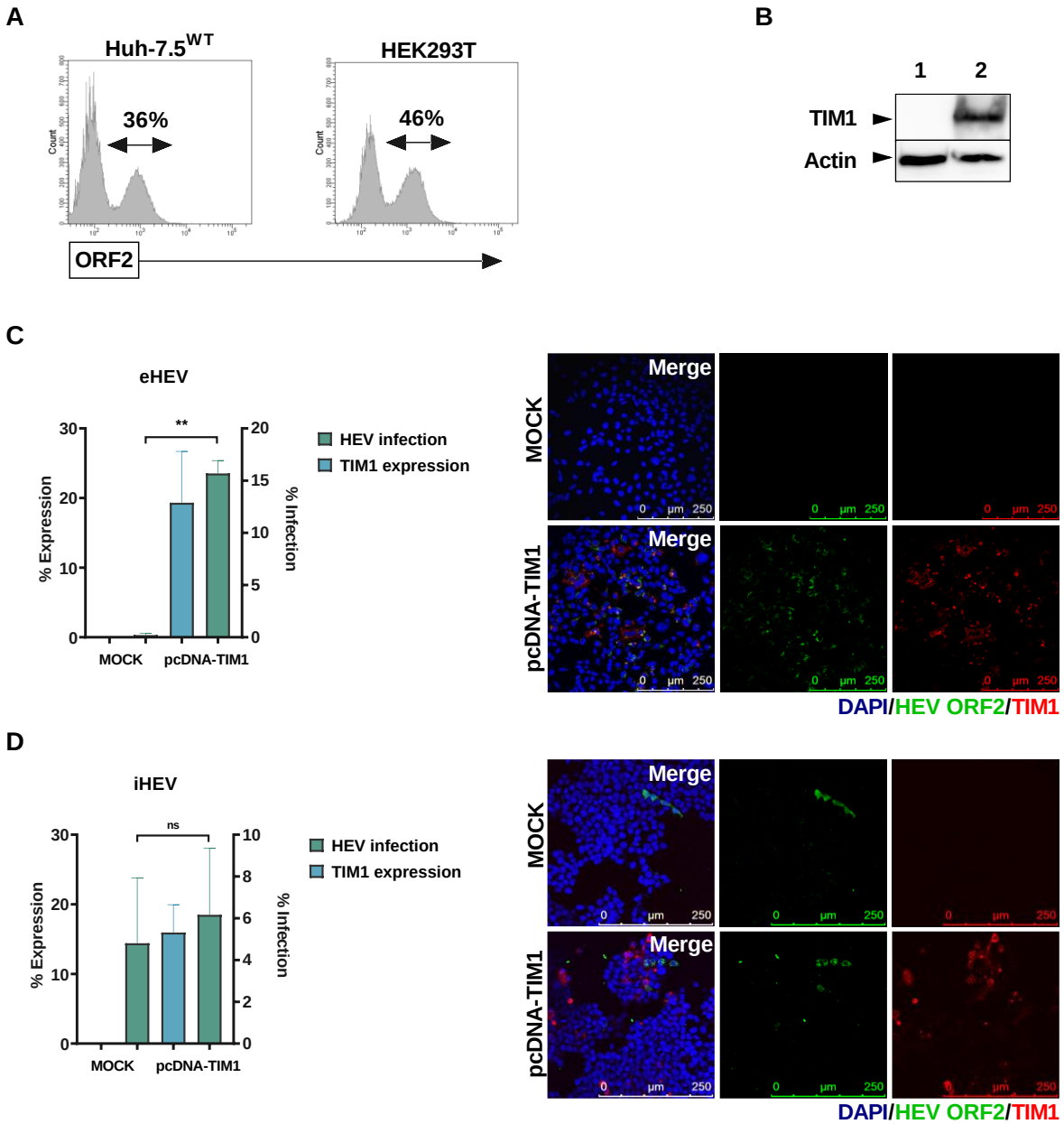


Fig. 3

Ectopic expression of TIM1 facilitates eHEV infection in HEK293T cells.

(A) HEK293T cells support HEV replication after transfection with HEV RNA, comparison made with electroporated Huh-7.5 cells. Percentages indicate expression of HEV, as determined by flow cytometry after intracellular staining using an HEV ORF2 monoclonal antibody, followed by labeling with an Alexa Fluor 488-conjugated secondary antibody.

(B) HEK293T were transiently transfected with an empty plasmid (MOCK) or pcDNA3.1-TIM1 expression vector and expression was verified via western blot. 1= HEK293T/MOCK 2=HEK293T/pcDNA-TIM1

(C) HEK293T were transfected with an empty plasmid (MOCK) or pcDNA3.1-TIM1 expression vector one day before eHEV infection. left panel shows results with eHEV infection percentage on the right y-axis 3 days after, as measured by HEV ORF2 immunofluorescence staining. TIM1 expression on the same day is depicted on the left y-axis and was measured by TIM1 immunofluorescence staining. Right panel are representative confocal images of eHEV infected HEK293T/MOCK (parental) compared to the HEK293T/pcDNA-TIM1, DAPI staining in blue, HEV ORF2 in green and TIM1 in red.

(D) HEK293T cells transfected the same way as described and infected with iHEV. iHEV percentage on the right y-axis on day 3 and TIM1 expression on the same day depicted on the left y-axis. Right panel shows representative confocal images of iHEV infected HEK293T/MOCK compared to HEK293T/pcDNA-TIM1.

Data represented as means ± SEM from two independent experiments. Each experiment consisted of at least 3 technical replicates. Significance was calculated using a t-test. **p<0.01 ns non-significant

3.1 TIM1 facilitates eHEV internalization

The above-mentioned data clearly indicates the role of TIM1 during infection of eHEV. To examine the exact function of TIM1 during viral entry in more detail, we performed viral binding assays. eHEV or iHEV were allowed to adhere to Huh-7.5^{WT} and Huh-7.5$^{TIM1\text{-}KO}$ cells at 4°C. Perhaps unexpectedly, we could not observe a difference in eHEV binding between Huh-7.5^{WT} and Huh-7.5$^{TIM1\text{-}KO}$ (p=0.4697) (**Fig. 4a, left panel).** As a control, iHEV attachment assays were performed in parallel and also no difference in viral binding was observed (p=0.3728) (**Fig. 4a, left panel)**. In a next step, we carried out an internalization assay, where the virus was first attached, followed by a shift to 37°C to permit virus internalization. Cell-surface bound virions were removed by trypsinization and after RNA extraction, qPCR showed a marked decrease of almost 1-log in internalized eHEV in the Huh-7.5$^{TIM1\text{-}KO}$, compared to Huh-7.5^{WT} (p=0.0046), while internalized iHEV was similar in the two cell types (p=0.4462) (**Fig. 4a, right panel)**. These findings were confirmed in 293T stably transduced with TIM1 and compared to parental 293T. For both eHEV and iHEV, no difference in viral binding could be observed (p=0.1254 and p=0.1864 respectively), but a decrease in internalized eHEV was observed (p=0.0463), while iHEV internalization was the same (p=0.0911) (**Fig. 4b**). Similarly, we verified our observations with another gt-3 isolate, 83-2-27. Again no differences in attachment between Huh-7.5^{WT} and Huh-7.5$^{TIM1\text{-}KO}$ could be observed (p=0.9684 for eHEV and p=0.1230 for iHEV) but, similar as with the p6 clone, less enveloped virus internalizes in TIM1 KO cells (p=0.0139) (**Supplementary fig. 6**).

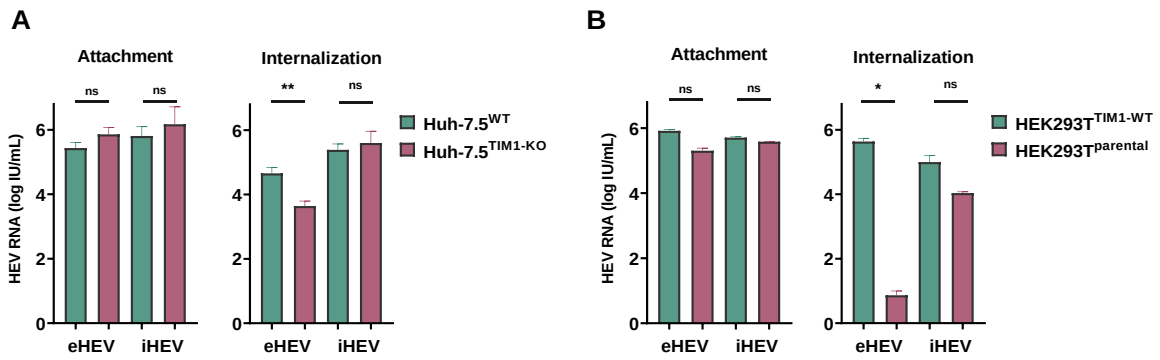


Fig. 4

TIM1 facilitates eHEV entry

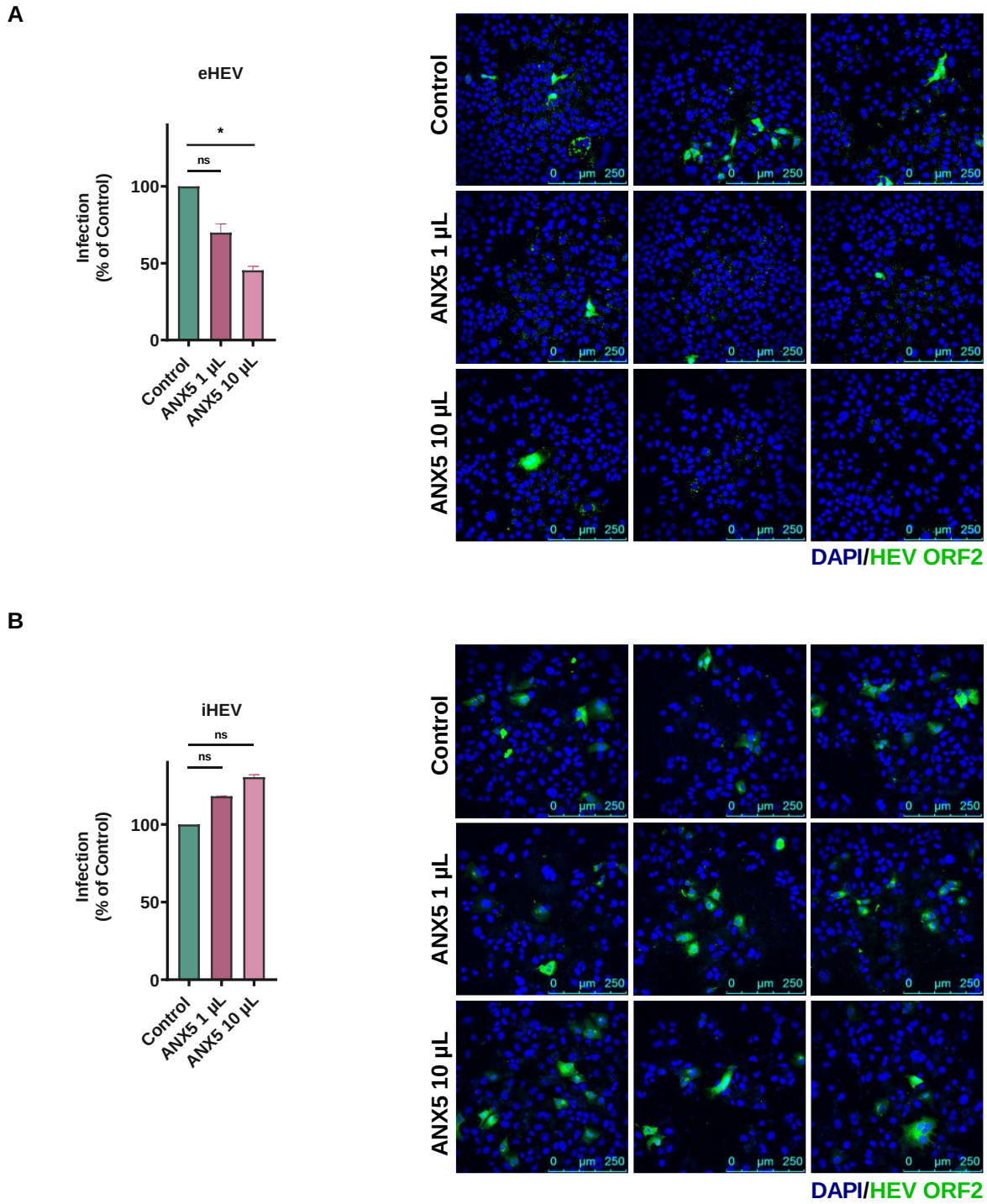
(A) Adherence of eHEV and iHEV to Huh-7.5^{WT} and Huh-7.5$^{TIM1\text{-}KO}$ at 4°C for 1 hour. After washing, RNA was extracted and determined by qRT-PCR (left panel). Right panel shows viral binding of eHEV and iHEV to Huh-7.5^{WT} and Huh-7.5$^{TIM1\text{-}KO}$, followed by a shift to 37°C for 2 hours. Cells were washed, trypsinized and total RNA was extracted and measured by qRT-PCR.

(B) Adherence of eHEV and iHEV to HEK293T^{parental} and HEK293T$^{TIM1\text{-}WT}$ at 4°C for 1 hour. After washing, RNA was extracted and determined by qRT-PCR (left panel). Right panel shows viral binding of eHEV and iHEV to Huh-7.5^{WT} and Huh-7.5$^{TIM1\text{-}KO}$, followed by a shift to 37°C for 2 hours. Cells were washed, trypsinized and total RNA was extracted and measured by qRT-PCR.

Data represented as means ± SEM from two independent experiments. Each experiment consisted of at least 2 technical replicates. Significance was calculated using a t-test. **p<0.01 *p<0.05 ns non-significant

3.2 The PS-binding cavity of TIM1 is important for eHEV viral infection

TIM proteins bind phosphatidylserine (PS) present on the surface of apoptotic cells or viruses [12]. eHEV as well has been described to be associated with PS [11]. To further explore the potential role of eHEV-associated PS and the effect on viral infection, we pre-incubated eHEV or iHEV with Annexin V (ANX5), a PS-binding protein that has often been used to explore the role of TIM1 in viral entry and infection. Using the highest amount of ANX5, a reduction in eHEV infection of more than 50 % could be observed (p=0.0101), while a 10-fold less amount of ANX5 had an intermediate effect (p=0.1502) (**Fig. 5a**), indicating dose-dependent inhibition. In contrast, control infections with iHEV showed similar infection levels in all tested conditions (p=0.3321 for 10 µL and p=0.6465 for 1µL)



359

360 **Fig. 5**

361 Annexin V inhibits eHEV infection.

362 (A) eHEV containing viral supernatant was preincubated with different amounts of ANX5 (1 µL, 10 µL) followed by
363 addition to Huh-7.5 cells. Infection normalized to infection without ANX5. On the right, representative confocal
364 microscopy images of the different conditions are depicted.
365 (B) iHEV containing viral lysate was preincubated with different amounts of ANX5 (1 µL, 10 µL) followed by addition
366 to Huh-7.5 cells. Infection normalized to infection without ANX5. On the right, representative confocal microscopy
367 images of the different conditions are depicted.

Data represented as means ± SEM from two independent experiments. Each experiment consisted of at least 3 technical replicates. Significance was calculated using a two-way ANOVA. *p<0.05 ns non-significant

To study the role of PS in greater detail in the context of eHEV, we further focused on the metal ion-dependent ligand-binding site (MILIBS) situated in a conserved cavity that is part of the extracellular IgG-V-like domain of TIM receptors, which has been well characterized as the binding site for PS, including in the context of viral entry and infection [12]. A HEK293T cell line was generated that expresses a previously characterized mutated TIM1 receptor [13], harboring two mutations (N114A and D115A; termed TIM1-ND) in the conserved cavity, rendering it unable to bind PS. TIM1 was correctly expressed at the cell surface, as confirmed via flow cytometry and protein expression was also verified via western blot (**Fig. 6a**). We determined the level of HEV infection compared to a HEK293T cell line stably expressing TIM1-WT and included parental HEK293T cells as a control. eHEV infection in the parental cells was almost non-existent compared to the HEK293T$^{TIM1\text{-}WT}$ ($p<0.0001$)(**Fig. 6b, left panels**), but infection decreased almost 95% in HEK293T$^{TIM1\text{-}ND}$ compared to HEK293T$^{TIM1\text{-}WT}$ ($p<0.0001$) **(Fig. 6b**). We compared our results to iHEV infection. As with transient expression in HEK293T, infection in parental cells was low, though remained the same in HEK293T$^{TIM1\text{-}WT}$ or HEK293T$^{TIM1\text{-}ND}$ expressing cells (**Fig 6b, right panels**).

To confirm these findings, Huh-7.5$^{TIM1\text{-}KO}$ cells were also transduced with the TIM1-ND mutant and infection levels were compared to Huh-7.5$^{TIM1\text{-}KO}$ stably transduced with TIM1-WT and control Huh-7.5$^{TIM1\text{-}KO}$ (Fig. 7a). We observed a decrease of 99% in Huh-7.5$^{TIM1\text{-}ND}$ compared to Huh7.5$^{TIM1\text{-}WT}$ upon eHEV infection (**Fig. 7b, left panels**). iHEV infection levels were the same in all three conditions (**Fig. 7b, right panels**), indicating that the PS-binding pocket is required only for the TIM1-mediated eHEV infection.

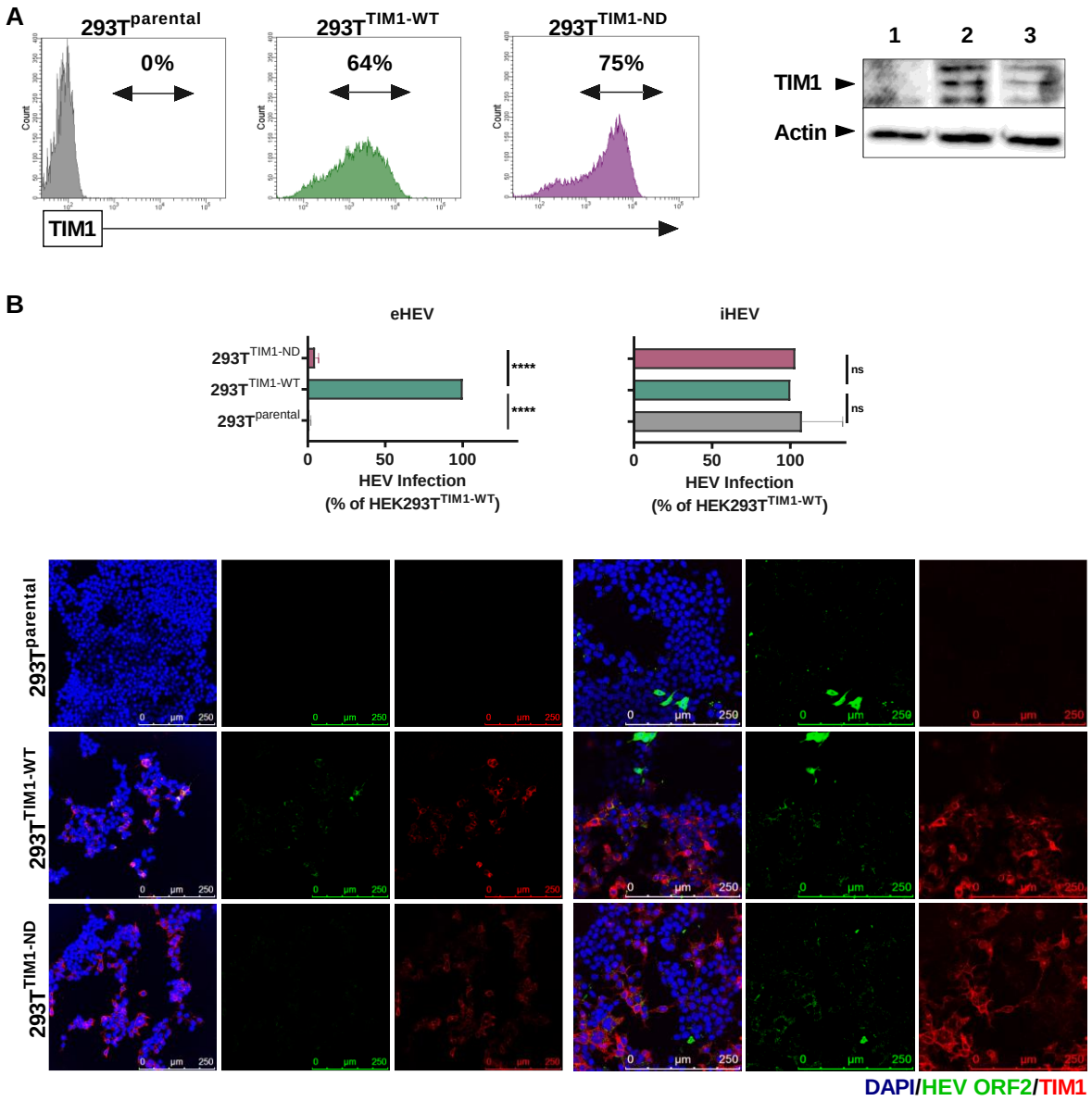
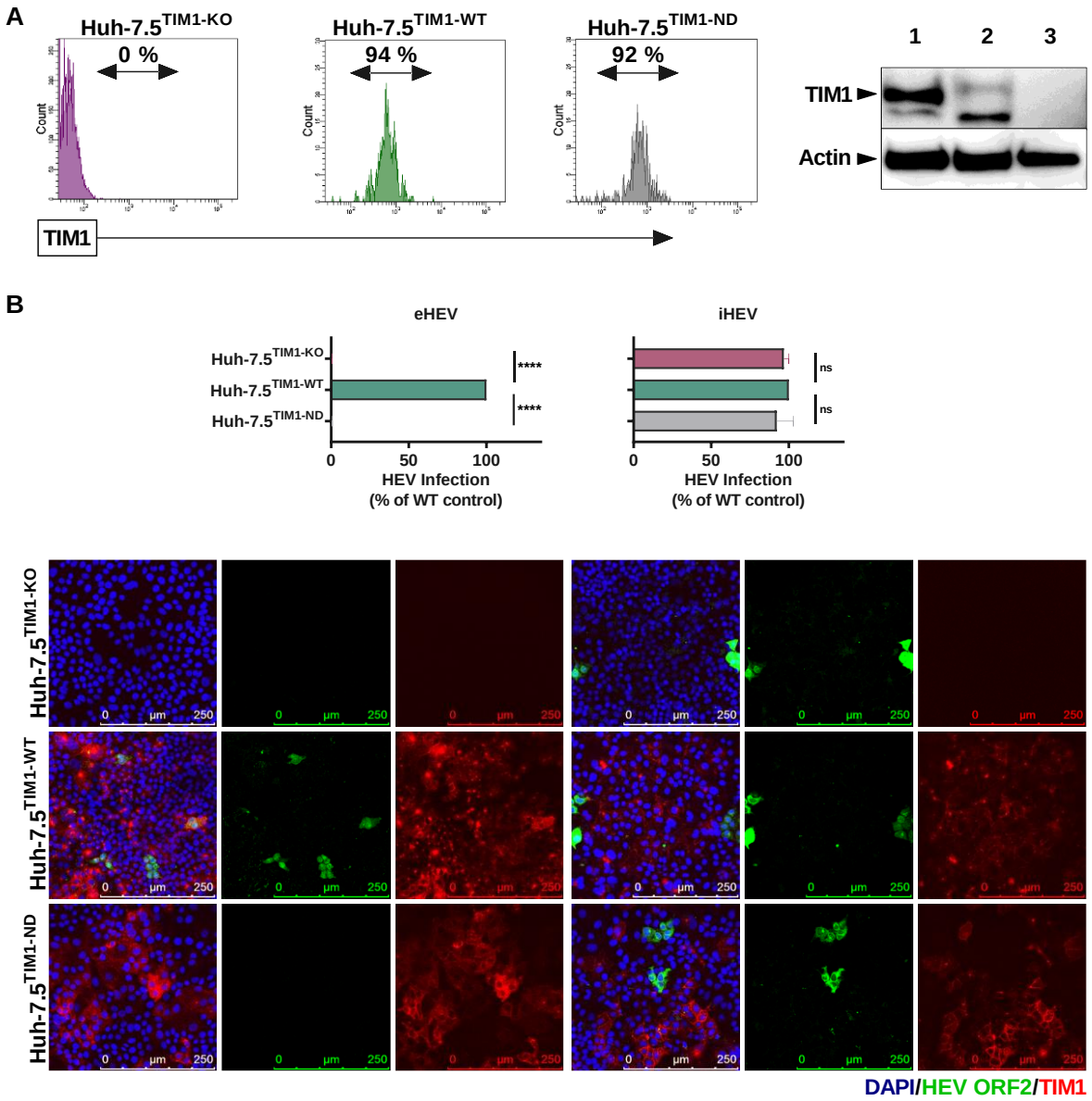


Fig. 6

The PS-binding cavity is important for viral binding to TIM1.
(A) TIM1 cell surface expression of parental HEK293T (purple) compared to TIM1-WT transduced HEK293T (green) and TIM1-ND mutant transduced HEK293T (grey). Expression was evaluated by flow cytometry using a TIM1 recombinant monoclonal antibody (left panel). Right panel shows expression verified by western blot. 1=HEK293T^{parental} 2=HEK293T$^{TIM1\text{-}WT}$ 3=HEK293T$^{TIM1\text{-}ND}$
(B) Stable transduced HEK293T$^{TIM1\text{-}WT}$ and HEK293T$^{TIM1\text{-}ND}$ mutant as well as parental HEK293T cells were challenged with eHEV (left panels) or iHEV (right panels) and analyzed by HEV ORF2 immunofluorescence staining three days later. Lower panels are representative confocal images of the different conditions, DAPI staining in blue, HEV ORF2 in green and TIM1 in red.

Data represented as means ± SEM from two independent experiments. Each experiment consisted of at least 3 technical replicates. Significance was calculated using a two-way ANOVA. ****p<0.0001 ns non-significant



407

408 **Fig. 7**
409
410 The PS-binding cavity is important for viral binding to TIM1.
411 (A) TIM1 cell surface expression of Huh-7.5$^{TIM1-KO}$ (purple) compared to Huh-7.5$^{TIM1-WT}$ (green) and Huh-7.5$^{TIM1-ND}$ (grey).
412 Expression was evaluated by flow cytometry using a TIM1 recombinant monoclonal antibody (left panel). Right panel
413 shows expression verified by western blot. 1=Huh-7.5$^{TIM1-WT}$ 2= Huh-7.5$^{TIM1-ND}$ 3= Huh-7.5$^{TIM1-KO}$
414
415 (B) Huh-7.5$^{TIM1-KO}$,Huh-7.5$^{TIM1-WT}$, and Huh-7.5$^{TIM1-ND}$ were challenged with eHEV (left panels) or iHEV (right panels) and
416 analyzed by HEV ORF2 immunofluorescence staining three days later. Lower panels are representative confocal images
417 of the different conditions, DAPI staining in blue, HEV ORF2 in green and TIM1 in red.

418 Data represented as means ± SEM from two independent experiments. Each experiment consisted of at least 3 technical
419 replicates. Significance was calculated using a two-way ANOVA. ****p<0.0001 ns non-significant

420

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4. Discussion

In this study, we provide the first experimental evidence that TIM1 plays a role in the infectivity of enveloped HEV particles. We found that absence of TIM1 significantly decreased eHEV infection in Huh-7.5$^{TIM1-KO}$ cells, but not iHEV infection **(Fig. 1a)**, indicating that the inhibition of infection is probably limited to viral particles surrounded by a host membrane-derived envelope. This finding was further corroborated by the fact that infection by deoxycholate/trypsin-treated eHEV (tHEV) was less dependent on TIM1 (only 50% inhibition) compared with non-treated eHEV (more than 90% inhibition) **(Fig. 1a)**. Of note, tHEV infection levels were intermediate between those of eHEV, consisting merely of enveloped particles, and those of iHEV, comprising mainly non-enveloped virus (**Supplemental Fig. 1**). The fact that we observed a significant difference in infection between WT and TIM1-KO cells using tHEV may be explained by a partial loss of the viral membrane upon deoxycholate/trypsin treatment. This has been illustrated by tHEV density profile analysis, showing a mixture of both enveloped and non-enveloped particles [18]. We as well observed a peak shift for the density profile (**Supplemental Fig. 1B**).

We confirmed eHEV infection inhibition in A549$^{TIM1-KO}$ cells **(Supplemental Fig. 2),** but unfortunately, iHEV or tHEV preparations failed to establish robust infection levels in these cells. Therefore, we focused the rest of our infection studies mainly on Huh-7.5 cells and subsequently on HEK293T cells. We also confirmed eHEV infection inhibition for the gt-3 83-2-27 isolate in Huh-7.5$^{TIM1-KO}$ cells (**Supplemental Fig. 3**).

A reduced virus secretion from Huh-7.5$^{TIM1-KO}$ cells when they were infected with eHEV could be detected **(Fig. 1b)**.

We also showed that an antibody targeting TIM1 blocks eHEV infection but not iHEV infection in cells naturally expressing the receptor (**Fig. 1c**). Furthermore, eHEV infection could be restored in Huh-7.5$^{TIM1-KO}$ cells upon transfection with a TIM1-expressing plasmid **(Fig. 2a);** and ectopic TIM1 expression in HEK293T cells enhances eHEV infection **(Fig. 3c)**. Taken together, it is clear that TIM1 is involved in the infectious cycle of enveloped HEV particles.

In context of viral infections, TIM1 has been shown to enhance entry by a process called viral apoptotic mimicry where PS exposed on viral envelope facilitate viral engulfment, either by directly interacting with TIM1 or via bridging molecules [12]. The MILIBS of TIM1 is well characterized as the essential part of PS recognition [19, 20]. It was previously shown that HEV particles exit cells as exosome-like vesicles and contain PS, possibly acquired from membranes derived from multivesicular bodies, key players of the endosome fraction that play a central role during enveloped particle biogenesis and viral egress [11, 21]. Using Annexin V, which binds PS with

high affinity, we showed that eHEV infection decreases (**Fig. 5a**). Mutation of the critical residues in the PS-binding pocket inhibited eHEV infection by more than 90% **(Fig. 6b** and **7b)**. Both experiments indicate the importance of this cavity. Based on current knowledge about the interaction between PS and the binding pocket of TIM1 in context of viral infection, one could assume that PS present on enveloped HEV viral particles would act as such. In general, it is not always clear whether TIM1 only enhances virus attachment or serves as an authentic receptor. For the latter a direct interaction between a viral protein and TIM1 is needed, as has been described for Dengue virus and Ebola virus [22, 23]. Our data suggests the role of PS in entry **(Fig. 5, Fig. 6** and **Fig. 7)**, which would favor a role of TIM1 as attachment factor. However, our attachment assays in both Huh-7.5 and HEK293T cells show no difference in viral binding in absence of TIM1. A difference between WT and KO only became apparent upon analysis of viral internalization (**Fig. 4** and **Supplementary Fig. 6**). A similar finding was observed for hepatitis A virus (HAV) where an analogous distinction of both enveloped and non-enveloped viral particles is made [17]. TIM1-KO reduces entry of enveloped HAV, but not non-enveloped HAV, and the role of TIM1 in enveloped HAV entry is considered to involve interaction with PS present in the viral envelope [14, 24]. A comparison between the two viruses might therefore seem conceivable with an analogous role for PS present in the viral envelope of HEV. Interestingly, in the context of HAV, no difference in attachment to Huh-7.5$^{TIM1\text{-}KO}$ cells compared to WT was detected either. Only when a kidney-derived cell line was used, a difference in attachment was observed, which was attributed to the fact that expression of TIM1 is higher in the kidney compared to the liver [14]. We used the kidney-derived HEK293T cell line but were unable to observe a difference in attachment upon TIM1 transduction. However, parental HEK293T cells do not express TIM1 as verified with flow cytometric staining and Western blotting (**Fig. 6a**) and reports from other groups [13], precluding a similar observation as with HAV. The fact that we do observe that TIM1 facilitates viral internalization, indicates that it is not merely acting as attachment factor but is involved in the process of viral entry of enveloped HEV. Our current understanding of this protein is incomplete to address all knowledge gaps. For example, the exact mechanism of virus internalization and following uncoating in context of TIM1 should be further explored. It was previously described that both eHEV and iHEV internalize via clathrin-and dynamin-dependent pathways but it seems that the uncoating process differs. A previous study showed that iHEV seems to uncoat in early endosomes, whereas eHEV entry is probably dependent on endosomal trafficking to early and late endosomes [25]. Further uncoating may be achieved by either fusion of the viral membrane with the cellular membrane or by removal of the envelope by lysosomal degradation. Previous work illustrated that lysosomal proteins Niemann-Pick disease type C1 (NPC1) and lysosomal acid lipase (LAL) are involved in eHEV infection, and therefore the second

hypothesis may be more likely [9]. It is believed that the virus must encode specific targeting signals, and that the interaction of a viral protein is possibly involved in trafficking [26]. Further study should explore a potential additional role of TIM1 in viral trafficking. Interestingly, it was previously shown that TIM1 can traffic between early endosomes as well as late endosome and lysosomal compartments, as they carry their cargo from the cell surface to the lysosome [27]. This role of TIM1 has briefly been explored during Dengue virus infection, where TIM1 seems to co-internalize with viral particles during endocytosis. Moreover, an interaction with STAM1 was observed [22], a component of ESCRT-0 necessary for endosomal sorting of ubiquitinylated membrane proteins [28].

Although infection decreased by more than 90% after TIM1-KO or antibody neutralization, a limited number of infected cells could still be observed. This may suggest that TIM1 is an important but not the only factor mediating entry for enveloped particles. On the other hand, we cannot exclude that some of the eHEV preparations present with a very low amount of naked HEV particles, which seem not to be dependent on TIM1 for entry. We were unable to use density-gradient purified virus, merely because the infectivity of those purified preps was too low to reveal potential significant differences in inhibition (data not shown). The in vivo significance of TIM1 during HEV infection remains to be further explored. If endogenous TIM1 molecules are important for HEV infection, one would expect a certain level of expression on hepatocytes. According to the Human Protein Atlas, hepatic TIM1 expression is rather low and no TIM1 expression is detected on hepatocytes. We corroborated these findings by cell surface staining of TIM1 on isolated primary human hepatocytes (PHH) and did not succeed to visualize cell surface TIM1 on PHH (data not shown). However, also according to Human Protein Atlas, there should be RNA expression in these cells in the liver. We sought out if this was the case by total RNA extraction from PHH, followed by TIM1 amplification and sequencing. Our data confirmed that TIM1 mRNA is present in these cells (data not shown). It is possible that TIM1 surface expression is indeed absent, as we observed, but natural surface expression could also be below the detection limit of our detection method, and/or visualization of TIM1 cell surface expression on isolated PHH is hampered by the proteinase digestion to isolate PHH from liver tissue. Interestingly, it has been described that renal TIM1 expression level increases upon kidney injury. This subsequently promotes transformation of the tubular epithelial cells into phagocytic cells that are able to recognize PS-associated apoptotic cells and execute apoptotic clearance necessary for resolution of inflammation [29]. To our knowledge, such effect has not yet been described in the context of virus infection, but it is an interesting speculation that a virus would upregulate TIM1 surface expression on target cells to enhance its entry and subsequent spread.

Even if hepatic TIM1 expression is low, this would not diminish the importance of the results we show as HEV infection is also associated with a variety of extrahepatic manifestations, leading to e.g. neurological or renal complications. In these tissues, TIM1 expression is notably high (Human Protein Atlas). During natural HEV infection, infection is likely mediated by fecal-oral transmission between individuals with an exposure to non-enveloped viral particles. However, after initial infection, enveloped particles are released from the infected liver into the blood circulation. It is possible that viral spread within the body mainly occurs through enveloped HEV particles that interact with a non-specific receptor such as TIM1, via a connection with PS incorporated in the viral envelope. The enveloped nature of HEV particles in the blood is of importance since it makes them less susceptible to ORF2-vaccine induced antibody mediated neutralization. Previously, PS-specific antibody therapy was able to cure guinea pigs lethally infected with Pichinde virus, illustrating the potential of targeting PS in the viral envelope [30]. On the other hand, given the importance of TIM1 in the entry of enveloped HEV, TIM1 might also constitute a target for antiviral therapy.

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Statements and Declarations

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Competing interests

The authors declare no conflict of interest.

Author Contributions

The study was conceived by PM. All authors contributed to the experimental design. LCor, IL, CM, XH, MF and FVH performed experiments. LCor, IL, CM, MF, KW, XH, LCoc and PM analyzed and interpreted the experimental data. LCor and PM wrote the manuscript. All authors critically reviewed and contributed to the manuscript.

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.