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SECRETION OF HEPATITIS C VIRUS REPLICATION INTERMEDIATES DAMPENS TOLL-LIKE RECEPTOR 3 ACTIVATION IN HEPATOCYTES

Oliver Grünvogel[a],Ombretta Colasanti[a], Ji-Young Lee[a], Volker Klöss[b], Sandrine Belouzard[c], Anna Reustle[a*], Katharina Esser-Nobis[a#], Jasper Hesebeck-Brinckmann[a], Pascal Mutz[a,i], Katrin Hoffmann[d], Arianeb Mehrabi[d], Ronald Koschny[e], Florian W. R. Vondran[f,g], Daniel Gotthardt[e$], Paul Schnitzler[h], Christoph Neumann-Haefelin[i], Robert Thimme[i], Marco Binder[j], Ralf Bartenschlager[a,j], Jean Dubuisson[c], Alexander H. Dalpke[b], Volker Lohmann[a]

Corresponding Author: Dr. Volker Lohmann, Department of Infectious Diseases, Molecular Virology, University of Heidelberg, Im Neuenheimer Feld 345, 69120 Heidelberg, Germany.

volker.lohmann@med.uni-heidelberg.de

Affiliations: Department of Infectious Diseases, Molecular Virology, University of Heidelberg, Heidelberg, Germany[α]; Department of Infectious Diseases, Medical Microbiology and Hygiene, University Hospital Heidelberg, Heidelberg, Germany[b]; Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 - UMR 8204 - CIIL- Centre d'Infection et d'Immunité de Lille, F-59000 Lille, France[c]; Department of General-, Visceral- and Transplantation Surgery, University Hospital Heidelberg, Heidelberg, Germany[d]; Department of Gastroenterology, Infectious Diseases and Intoxication, University Hospital Heidelberg, Heidelberg, Germany[e], Regenerative Medicine and Experimental Surgery (ReMediES), Department of General, Visceral and Transplant Surgery, Hannover Medical School, Hannover, Germany[f]; German Center for Infection Research (DZIF), partner site Hannover-Braunschweig, Hannover, Germany[g]; Department of Infectious Diseases, Virology, University of Heidelberg, Heidelberg, Germany[h]; Department of Medicine II, Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg[i]. Division of Virus-Associated Carcinogenesis (F170), German Cancer Research Center (DKFZ), Heidelberg, Germany[j].

Present address: Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany*; Department of Immunology, University of Washington, Seattle, Washington, USA[#]; Mediteo GmbH, Heidelberg, Germany[$]

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Short Title: HCV dsRNA secretion and TLR3 activation

Abbreviations: DMV, double-membrane vesicle; dsRNA, double-stranded RNA; EVs, extracellular vesicles; HCV, Hepatitis C virus; HCVcc, cell culture derived HCV; i.c., intracellular; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated protein 5; MMV, multi-membrane vesicle; MVB, multivesicular body; PRR, pattern recognition receptor; RIG-I, retinoic-acid inducible gene I; TLR3, toll-like receptor 3

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ABSTRACT

Background and aims

Hepatitis C virus (HCV) infections most often result in chronic outcomes, although the virus constantly produces replication intermediates, in particular double-stranded RNA (dsRNA), representing potent inducers of innate immunity. We aimed to characterize the fate of HCV dsRNA in hepatocyte cultures to identify mechanisms contributing to viral persistence in presence of an active innate immune response.

Methods

Various hepatocyte based cell culture models for HCV were analyzed for induction of innate immunity, secretion of viral positive/negative strand RNA and viral replication using different quantification methods and microscopy techniques. Expression of pattern recognition receptors was reconstituted in hepatoma cells by lentiviral transduction.

Results

HCV infected cells secrete substantial amounts of viral positive and negative strand RNA in extracellular vesicles (EVs), towards the apical and basolateral domain of hepatocytes. Secretion of negative strand RNA was independent from virus production and viral RNA secreted in EVs contained higher relative amounts of negative strands, indicating that mainly viral dsRNA is released. A substantial part of viral replication complexes and dsRNA was found in the endosomal compartment/multivesicular bodies, suggesting that secretion of HCV replication intermediates is mediated by the exosomal pathway. Block of vesicle release in HCV-positive cells increased intracellular dsRNA levels and led to an increased activation of toll-like receptor 3 (TLR3), inhibiting HCV replication.

Conclusion

Our results demonstrate that part of the HCV dsRNA intermediates are released from infected cells in EVs attenuating activation of TLR3. This mechanism represents a novel strategy to dampen intracellular innate immune responses, potentially contributing to the establishment of persistence.

Keywords: exosomes, escape

INTRODUCTION

Hepatitis C virus (HCV) presents a global health problem, as it persistently infects about 1 % of the world's population, and about 75% of all HCV infections will progress to a chronic stage[1]. HCV is a positive-strand RNA virus of the *flaviviridae* family, and primarily replicates in human hepatocytes. Upon infection, viral proteins induce formation of membranous replication organelles, consisting primarily of typical double membrane vesicles (DMVs), the presumed site of viral RNA replication, and multi-membrane vesicles (MMVs)[2]. HCV forms a double-stranded RNA (dsRNA) replication intermediate during its replication process, and persistently HCV-infected cells constantly produce new DMVs[3]. However, only about 30% of replication complexes are considered to actively support RNA replication in HCV-infected cells[4]. The fate of inactive replication complexes remains unknown.

Viral dsRNA in general is a major pathogen associated molecular pattern (PAMP) inducing innate immune responses. Hepatocytes express several pattern recognition receptors (PRRs) for detection of dsRNA, including the cytoplasmic sensors retinoic-acid inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5)[5], and the endosomal toll-like receptor 3 (TLR3)[6]. However, HCV counteracts the activation of these sensors by proteolytic cleavage of the mitochondrial antiviral signaling protein (MAVS), efficiently inhibiting the production of interferon (IFN) and interferon-stimulated genes (ISGs)[7]. In the TLR3 signaling pathway, the HCV protease NS3/4A cleaves the adaptor TIR domain-containing adapter molecule 1 (TRIF)[8], yet the efficiency of inhibiting TLR3-induced upregulation of IFNs and ISGs by this mechanism is still discussed controversially[6,9].

HCV-infected cells not only secrete regular infectious virions, but also infectious genomic RNA in exosomes, which are a subpopulation of extracellular vesicles (EVs)[10,11]. These vesicles with a diameter of 50-150 nm are formed by budding into the outer limiting membrane of the multivesicular body (MVB), a sorting vehicle of late endosomal origin[12]. The MVB content can then be degraded by fusion with a lysosome[13], a process in which the content could come into contact

with endo-lysosomal TLR3. On the other hand, if the MVB fuses with the plasma membrane, the intraluminal vesicles are released to the exterior as exosomes, transporting proteins, mRNAs and miRNAs to mediate cell-cell communication[14]. The release of HCV particles is linked to the exosomal release pathway[15,16], but HCV RNA is also released in exosomes in the absence of viral structural proteins[10,11]. HCV RNA released in exosomes can have a dual function, as it can activate innate immune cells, such as plasmacytoid dendritic cells (pDCs), to trigger production of antiviral IFN-α[10] or it can propagate the infection to naïve cells, while being shielded from neutralization[11,17].

Here, we determined the contribution of the late endosomal / exosomal RNA release pathway to the fate of HCV replication complexes and replication intermediates, and evaluated the potential impact on the activation of PRRs in HCV-infected cells. We found that dsRNA indeed was secreted from HCV-positive cells. Blocking of dsRNA release strongly increased the activation of TLR3 and its antiviral effects on HCV replication. Secretion of dsRNA therefore represents a novel immune evasion mechanism employed by HCV to dampen the activation of innate immune responses within the infected cell.

MATERIAL AND METHODS

Unless specified below, detailed information about used materials and methods can be found in the supplementary materials and methods section.

Cell Culture

All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum, non-essential amino acids (Life Technologies, Darmstadt, Germany), 100 U/ml penicillin and 100 ng/ml streptomycin (Life Technologies) and cultivated at 37°C and 5% CO_2. Huh7-Lunet cells and Huh7-Lunet-CD81high cells have been described before[18], as well as the Con1 subgenomic replicon clone 9-13 (gt 1b), cell lines with persistent reporter replicons of genotype 2a (LucubineoJFH1)[19] and HAV replicon cell lines[20]. Cells were kept under selection pressure by addition of 1mg/ml G418 (Geneticin, Life Technologies),

1µg/ml puromycin (Sigma-Aldrich, Steinheim, Germany) or 5µg/ml blasticidin (Sigma-Aldrich). Clones 15 and 1SC3 of the HepG2-CD81 cell line were described recently[21], and were grown on semi-permeable support (Corning) in William's medium supplemented with 2mM glutamax, 10 ng/ml gentamicin and 1% DMSO to induce cell polarity development.

Primary Human Hepatocytes (PHH)

Liver samples were obtained either after partial liver resection for medical reasons or from organ donors when the liver was considered unsuitable for transplantation. Primary human hepatocytes (PHH) were isolated as described before[22,23]. Tissue donors gave written informed consent for the experimental use of their specimens. The protocol was approved by the ethics commission of Hannover Medical School (#252-2008). Ethical approval further is covered with reference number # S-161/2007. Isolated PHH were cultured in Williams E medium (Life Technologies), supplemented with 10% FCS (Seromed, heat inactivated), 50 U/ml penicillin / 50 µg/ml streptomycin antibiotics, 50 µM hydrocortisone and 5 µg/ml insulin (SAFC, Sigma Aldrich), and incubated at 37° C and 5% CO_2.

Exosome Isolation

For Extracellular vesicle (EV)-isolation, cells were grown in EV-depleted medium, which was generated by ultracentrifugation of fetal bovine serum at 110,000 g for 16h at 4°C. EVs were isolated using Exo-spin Exosome Purification Kit (Cell Guidance Systems, Cambridge, UK) according to manufacturer's protocol, with the addition of a filtration step with 0.22 µm filters after the final pre-clearing centrifugation.

Statistical analysis

Independent biological replicates are denoted with n-numbers. To test for significance, two-tailed paired t-test or Welch's test was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

RESULTS

HCV RNA secreted in extracellular vesicles is enriched for negative strand

HCV genomic RNA has been described to be secreted in exosomes[10,11]. We therefore hypothesized that these EVs might also contain HCV negative strand RNA, indicative for the secretion of viral replication intermediates. We isolated EVs from HCV infected Huh7-Lunet CD81high cells [18] (strain Jc1, Fig. 1A), which likely also contained co-purified infectious virus due to similar biophysical properties[10] and investigated the composition of HCV RNA in these vesicles relative to intracellular viral RNA by use of a strand-specific RT-qPCR assay (Fig. S1A,B). Since negative strand RNA was used as a quantitative marker for dsRNA, we ensured that our assay reached a sensitivity of minus strand detection comparable to a previously published protocol ([24], Fig. S1C).We found substantial amounts of negative strands in the EV fraction of infected cells, with a far lower relative ratio of positive to negative strands than intracellularly (3.8 vs 29.8, respectively (Fig. 1B)). Secretion of negative strand RNA started at about 24h after infection and showed similar kinetics as the secretion of positive strand RNA (Fig. 1C). We further analyzed HCV infected primary human hepatocytes (PHH) to confirm the secretion of viral replication intermediates in a more physiological cell culture system. Amounts of secreted RNA were lower in PHH than in HCV-infected Huh-7 cells and varied between donors, which is most likely due to lower replication of HCV, because of the strong counteraction by innate immune responses in PHH[25]. However, negative strand RNA secretion in EVs again recapitulated secretion kinetics of positive strand (Fig. 1D, S1D, E). Importantly, also in PHH relative amounts of negative strand HCV RNA in EVs were strongly increased compared to intracellular levels (Fig. 1E). We next used cell lines harboring persistent subgenomic HCV replicons, lacking the coding region of structural proteins (Fig. 1A), to assess the impact of virion production on secretion of negative strand RNA. Cells harboring replicons of different genotypes secreted similar amounts of negative strand RNA in EVs as HCV infected cells, but lower levels of positive strands, likely due to the absence of virions (Fig. 1F,G), which co-purify with EVs due to similar biophysical properties[10]. Therefore, negative strand RNA was clearly enriched in EVs as compared to levels in HCV-infected

and replicon cells (Fig. 1H). We further assessed the kinetics of RNA release from replicon cells, which reached steady state levels 15 min. after medium exchange (Fig. S1F). This result suggested that, albeit steady state RNA levels are moderate compared to intracellular viral RNA, a higher amount of negative strand RNA might be secreted due to rapid secretion kinetics and high turnover. Overall these data argued for enrichment and secretion of substantial amounts of dsRNA in EVs.

Since subgenomic replicons allowed the analysis of HCV RNA secretion independent from virion production we mainly used this model throughout this study. We first verified that secreted HCV RNA was protected by membranes as deduced from RNAse resistance in the absence of detergent (Fig. S2A). We further verified that the exosomal fraction contained the marker CD63, as well as small amounts of NS5A (Fig. S2B), in line with a recent study[26], supporting the hypothesis that the secreted viral RNA might be contained in previous replication vesicles. The secretion of HCV RNA could be decreased by GW4869, an inhibitor of neutral sphingomyelinase 2, blocking exosome release as previously reported[10,27], as well as by knocking down Rab27a, an important host factor in the release pathway of exosomes (Fig. S2C-D)[28], with the combination of GW4869 and siRNA showing increased efficiency, probably due to targeting of different steps involved in the exosome release pathway. Knockdown of Rab27a was specific, as reconstitution of RAb27a expression rescued HCV RNA release (Fig. S2E).In addition, Rab27a KD had no detrimental effects on cell viability (Fig. S2F). In contrast to HCV, viral RNA was almost undetectable in extracellular vesicles purified from the supernatant of HAV replicon cells (Fig. S2G), suggesting that secretion of viral replication intermediates in EVs is not common for all hepatotropic RNA viruses.

Altogether, these data suggested that HCV replication intermediates are secreted in EVs/exosomes, as previously shown for positive strand RNA[10]. Moreover, HCV negative strand RNA was highly enriched in the secreted fraction as compared to intracellular levels.

HCV replication intermediates are detected in the late endosomal compartment

We next analyzed the intracellular localization of viral replication organelles in replicon cells. Since secretion of viral RNA in EVs was blocked by inhibitors of exosome release we particularly focused on detection of viral replication organelles and dsRNA in MVBs and endosomal compartments.

We used a replicon harboring an insertion of mCherry in nonstructural protein (NS)5A (Fig. 2A), and implemented correlative light and electron microscopy to assess the ultrastructure of NS5A-positive regions (Fig. 2B,C). About 25% of NS5A positive regions showed no distinct substructures (Fig. 2D, other). About 35% of NS5A-mCherry signals were associated with cytoplasmic regions representing the viral replication organelle, as judged by the presence of DMVs and MMVs (Fig. 2C, area 2; Fig. 2D, DMVs). In addition, NS5A signal could be detected at lipid droplets (LD), the endoplasmatic reticulum (ER), and the mitochondria (Fig. 2D, Fig. S3). Surprisingly, more than 20% of all NS5A positive regions per cell were in fact multivesicular bodies (MVBs) or lysosomes (Fig. 2C, area 3; Fig. 2D). Most of these NS5A-positive MVBs also contained DMVs and MMVs (Fig. 2E, Fig. S3). This observation was in line with our hypothesis that secreted HCV replication intermediates originate from the MVB, as previously reported for genomic RNA secreted in exosomes[10]. We next assessed the colocalization of dsRNA with markers of the MVB/lysosomes using immunofluorescence-based staining, confocal microscopy and 3D reconstruction. Indeed, dsRNA was detected in CD63-positive compartments and in association with the late endosomal/lysosomal marker Lamp1, adding up to about 20% and 15% of all dsRNA signals in HCV-positive cells, respectively (Fig. 2F-H).

To further support the mechanistic link between secretion of HCV replication intermediates and the late endosome/MVB, we blocked the exosomal release pathway by knockdown of Rab27a. While release of HCV RNA in EVs was decreased in Rab27a-KD cells (Fig. 3A), intracellular dsRNA levels were significantly increased (Fig. 3C), which was confirmed by strand-specific RT-qPCR, using negative strand as a marker of dsRNA (Fig. S4). More specifically, knockdown of Rab27a increased the dsRNA amounts associated with CD63+ exosome release compartments and Lamp1+ lysomomal

compartments (Fig. 3E, G). In contrast, when lysosomal acidification was inhibited by Bafilomycin A (BafA), significantly higher amounts of HCV RNA were secreted (Fig. 3B), while the number of intracellular dsRNA positive signals decreased (Fig. 3D). Consequently, also a lower number of dsRNA signals was found associated with CD63+ and Lamp1+ compartments.

(Fig. 3F, H).

Altogether, our data suggested that HCV dsRNA, engulfed in vesicular structures, entered the endosomal pathway, from where it was either secreted in EVs or entered the lysosomal compartment.

TLR3 can be activated within HCV-positive cells

As dsRNA is a strong inducer of innate immune responses, we next studied the impact of dsRNA release on the activation of intracellular PRRs. Hepatocytes constitutively express the cytosolic dsRNA sensors RIG-I and MDA5, as well as the endosomal dsRNA sensor TLR3[8]. Indeed, ISG expression was strongly induced in PHH upon transfection of poly(I:C) and by delivery of poly(I:C) to the supernatant, thus only accessible for uptake in endo-lysosomes and exclusively activating TLR3 (Fig. 4A, PHH). In contrast, Huh7-Lunet cells barely responded to either of these stimuli (Fig. 4A, empty), indicating low expression of the PRRs. We therefore reconstituted expression of individual dsRNA sensor, leading to rescue of dsRNA recognition upon poly(I:C) stimulation (Fig. 4A, S5A). We chose IFIT1 mRNA levels as a robust measure of ISG responses induced by all PRRs[5], whereas IFNβ mRNA was not detectable in Huh7 cells expressing TLR3 (Fig. S5C) and not induced in PHH by addition of poly(I:C) to the supernatant (Fig. S5D), as reported[9].

We next assessed ISG induction by each PRR and its corresponding impact on HCV replication by transfection of a subgenomic HCV reporter replicon (Fig. 4B). Expression of RIG-I resulted in an early and transient induction of IFIT1 mRNA levels (Fig. 4C), likely by the incoming RNA and blocked by cleavage of MAVS at later time points[7]. Expression of MDA5 did not result in induction of ISG response and neither RIG-I nor MDA5 expression had any significant impact on HCV replication (Fig.

4D). In contrast, presence of TLR3 induced strong expression of IFIT1 mRNA, starting 48 h after transfection of the replicon (Fig. 4C), correlating with impaired viral replication, with a 24 h delay (Fig. 4D). TLR3 expression was similar to PHH (Fig. S5B), indicating that TLR3 activation was likely not an artefact of overexpression. The induction of ISG responses and its negative impact on HCV replication upon TLR3 activation was further confirmed in cell lines containing a persistent reporter replicon (Fig. 4E). Transient transduction with a lentiviral vector expressing TLR3 induced a strong ISG response and decreased HCV replication also in this case (Fig. 4F,G). In contrast, HCV infection did result in a delayed, slightly less pronounced activation of TLR3[5], which did not result in a clear reduction of HCV replication in the time frame of the experiment (Fig. S6), either due to differences in the kinetics of dsRNA production and/or by the increased levels of secreted dsRNA found upon infection, dampening TLR3 response.

TLR3 is typically activated by endocytic uptake of extracellular dsRNA and it has been shown that HCV can cause TLR3 activation in uninfected cells by extracellular transfer of dsRNA[29]. We performed co-culture experiments to investigate the contribution of secreted dsRNA to trans-activation of neighboring cells versus the induction of TLR3 upon intracellular transfer of replication intermediates. We either transfected subgenomic HCV replicons into Huh7-Lunet-TLR3 cells and co-cultured them with the same cell type lacking TLR3, resulting in a strong induction of IFIT1 mRNA expression (Fig. 4H, bottom). In contrast, the same replicons transfected into Huh7-Lunet cells lacking TLR3 and co-cultured with Huh7-Lunet-TLR3 only caused minimal IFIT1 induction (Fig. 4H, top). This result showed that TLR3 is activated mostly within HCV-positive cells and that transfer of dsRNA played a minor role.

In summary, TLR3 elicits antiviral ISG responses in HCV-positive cells, despite the presence of viral protease. The activation of TLR3 did not depend on extracellular but on intracellular transfer of dsRNA.

HCV dsRNA secretion decreases activation of TLR3

TLR3 resides in the late endosome, in which HCV dsRNA replication intermediates were detected. Therefore, we investigated the effect of blocking dsRNA release in EVs on the activation of TLR3.

Huh7-Lunet-TLR3 or empty control cells were transfected with HCV reporter replicon RNA and release of EVs was inhibited by knockdown of siRab27a (Fig. S7A) and/or treatment with GW4869, which in combination was most efficient in preventing HCV RNA secretion (Fig. S2C). GW4869 treatment itself had no impact on poly(I:C)-induced TLR3 activation (Fig. S7B), and showed no detrimental effects on cell viability (Fig. S7C, D).

Neither treatment affected ISG induction or HCV replication in control cells (Fig. 5A, C), or impacted HCV replication in cells expressing RIG-I or MDA5 (Fig. S8). In addition, replication of DENV reporter replicons was not influenced by blocking EV secretion in any of the dsRNA-sensor-expressing cell lines, while HAV replication was inhibited by Rab27a knockdown independently of PRR expression (Fig. S9). In contrast, siRab27a and GW4869 treatment both lead to higher ISG induction at earlier time points in TLR3-expressing cells upon HCV replication than the non-treated controls (Fig. 5B). Consequently, the increased induction of ISGs resulted in a significant decrease in HCV replication upon blocking of EV secretion (Fig. 5D). This result indicated that secretion of HCV dsRNA indeed reduced the TLR3 response induced by HCV replication, albeit we cannot formally rule out the possibility that other exosomal content might additionally regulate TLR3 response. Increased induction of ISG expression was also observed in HCVcc-infected cells expressing TLR3 upon EV release inhibitor treatment (Fig. 5E,F). However, the release of infectious virus is at least partially dependent on the exosome release pathway[15,16], therefore Rab27a knockdown and GW4869 treatment resulted in decreased secretion of infectious virus and slightly increased intracellular RNA levels in presence and absence of TLR3, precluding an unequivocal interpretation of the replication data (Fig. S10).

In summary, release of HCV dsRNA in EVs decreased the activation of TLR3 in HCV-positive cells. Therefore, secretion of dsRNA represents an escape mechanism dampening cell intrinsic innate immune responses.

HCV negative strand RNA is secreted at both sides of polarized hepatocytes and is lowly abundant in serum of chronically-infected patients

Our work so far demonstrated that secretion of dsRNA by HCV infected cells weakens intracellular TLR3 responses, thus facilitating viral replication. However, previous work has shown that secreted HCV positive strand RNA might activate pDCs to produce IFN, counteracting HCV replication[10]. Since hepatocytes are highly polarized cells, EVs are secreted not only at the basolateral, sinusoidal side, but also at the apical side, into bile canaliculi. Therefore, we wondered whether all HCV negative strand RNA indeed is secreted to the basolateral surface, potentially capable of activating pDCs, or at least in part to the apical side, thereby avoiding activation of innate immune responses. We therefore employed a cell culture system based on HepG2 cell clones with polarization capabilities. These recently described cell clones are the so far only cell lines permissive for HCV infection that can be polarized on transwell inserts to form an intact apical and basolateral compartment[21]. Only this experimental setting allowed collecting and analyzing media from both compartments separately. After infection with HCV, these cells secrete infectious HCV particles as well as EVs on both their apical and their basolateral side. We isolated RNA from the apical and basolateral supernatants from two independent cell clones infected with HCVcc and analyzed their HCV RNA composition. Both cell clones secreted the majority of HCV RNA at the basolateral side (Fig. 6A, 6C). This correlated with the preferential secretion of infectious HCV particles at the basolateral side[21]. However, HCV RNA from apical supernatants was enriched for negative strand RNA in both clones (Fig. 6B, D), suggesting a substantial secretion of dsRNA at the apical side.

Finally, we analyzed the abundance of positive and negative strand RNA in EVs purified from the serum of 5 patients with chronic HCV infection. Interestingly, we could detect significant amounts of

negative strand RNA in all sera, albeit at far lower and highly variable ratios compared to cell culture supernatants (Fig. 6E), indicating that serum predominantly contains infectious virus and that the majority of dsRNA secreted in EVs does not reach the systemic circulation. Since a previous study has observed increased levels of dsRNA in the liver of chronic HCV patients depending on their IL28B genotype[24], it will be interesting to analyze in future studies whether this correlates with negative strand levels in the serum.

In summary, polarized HepG2 cells infected with HCV secreted negative strand RNA partly at their apical side, away from systemic circulation. In line with these results, we could detect negative strand viral RNA in the sera of HCV infected patients, but to a far lower extent than in cell culture.

In conclusion, our results demonstrate that HCV dsRNA enters the endosomal compartment, where it is either released in EVs, or activates TLR3. Release of dsRNA replication intermediates attenuates TLR3 responses, thereby representing a mechanism to avoid excessive activation of cell intrinsic innate immunity. In addition, dsRNA replication intermediates are partly secreted at the apical side of polarized hepatocytes, away from access to systemic circulation and detection by professional immune cells. Our findings add another mechanism to the repertoire of immune evasion strategies employed by HCV.

DISCUSSION

This study shows that viral replication vesicles containing dsRNA enter the endosomal pathway and are either secreted in EVs via the MVB or transported into lysosomes, activating TLR3 (Fig. 7A). Blocking EV secretion by the MVB pathway strongly increases TLR3 activation, mounting a stronger antiviral response, suggesting that secretion of dsRNA and lysosomal degradation are governed by the same mechanism (Fig. 7B). Therefore, secretion of dsRNA replication intermediates in EVs alleviates induction of TLR3 responses in HCV-infected hepatocytes, representing a novel viral immune escape mechanism.

Our results demonstrate that substantial amounts of HCV negative strand are secreted in extracellular vesicles, likely exosomes, by HCV infected cells. The interpretation that these negative strands indeed are dsRNA replication intermediates engulfed in replication vesicles is supported by the high ratio of negative to positive strand RNA found in the secreted fraction and presence of NS5A positive DMVs/MMVs, as well as dsRNA in late endosomes/MVBs. Secretion of these viral replication vesicles is independent from presence of structural proteins; however, egress of a subfraction of infectious virions also follows this route[15,16]. A mechanistic link between both processes is suggested by an increased efficiency of viral positive strand RNA release in exosomes in the context of full viral replication[30], which was also found in our analysis of negative strand RNA secretion. The detailed mechanism underlying transport to the endosome and secretion of viral replication intermediates in EVs, as well as infectious virus, remains to be determined. Previous studies suggested involvement of the ESCRT complex[10,31]. Alternatively, autophagy has been suggested to play a role in generation of viral replication complexes as well as release of infectious HCV virions[16,32]. We can furthermore not rule out that replication vesicles are directly formed at the MVB.

HCV is known to activate TLR3, which is expressed constitutively in hepatocytes[6]. Conversely, HCV is reported to cleave the TLR3 adaptor TRIF to blunt this pathway[8], whereas other reports claim that the TLR3 pathway is not efficiently shut off during HCV replication[6,9]. Our data strongly support the latter studies, since selected replicon cells, by definition all containing replicating viral RNA and expressing the NS3/4A protease, remained fully susceptible to TLR3 signaling. Our results further suggest that TLR3 is mostly activated upon intracellular delivery of viral replication vesicles to the endosomal/lysosomal compartment within the HCV-replicating cell itself, and not by transfer of naked RNA to neighbouring cells[29], although we cannot formally rule out, that TLR3 is activated by autocrine secretion and re-entry of viral dsRNA. Overall TLR3 seems as important in detecting intracellular HCV RNA as the cytosolic sensors RIG-I and MDA5, since we only found a moderate, transient RIG-I activation at early time points, compared to a sustained TLR3 response starting at 48h

after infection. For several reasons, it is currently difficult to evaluate to which extent TLR3 indeed contributes to high ISG expression in hepatocytes even in chronic patients[33-35]. First, ISG responses mounted by cytosolic PRRs, TLR3 and paracrine Type I or Type III IFNs are very similar and hard to differentiate [9]. Second, in PHH, early secretion of IFNs, likely induced by RIG-I activation, masks possible late TLR3 responses. In future studies, the contribution of TLR3 to intrahepatic ISG responses against HCV might be addressed upon availability of fully immunocompetent animal models by selective knockdown/knockout of PRRs.

Secretion of HCV RNA in exosomes/EVs has been described in previous studies as an antiviral sensing process activating innate immune responses in DCs, NK cells and monocytes[10,36,37], inducing interferon responses and inhibiting viral replication. However, activation requires close cell to cell contact. In addition, several studies indicate that elevated ISG expression in the liver of chronic HCV patients is found primarily in HCV positive hepatocytes and their surrounding cells, suggesting that intracellular and not immune cell-derived innate immune responses might substantially contribute to ISG induction[34,38,39]. Our work now shows that mainly double stranded HCV RNA is secreted in EVs, dampening intracellular detection by TLR3. We further demonstrate significant secretion of dsRNA, but not virions, into the apical compartment, towards the bile duct, using a new model of polarized hepatocytes in transwells[21]. This result is in line with previous studies detecting HCV RNA in bile[40] and stool[41] of chronic HCV patients and strongly argues for an escape mechanism evolved by HCV, reducing antiviral innate immune responses in infected cells and preventing detection of secreted RNA by the cellular innate immune response. Interestingly, we found no evidence for substantial secretion of viral RNA in case of HAV and Dengue virus replicon cells, nor did blocking of exosome release inhibit viral replication, as was the case for HCV. Since HAV and Dengue virus cause only acute infections it is tempting to speculate that secretion of dsRNA was established as a disposal mechanism by HCV allowing persistent replication in the same hepatocyte.

In summary, we report a novel immune escape mechanism employed by HCV to decrease the activation of TLR3 in HCV-infected cells by secreting dsRNA. This mechanism underlines the importance of TLR3-dependent responses in the course of HCV infection and might contribute to the establishment and maintenance of chronic HCV infections.

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Author names in bold designate shared co-first authorship.

FIGURE LEGENDS

Figure 1: Strand-specific detection of HCV RNA in cells and EVs

(A) Schematic of the HCV genome used for infection (upper) and the subgenomic replicon used to select stable cell lines (lower). HCVcc was derived from the genotype 2a (chimera Jc1), while subgenomic replicons were of genotype 1b (isolate Con1) and genotype 2a (isolate JFH-1). (B) Huh7-Lunet-CD81high cells were infected with HCVcc at MOI = 0.1. After 72h, HCV positive and negative strand RNA was quantified in intracellular (i.c.) RNA and RNA from EVs by strand-specific RT-qPCR. Bars represent mean values with range from independent experiments (n=2). (C) Huh7-Lunet-CD81high cells were infected with HCVcc at MOI = 0.1. At the respective time points, positive and negative strand RNA was quantified in EVs isolated from infected cells by strand-specific RT-qPCR. Shown are mean values with range from independent experiments (n=2). (D) PHH were infected with HCVcc at MOI = 0.1. At the respective time points, positive and negative strand RNA was quantified in EVs isolated from infected cells by strand-specific RT-qPCR. Shown are mean values with range from two wells of one representative donor. (E) HCV positive and negative strand RNA was quantified in i.c. RNA and RNA from EVs isolated from HCV-infected PHH 72h after infection by strand-specific RT-qPCR. Bars represent mean values with SD from 3 independent donors (n=3). (F),

(G) HCV positive and negative strand RNA was quantified in i.c. RNA and RNA from EVs isolated from HCV replicon cells (F: genotype 1b, isolate Con1; G: genotype 2a, isolate JFH-1) by strand-specific RT-qPCR. Bars represent mean values with SD from independent experiments (n=3). (H) The data from (B), (E), (F), and (G) was used to calculate the ratio of negative to positive HCV strands. Bars represent mean values with SD. Statistical significance was assessed by Welch's test.

Figure 2: Detection of HCV replication complexes in the late endosomal compartment

(A) Schematic of HCV NS5A-mCherry reporter replicon RNA used for CLEM. (B),(C) Huh7-Lunet cells were electroporated with replicon RNA and 72 hours after electroporation, CLEM was performed to correlate the NS5A-mCherry signal with the ultrastructure detected by EM. Scale bar represents 200 nm. Asterisks indicate DMVs. Areas in (C) were chosen to display the most commonly observed NS5A positive ultrastructural features, DMVs (area 2) and MVB with DMV (area 3, 4). (D) NS5A-positive areas were scored for correlation with cellular structures. LD: lipid droplet. Bars represent mean values with SD of 5 cells from 2 different dishes. (E) NS5A-mCherry-positive MVB/lysosomes or unmarked MVB/lysosomes from the same cells were scored for their content. If a DMV or MMV was present in the structure, it was scored as positive. Bars represent mean values with SD of 5 cells from 2 independent experiments. Statistical significance was assessed by paired t test. (F), (G) HCV subgenomic replicon cell lines were stained with antibodies against dsRNA (J2) and late endosomal markers. Scale bar represents 5 µm. (H) Percentage of dsRNA signal colocalizing with late endosomal markers was determined after 3D reconstruction of HCV replicon cells. 8 cells from two independent experiments were analyzed. Error Bars represent SD.

Figure 3: Impact of EV release block on dsRNA levels

HCV replicon cell lines were transfected with siRNA against Rab27a, or left untreated. 40 hours after transfection, untreated cells were treated with 1 µM Bafilomycin A (BafA). 8h later, EVs were isolated from the supernatants, and cells were processed for immunofluorescence and microscopy. (A) HCV RNA copies in EVs were detected by RT-qPCR. Bars represent mean values with SD from

554 independent experiments (n=4). (B) HCV RNA copies in EVs were detected by RT-qPCR. Bars
555 represent mean values with SD from independent experiments (n=3). (C) dsRNA in Rab27a-
556 transfected cells was detected by IF staining for dsRNA. 10 cells were analyzed from 2 independent
557 experiments. . (D) dsRNA in BafA-treated cells was detected by IF staining for dsRNA. 11 cells were
558 analyzed from 2 independent experiments. (E, G) dsRNA colocalized with CD63 (E) or Lamp1 (G) was
559 detected in siRab27a- or siNT transfected cells by IF staining and confocal microscopy. At least 11
560 cells were analyzed from 2 independent experiments. (F, H) dsRNA colocalized with CD63 (F) or
561 Lamp1 (H) was detected in BafA-treated or control cells by IF staining and confocal microscopy. At
562 least 11 cells were analyzed from 2 independent experiments. Statistical significance was assessed
563 by Welch's test.

564 **Figure 4: Activation of TLR3 by HCV replication**

565 (A) Huh7-Lunet cells overexpressing PRRs and PHH were transfected with 100 ng/well poly(I:C) or
566 stimulated with 50 µg/ml poly(I:C) in the supernatant (SN) for endosomal delivery. 8h after
567 stimulation, total RNA was isolated and IFIT1 mRNA levels were quantified by RT-qPCR as readout
568 for PRR activation. Bars represent mean values with SD from technical triplicates from one
569 experiment (n=1). (B) Schematic representation of HCV luciferase reporter replicons. (C) Huh7-Lunet
570 cells stably transduced with respective PRRs were electroporated with HCV luciferase reporter
571 replicon RNA. At the respective timepoints, RNA was isolated and IFIT1 mRNA was quantified by RT-
572 qPCR. Values shown are mean values with SD from independent experiments (n=3). HCV replication
573 was monitored in parallel by luciferase assay. (D) HCV replication in Huh7-Lunet empty/RIG-
574 I/MDA5/TLR3 cells was determined by luciferase assay. Mean values with SD from independent
575 experiments are shown (n=3). (E) Schematic representation of selectable HCV reporter replicons. (F),
576 (G) HCV reporter replicon cell lines were transduced with lentiviruses coding for TLR3 or a control
577 vector at MOI = 5. 48 hours after transduction, IFIT1 mRNA was measured by RT-qPCR to determine
578 TLR3 activation and luciferase assay was performed to determine replication of HCV. (F) IFIT1 mRNA
579 levels. Bars represent mean values with range from independent experiments (n=2). (G) HCV

replication was determined by luciferase assay. Bars represent mean values with SD from independent experiments (n=3). (H) Huh7-Lunet-empty or -TLR3 cell lines were electroporated with HCV reporter replicon RNA and co-cultured with mock-electroporated Huh7-Lunet-TLR3 or -empty cells, respectively. 72h after electroporation, RT-qPCR was performed to determine IFIT1 mRNA levels. Values were normalized a co-culture of mock-electroporated Huh7-Lunet-empty and -TLR3 cells. Bars represent mean values with SD from independent experiments (n=4). Statistical significance was assessed by Welch's test.

Figure 5: Impact of EV release on TLR3 activation

(A),(B),(C),(D) Huh7-Lunet-empty or -TLR3 cell lines were electroporated with HCV reporter replicon RNA and indicated siRNAs. 4h after electroporation, cells were treated with 10 µM GW4869 or DMSO. At the indicated timepoints, total RNA was isolated and IFIT1 mRNA was quantified by RT-qPCR. HCV replication was monitored by luciferase activity. Small panels below the graphs show blow-ups of IFIT1 mRNA levels at 48h ((A),(B)) or luciferase activity at 96h after electroporation ((C),(D)). (A) IFIT1 mRNA levels in Huh7-Lunet-empty cells. (B) IFIT1 mRNA levels in Huh7-Lunet-TLR3 cells. (C) HCV replication as determined by luciferase assay in Huh7-Lunet-empty cells. (D) HCV replication as determined by luciferase assay in Huh7-Lunet-TLR3 cells. (E), (F) Huh7-Lunet-CD81high-empty or -TLR3 cells were transfected with siRNAs against Rab27a or a control siRNA. 16 hours after transfection, cells were infected with HCVcc at MOI=5. 6h after infection, treatment with GW4869 or DMSO was started. At the indicated timepoints, total RNA was isolated and IFIT1 mRNA was quantified by RT-qPCR. IFIT1 mRNA levels of empty cells are shown in (E), IFIT1 mRNA levels of TLR3 cells are shown in (F). All values shown are mean values with SD from independent experiments (n=3 for A,B,C,D; n=2 for E,F). Statistical significance was assessed by Welch's test.

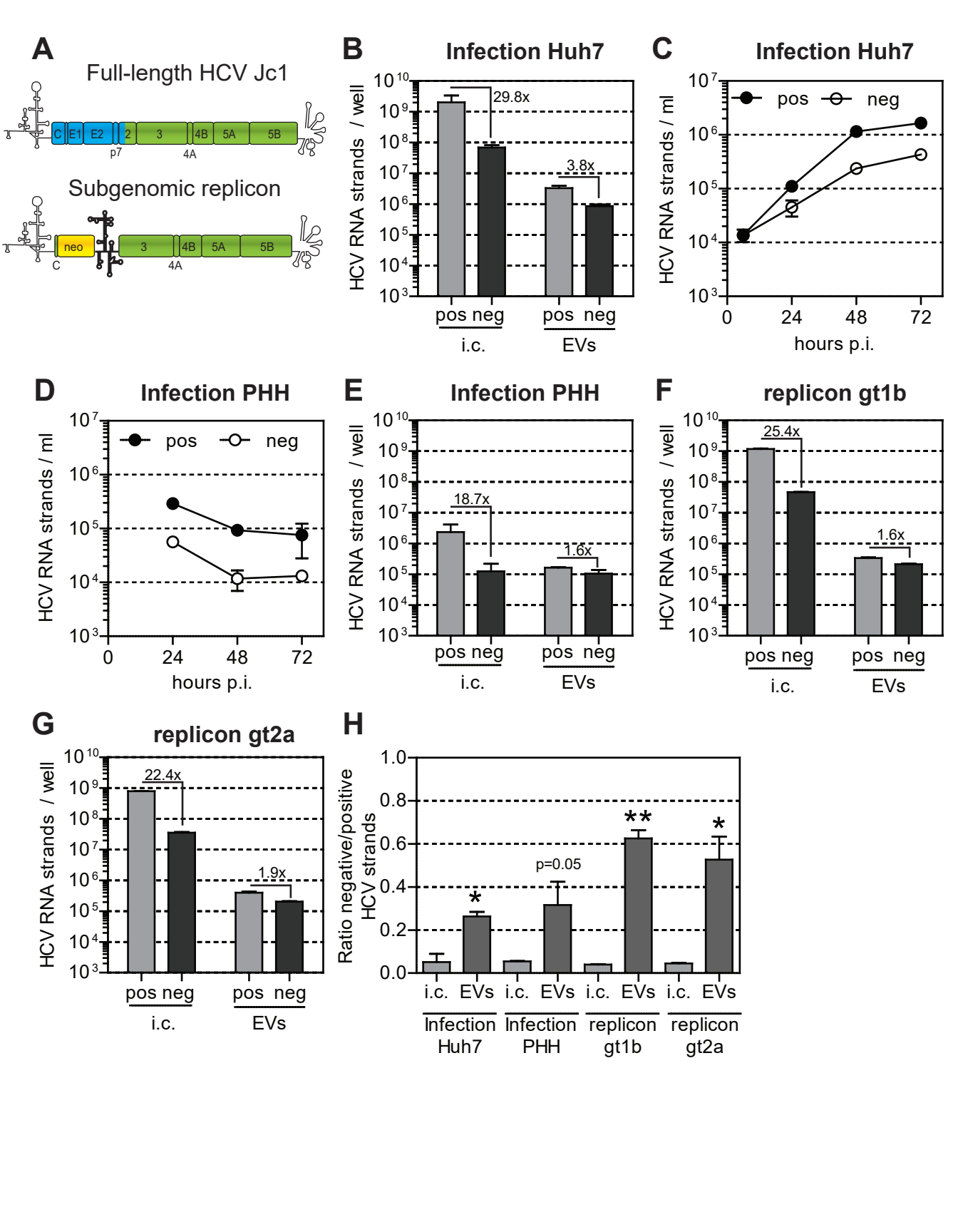
Figure 6: Polarized secretion of HCV RNA

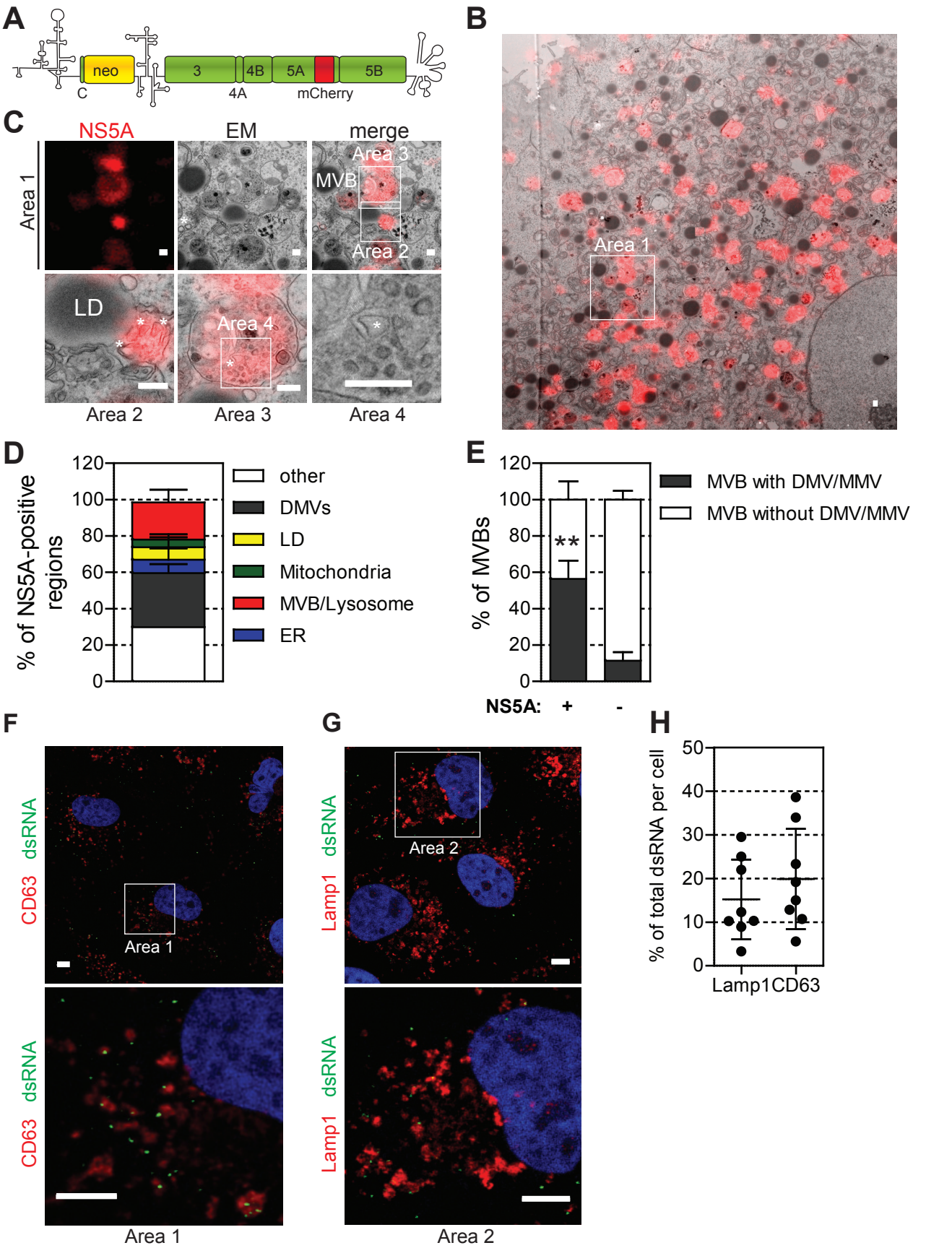
HepG2-based cell clones Clone 15 and 1SC3 were polarized on transwell dishes and infected with HCVcc. 48 hours after infection, RNA was isolated from apical and basolateral supernatants. (A,C)

HCV positive and negative strand RNA was detected by strand-specific RT-qPCR in supernatants from Clone 15 and Clone 1SC3, respectively. (B,D) Ratio of negative to positive stand HCV RNA in supernatants from Clone 15 and Clone 1SC3, respectively. All bars represent mean values with SD from independent experiments (n=3 for Clone 15, n=2 for clone 1SC3). (E) EVs were isolated from 1 ml of serum from chronic HCV patients, and used for strand-specific RT-qPCR for HCV RNA. Shown are mean values of technical triplicates with SD from each donor. Numbers indicate ratios between positive and negative strand HCV RNA. Statistical significance was assessed by Welch's test.

Figure 7: Model of novel immune attenuation mechanism.

(A) HCV dsRNA from the replication complexes is transported to the LE/MVB. From there, it can be degraded in the lysosome, secreted at the apical or basolateral compartments in EVs or activate TLR3 in the late endosome, inducing the induction antiviral ISGs that inhibit HCV replication. (B) Blocking of EV release results in increased dsRNA levels, enhancing TLR3 activation. This leads to a stronger production of antiviral ISGs, and a stronger inhibition of HCV replication.


A
Full-length HCV Jc1
C
E1
E2
2
3
4B
5A
5B
p7
4A
Subgenomic replicon
neo
C
B
Infection Huh7
HCV RNA strands / well
29.8x
3.8x
pos neg
pos neg
i.c.
EVs
C
Infection Huh7
HCV RNA strands / ml
pos
neg
hours p.i.
D
Infection PHH
HCV RNA strands / ml
pos
neg
hours p.i.
E
Infection PHH
HCV RNA strands / well
18.7x
1.6x
pos neg
pos neg
i.c.
EVs
F
replicon gt1b
HCV RNA strands / well
25.4x
1.6x
pos neg
pos neg
i.c.
EVs
G
replicon gt2a
HCV RNA strands / well
22.4x
1.9x
pos neg
pos neg
i.c.
EVs
H
Ratio negative/positive HCV strands
p=0.05
i.c. EVs i.c. EVs i.c. EVs i.c. EVs
Infection Huh7
Infection PHH
replicon gt1b
replicon gt2a


A
neo
3
4B
5A
5B
C
4A
mCherry
B
C
NS5A
EM
merge
Area 1
Area 3
MVB
Area 2
LD
Area 4
Area 2
Area 3
Area 4
D
% of NS5A-positive regions
120
100
80
60
40
20
0
other
DMVs
LD
Mitochondria
MVB/Lysosome
ER
E
% of MVBs
**
MVB with DMV/MMV
MVB without DMV/MMV
NS5A: + -
F
CD63 dsRNA
Area 1
CD63 dsRNA
Area 1
G
Lamp1 dsRNA
Area 2
Lamp1 dsRNA
Area 2
H
% of total dsRNA per cell
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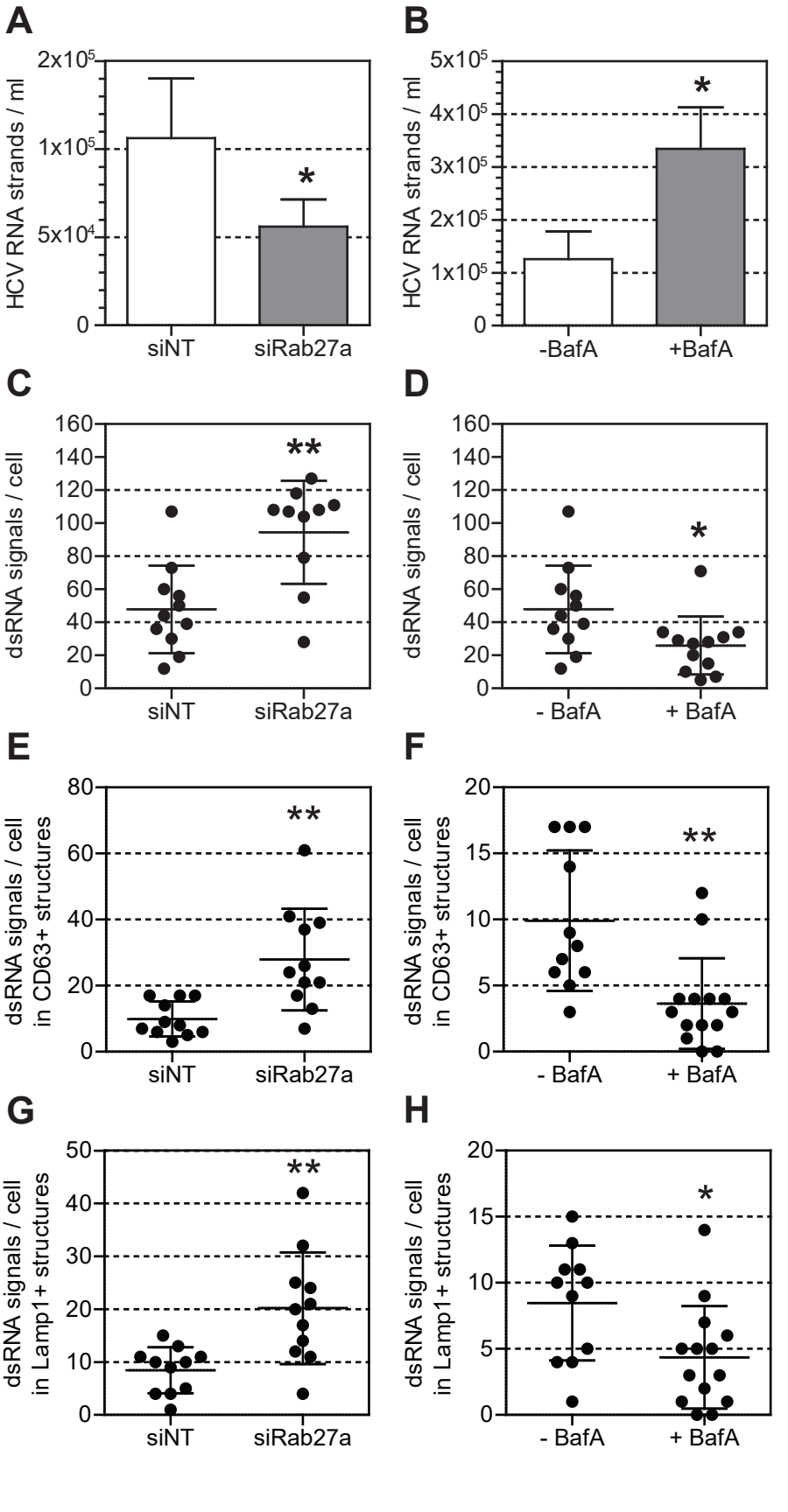
40
30
20
10
0
Lamp1
CD63


A
HCV RNA strands / ml
2x10^5
1x10^5
5x10^4
0
*
siNT
siRab27a
B
HCV RNA strands / ml
5x10^5
4x10^5
3x10^5
2x10^5
1x10^5
0
*
-BafA
+BafA
C
dsRNA signals / cell
160
140
120
100
80
60
40
20
0
**
siNT
siRab27a
D
dsRNA signals / cell
160
140
120
100
80
60
40
20
0
*
- BafA
+ BafA
E
dsRNA signals / cell in CD63+ structures
80
60
40
20
0
**
siNT
siRab27a
F
dsRNA signals / cell in CD63+ structures
20
15
10
5
0
**
- BafA
+ BafA
G
dsRNA signals / cell in Lamp1+ structures
50
40
30
20
10
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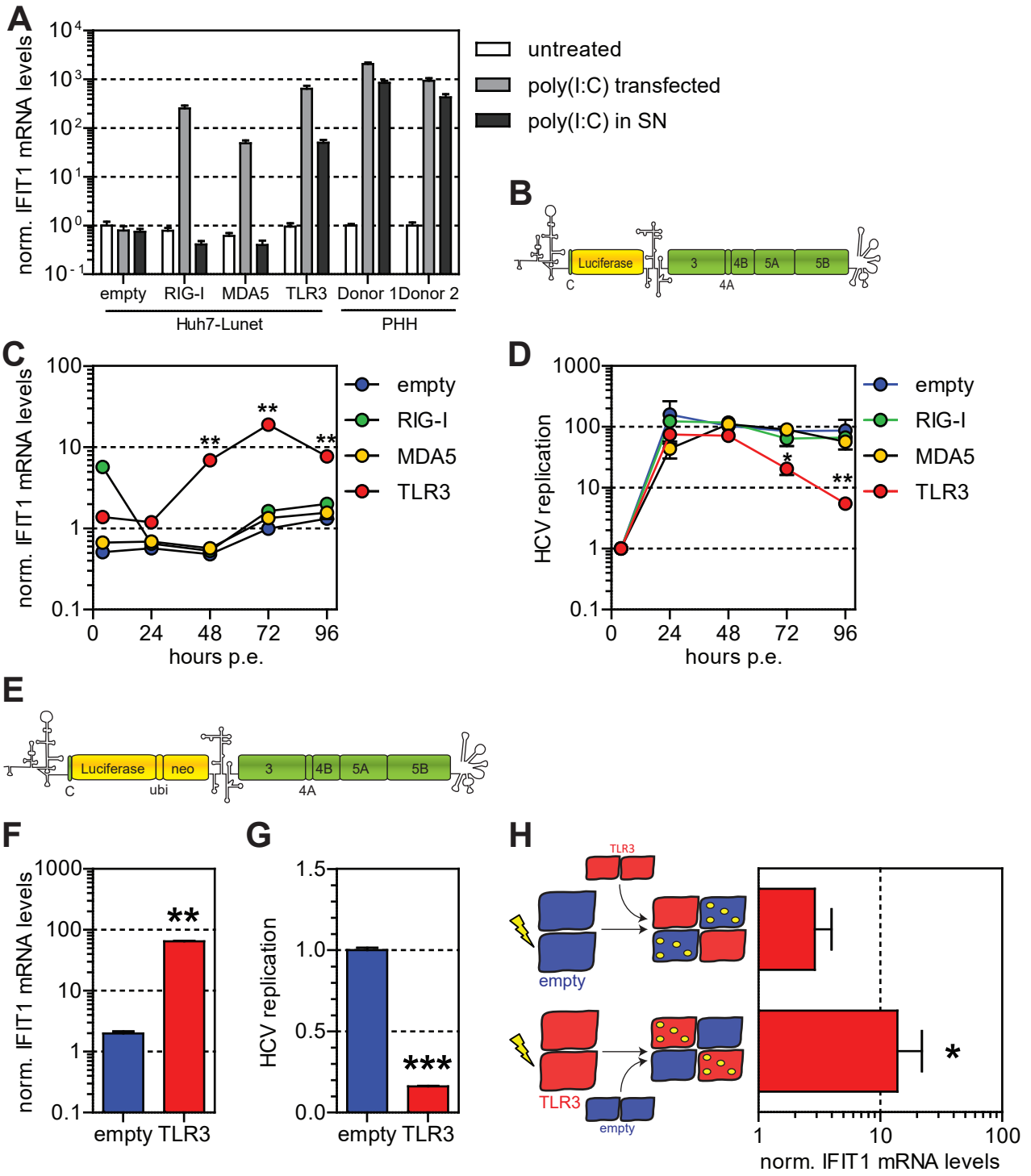
0
*
- BafA
+ BafA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norm. IFIT1 mRNA levels
untreated
poly(I:C) transfected
poly(I:C) in SN
empty
RIG-I
MDA5
TLR3
Donor 1
Donor 2
Huh7-Lunet
PHH
B
Luciferase
3
4B
5A
5B
C
4A
C
norm. IFIT1 mRNA levels
hours p.e.
empty
RIG-I
MDA5
TLR3
D
HCV replication
hours p.e.
E
Luciferase
neo
ubi
3
4B
5A
5B
C
4A
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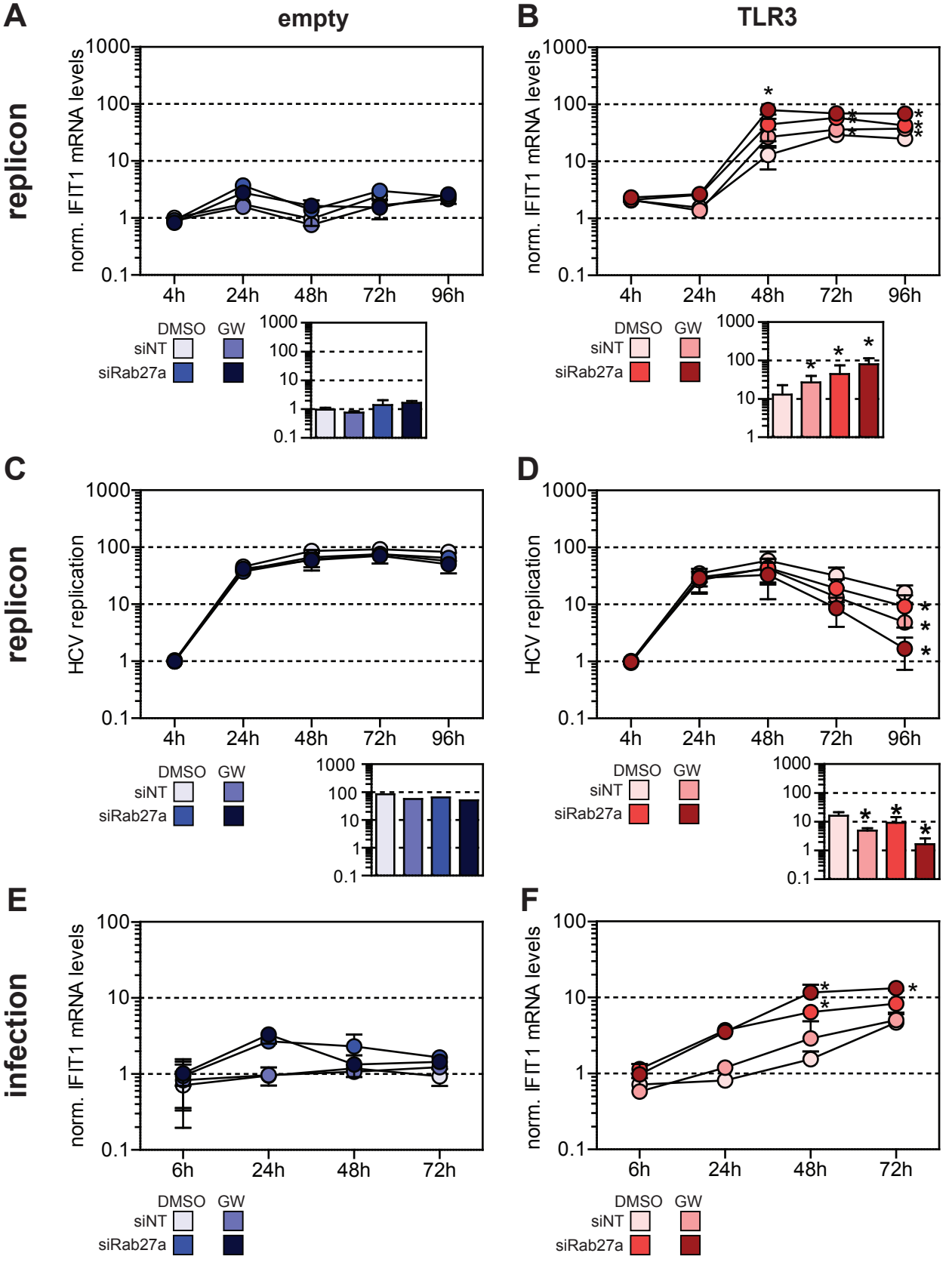
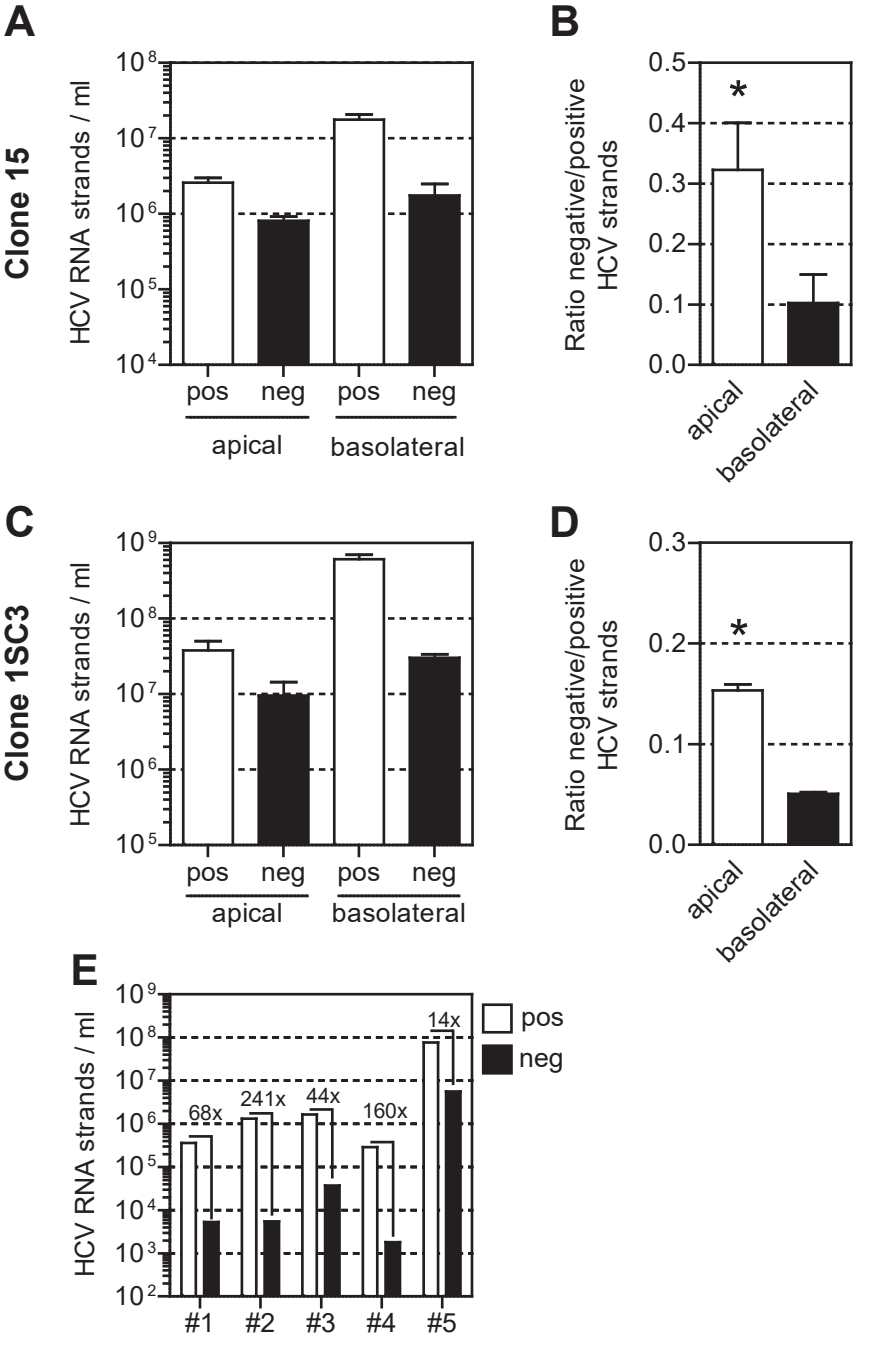
empty TLR3
G
HCV replication
empty TLR3
H
TLR3
empty
norm. IFIT1 mRNA levels


A
empty
B
TLR3
replicon
norm. IFIT1 mRNA levels
1000
100
10
1
0.1
4h
24h
48h
72h
96h
DMSO
GW
siNT
siRab27a
C
D
HCV replication
E
F
infection
6h


A
Clone 15
HCV RNA strands / ml
pos
neg
pos
neg
apical
basolateral
B
Ratio negative/positive HCV strands
*
apical
basolateral
C
Clone 1SC3
HCV RNA strands / ml
pos
neg
pos
neg
apical
basolateral
D
Ratio negative/positive HCV strands
*
apical
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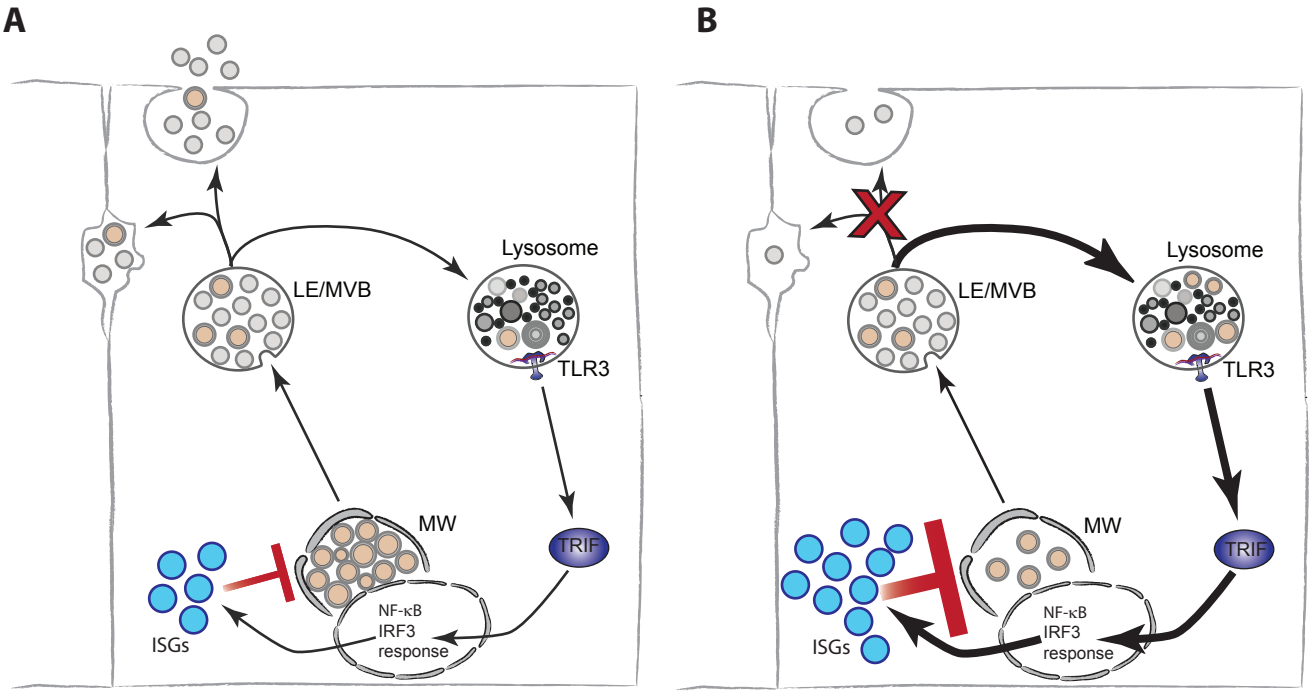
#1
#2
#3
#4
#5



Normal state **EV release blocked**