Research Article
Viral Hepatitis

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Comparison of HAV and HCV infections *in vivo* and *in vitro* reveals distinct patterns of innate immune evasion and activation

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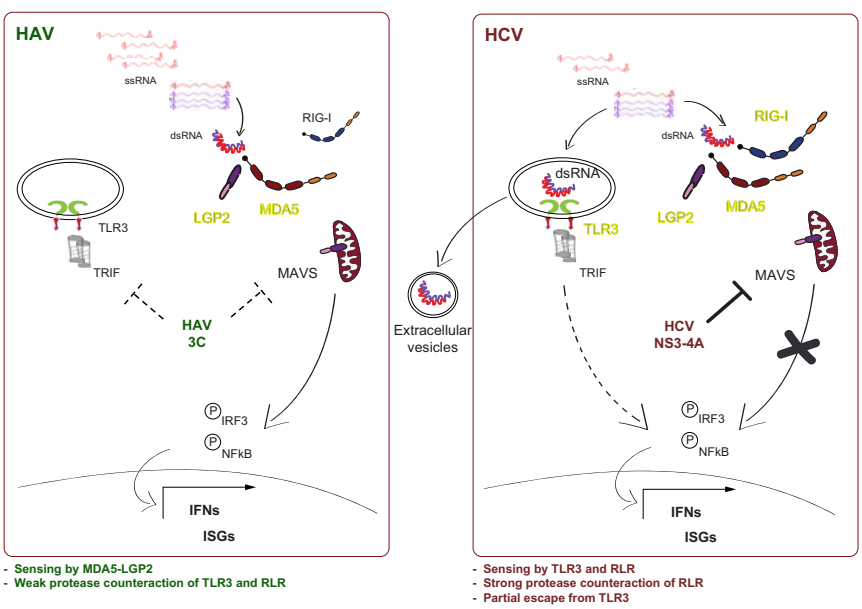
Graphical abstract



Highlights

- Our data indicate that HAV induces an innate immune response *in vitro* and *in vivo*.
- HAV 3C precursor proteases do not fully abolish the host response.
- The HCV NS3-4A protease instead disrupts the RLR pathways, but cannot cleave TRIF and has no impact on TLR3 responses.
- In systems devoid of adaptive immunity, HAV and HCV induce innate immune responses to similar degrees.

Impact and Implications

Understanding the mechanisms that determine the differential outcomes of HAV and HCV infections is crucial for the development of effective therapies. Our study provides insights into the interplay between these viruses and the host innate immune response *in vitro* and *in vivo*, shedding light on previously controversial or only partially investigated aspects. This knowledge could tailor the development of new strategies to combat HCV persistence, as well as improve our understanding of the factors underlying successful HAV clearance.

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Comparison of HAV and HCV infections *in vivo* and *in vitro* reveals distinct patterns of innate immune evasion and activation

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Background & Aims: Hepatitis A virus (HAV) infections are considered not to trigger innate immunity *in vivo*, in contrast to hepatitis C virus (HCV). This lack of induction has been imputed to strong interference by HAV proteases 3CD and 3ABC. We aimed to elucidate the mechanisms of immune activation and counteraction by HAV and HCV *in vivo* and *in vitro*.
Methods: Albumin-urokinase-type plasminogen activator/severe combined immunodeficiency (Alb/uPA-SCID) mice with humanised livers were infected with HAV and HCV. Hepatic cell culture models were used to assess HAV and HCV sensing by Toll-like receptor 3 and retinoic acid-inducible gene I/melanoma differentiation-associated protein 5 (RIG-I/MDA5), respectively. Cleavage of the adaptor proteins TIR-domain-containing adapter-inducing interferon-β (TRIF) and mitochondrial antiviral-signalling protein (MAVS) was analysed by transient and stable expression of HAV and HCV proteases and virus infection.
Results: We detected similar levels of interferon-stimulated gene induction in hepatocytes of HAV- and HCV-infected mice with humanised liver. In cell culture, HAV induced interferon-stimulated genes exclusively upon MDA5 sensing and depended on LGP2 (laboratory of genetics and physiology 2). TRIF and MAVS were only partially cleaved by HAV 3ABC and 3CD, not sufficiently to abrogate signalling. In contrast, HCV NS3-4A efficiently degraded MAVS, as previously reported, whereas TRIF cleavage was not detected.
Conclusions: HAV induces an innate immune response in hepatocytes via MDA5/LGP2, with limited control of both pathways by proteolytic cleavage. HCV activates Toll-like receptor 3 and lacks TRIF cleavage, suggesting that this pathway mainly contributes to HCV-induced antiviral responses in hepatocytes. Our results shed new light on the induction of innate immunity and counteraction by HAV and HCV.

Introduction

Hepatitis A virus (HAV) and hepatitis C virus (HCV) are both hepatotropic, (+)-sense RNA viruses causing remarkably different infection outcomes. Although HAV infections always result in a self-resolving, acute infection, HCV infection mainly evolves to chronic, persistent hepatitis. The reasons underlying these strikingly opposed outcomes are incompletely understood.

The replication cycles of HCV and HAV share distinct similarities, characterised by the formation of cytoplasmic membranous replicase complexes[1,2] and the synthesis of a double-stranded (ds) RNA intermediate, which can potentially trigger the cytosolic RIG-I-like receptors (RLRs) retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) (reviewed in Xu *et al.*[3]), both sensing cytosolic dsRNA. The MDA5 response to several (+)RNA viruses was described to further depend on laboratory of genetics and physiology 2 (LGP2).[4] Both RLRs are involved in HCV sensing,[5] whereas MDA5 is the sole cytoplasmic pattern recognition receptor (PRR) for HAV.[6] Both receptors recruit the adaptor protein mitochondrial antiviral-signalling protein (MAVS) (reviewed in Xu *et al.*[3]) and activate a signalling cascade which culminates in the establishment of an antiviral state, based on interferon (IFN) production and expression of IFN-stimulated genes (ISGs). Moreover, HCV dsRNA was reported to trigger the endosomal PRR Toll-like receptor 3 (TLR3),[7,8] which also activates IFN responses through recruitment of the adaptor

Keywords: HAV; HCV; Innate immunity; Mice with humanised liver; Hepatocytes; dsRNA; TLR3; TRIF; MDA5; RIG-I; MAVS; LGP2.
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protein TIR-domain-containing adapter-inducing interferon-β (TRIF) (reviewed in Xu *et al.*[3]).

Previous studies identified various interference mechanisms counteracting the establishment of an antiviral state. HCV NS3-4A and HAV 3ABC proteases were shown to disrupt RLRs through MAVS cleavage.[3] Degradation of TRIF by HAV 3CD was reported[9] but remains controversial for HCV NS3-4A.[10,11] Alternative mechanisms of innate immune interference, involving different HAV proteins, were described as well.[3] In case of HCV, secretion of dsRNA was furthermore found to weaken TLR3 responses.[8]

Particularly for HAV, only limited sets of *in vivo* data are available. A study investigating intrahepatic innate immune response in HAV and HCV infected chimpanzees found low ISG induction in case of HAV, whereas HCV infection was associated to robust ISG expression,[12] comparable to what is found in the majority of chronic HCV patients.[13,14] Yet, chemokines are robustly increased in the serum of patients with acute hepatitis A (AHA).[15] Also, in the murine model productive HAV infection requires the absence of MAVS or type I IFN receptors,[16] suggesting a robust innate immune activation in this model, which cannot be overcome by HAV interference mechanisms.[17] Hence, it still remains unclear to which extent the various counteraction mechanisms reported in literature contribute to the infection outcome.

Here, we aimed at comprehensively clarifying how HAV and HCV trigger and counteract innate immunity in physiologically relevant *in vivo* and *in vitro* models. We show that HAV and HCV infections induce to a similar extent ISGs and IFNs in albumin-urokinase-type plasminogen activator/severe combined immunodeficiency. (Alb/uPA-SCID) mice with humanised liver, demonstrating that both viruses trigger innate immunity in the infected human hepatocytes. HAV sensing by MDA5 and LGP2 was the main driver of ISG induction, with only limited MAVS cleavage not causing interference. In contrast, we confirmed that HCV sensing by RIG-I and MDA5 is completely blocked by cleavage of MAVS; therefore, the ISG response induced by HCV in hepatocytes appears to primarily originate from TLR3. Consistently, we were not able to detect TRIF cleavage by HCV NS3-4A.

Materials and methods

Viruses

For HCV infection of Huh7.5 cells, strain Jc-1 was used. For infection of Alb/uPA-SCID mice with humanised liver HCV wild-type (WT) isolates from sera of an infected patient after liver transplantation (gt1b, strain GLT1[18]) or of strain mH77c, originating from chimpanzee serum, amplified in naïve liver chimeric mice (gt1a[19]), were used. For HAV, genotype IA WT strains were isolated from patient stool samples. Huh7.5, HepG2, and HepaRG cells were infected with HAV strain HM175/18f. The use of serum derived HCV and HAV WT from anonymous stool donors was approved by local ethics committees (ethics vote: S-677/2020, Heidelberg University, medical faculty and DGR n. 565 from 2/04/2014, Apulia Region, Italy).

PRR stimulation

For TLR3 exclusive stimulation, poly (I:C) high molecular weight (HMW; Invivogen, San Diego, CA, USA) was added to the supernatant of cells at 10 or 50 μg/ml. Six hours after stimulation, cells were harvested for RNA extraction and RT-qPCR. For stimulation of cytosolic PRRs (RIG-I, MDA5) and TLR3 as well, poly (I:C) was transfected into cells using Lipofectamine2000 (Life Technologies, Karlsruhe, Germany). For transfection of one well in a 24-well format, different dilutions from 0.001 to 1 μg poly (I:C) were incubated at room temperature with 0.1–0.5 μl Lipofectamine2000 reagent in 100 μl OptiMEM (Gibco) for 5 min, and then added to the cells. RNA was also isolated from these cells 6 h after transfection.

Statistical analysis

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

Results

Upregulation of ISGs and chemokines upon HAV and HCV infection of Alb/uPA-SCID mice with humanised liver

Little is known so far on the contribution of innate immunity to the mechanisms of clearance *vs.* persistence of HAV and HCV infections. Alb/uPA-SCID mice with humanised liver, which lack functional murine B and T lymphocytes,[20] are permissive for both viruses and therefore represent the only *in vivo* model allowing a side-by-side comparison of the cell intrinsic innate immune responses in absence of adaptive immunity. We used two different WT-virus variants each to exclude major strain-specific differences. For HCV we chose the prototype strain H77 (gt1a)[19] and a high-titre post-transplant serum of the gt1b strain GLT1.[18] Because of the lack of availability of well characterised HAV WT strains, we extracted virus from the stool samples of two anonymous donors and used cell-culture grown HM175/18f as a reference.

We found on average higher HAV RNA amounts in the liver compared with HCV (Fig. S1A), despite comparable repopulation efficiency with human hepatocytes (Fig. S1B). Single HAV and HCV positive cells were detected by immunohistochemistry (IHC) in all areas with human hepatocytes, in correlation to ISGs (interferon induced protein with tetratricopeptide Repeats 1 [IFIT1]) and chemokine (C-X-C motif chemokine ligand 10 [CXCL10]) signals, prevalently confined to infected cells (Fig. 1A and B; Fig. S1C and E), but no signals in uninfected mice (Fig. 1C). In one of the HCV GLT1-infected mice we detected lower IFIT1 levels in HCV-positive human hepatocytes by IHC, together with aggregated clusters of murine cells and CXCL10 positivity surrounding infected cells, suggesting an inflammatory response (Fig. S1D). We then measured common ISGs activated by viral dsRNA downstream of the main PRRs in hepatocytes[6,8] and detected in almost all cases substantial upregulation of ISGs and chemokines by RT-qPCR (Fig. 2A–H) upon both HAV and HCV infection. Lower innate immune induction by HAV HM175/18f (Fig. 2A, C, and F) correlated to its lower replication (Fig. S1A). Fluctuations among the different samples (Fig. 2A and C) were likely associated with different sacrification timings (Table S1) and the individual condition of the mice upon tissue engraftment.[20]

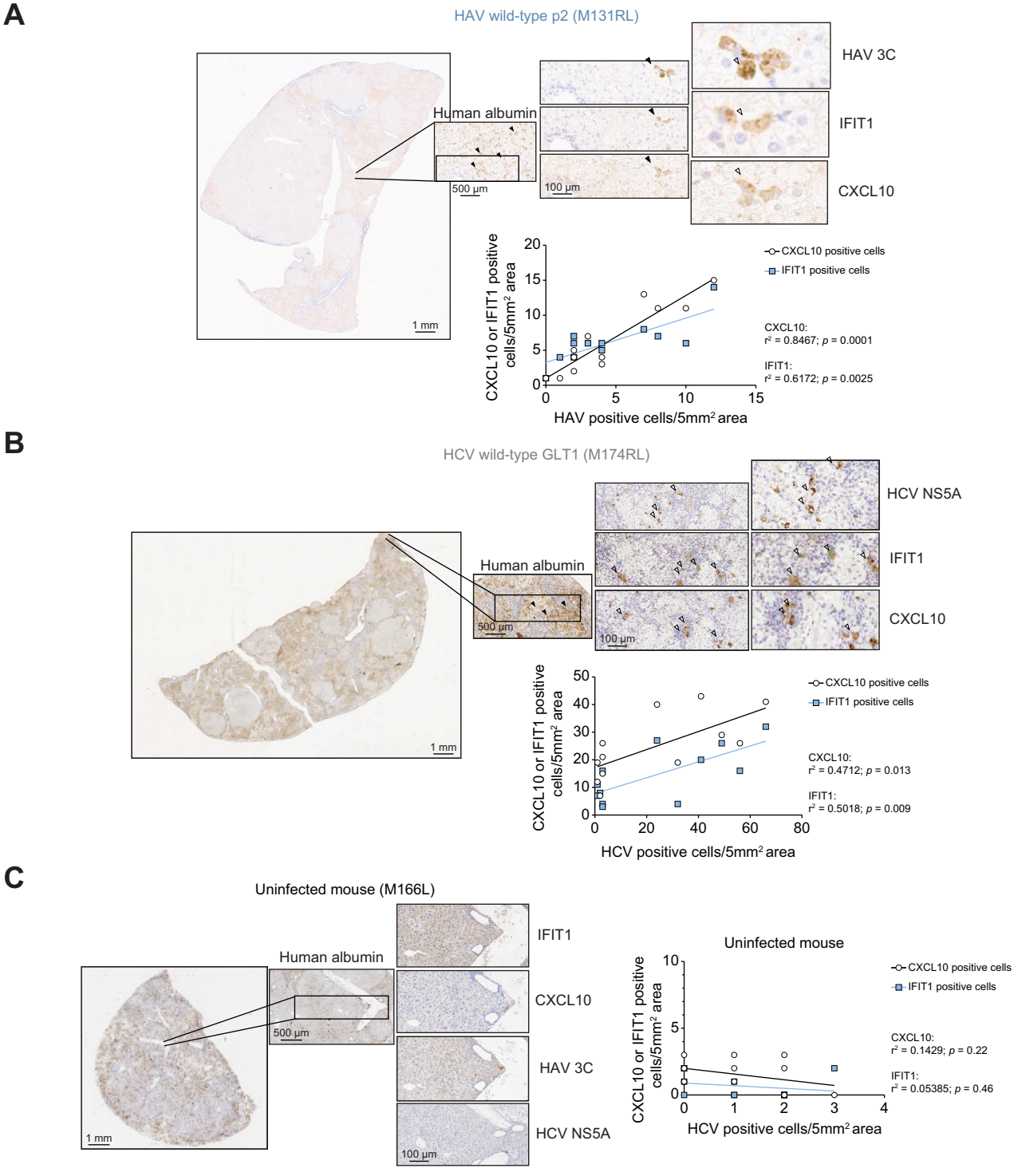

Fig. 1. Upregulation of ISGs and chemokines upon HAV and HCV infection of Alb/uPA-SCID mice. (A, B, C) Sections from the liver of Alb/uPA-SCID mice with humanised liver infected with wild-type HAV (A), HCV (B), or uninfected (C), and subjected to IHC, using human albumin, HAV, HCV, or ISG-specific antibodies, as indicated. Shown are representative examples with abundant albumin signal (A, B, C left and above panels). For each mouse, six albumin-rich view fields were quantified. Black arrowheads indicate a triple positive cell. Linear regression analysis was performed on CXCL10 or IFIT1 positive cells and HAV 3C or HCV NS5A positive cells, and statistical significance was assessed through Welch's unpaired *t* test (A, B, lower right panels). IHC, immunohistochemistry; ISG, IFN-stimulated gene; Alb/uPA-SCID, albumin (Alb)-urokinase plasminogen activator/severe combined immunodeficiency; ISGs, Interferon stimulated gene.

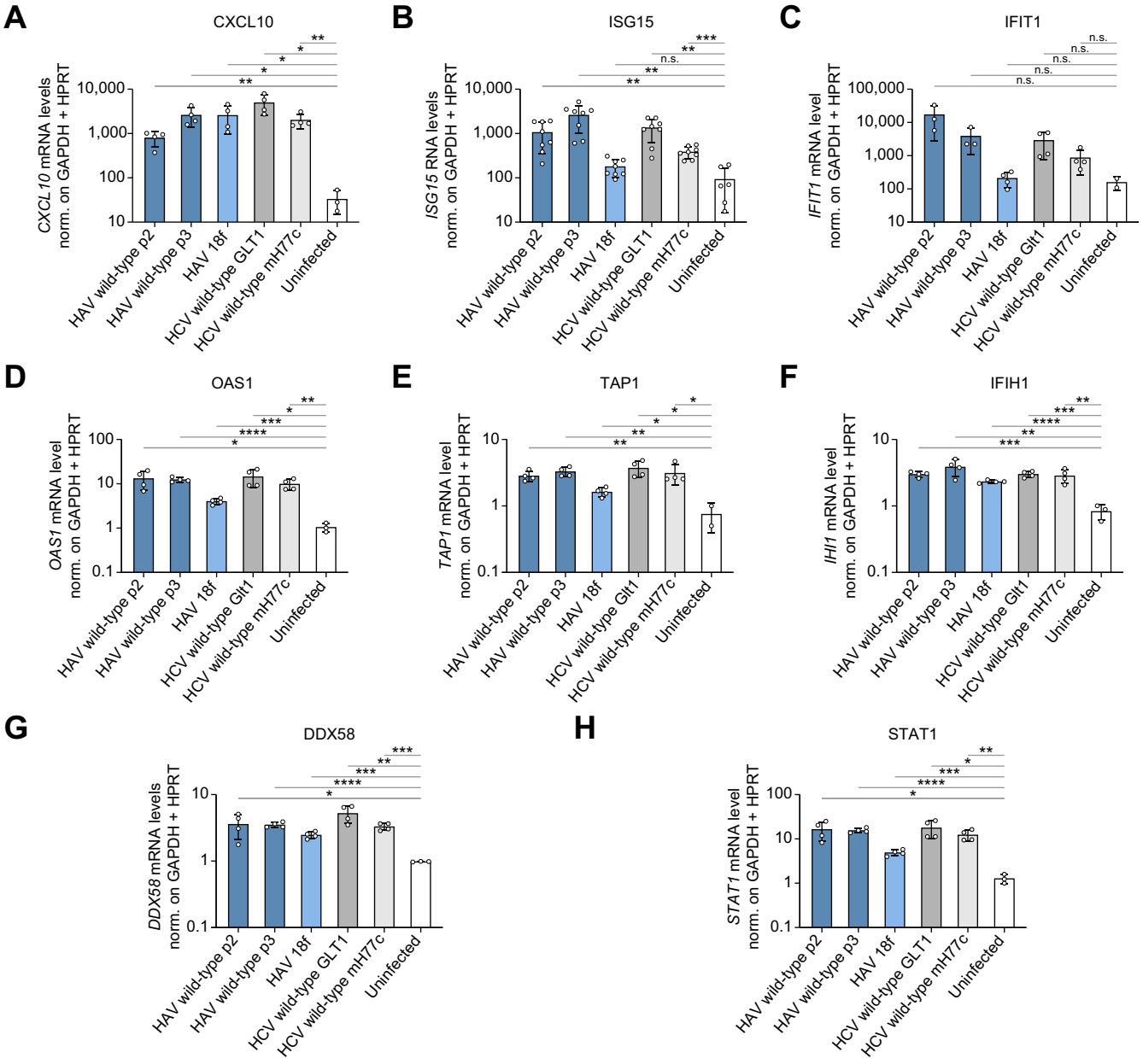

Fig. 2. ISG induction in livers of HAV or HCV infected Alb/uPA-SCID mice with humanised liver compared to non-infected controls. (A–H) Total RNA was isolated from liver of indicated mice and mRNA expression levels were quantified by a Taqman gene expression array. Statistical significance was assessed through Welch's unpaired *t* test.Alb/uPA-SCID, albumin-urokinase-type plasminogen activator/severe combined immunodeficiency; ISGs, Interferon stimulated gene.

Overall, these data suggested a substantial cell intrinsic innate immune activation upon both HAV and HCV infections in the livers of humanised mice devoid of adaptive immunity.

HAV does not trigger an innate immune response in Huh7 cells with reconstituted TLR3, MDA5, and RIG-I expression

Next, we sought to determine the source of the strong innate immune induction detected by us in the HAV-infected human liver chimeric mice. To allow direct comparison with HCV we used Huh7 cells, permissive for both viruses. All Huh7 variants lack detectable expression of TLR3 and MDA and contain little, or non-functional, RIG-I in the case of subclone Huh7.5.[21] Therefore, reconstitution of PRRs is required to restore their reactivity (Fig. 2A and B). To validate induction of cell intrinsic innate immune responses by authentic viral replication intermediates we used Huh7 cells selected for the presence of HAV or HCV subgenomic

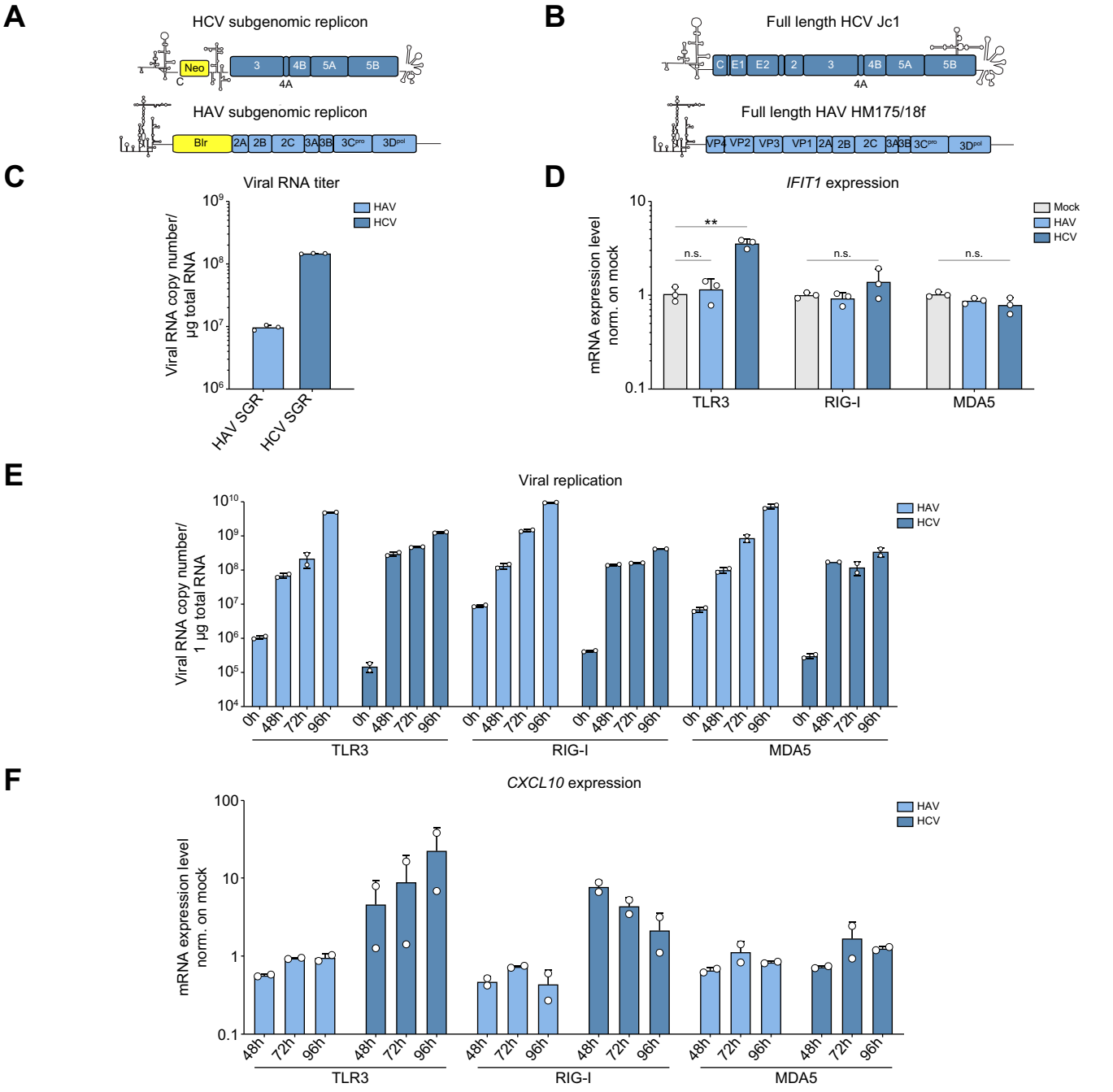

Fig. 3. Innate immune response to HAV and HCV in Huh7 cells with reconstituted TLR3, MDA5, and RIG-I expression. (A, B) Schematic of selectable subgenomic HCV/HAV replicons (A) and HCV/HAV genomes (B). (C) HAV or HCV RNA was quantified by RT-qPCR in cells harbouring persistent subgenomic replicons. (D) PRRs, as indicated, were transiently expressed in persistent replicon cell lines using lentiviral vectors. Twenty-four hours after transduction *IFIT1* mRNA was quantified by RT-qPCR and normalised to *GAPDH* expression. Huh7.5 cells stably expressing either TLR3, RIG-I, or MDA5, were infected with HAV (HM175/18f) or HCV (Jc1), respectively. *CXCL10* mRNA (F), and viral RNA (E), were quantified as indicated. *CXCL10* mRNA levels are normalised to *GAPDH* expression and shown as fold expression relative to uninfected cells. Statistical significance was assessed by Welch's unpaired *t* test. (C, E, F). All values shown are mean values with SD from independent experiments (n = 2). MDA5, melanoma differentiation-associated protein 5; PRR, pattern recognition receptor; RIG-I, retinoic acid-inducible gene I; SGR, subegnomic replicon; TLR3, Toll-like receptor 3. (This figure appears in color on the web.)

replicons[22] (Fig. 3A), in which abundant viral RNA was detectable (Fig. 3C) and transiently expressed TLR3, RIG-I, or MDA5 (Fig. 3D). *IFIT1* expression was induced in HCV replicon cells expressing TLR3, but not RIG-I and MDA5, in line with previous results,[8] whereas no induction by HAV was observed for any of the PRRs (Fig. 3D). In addition, we investigated the innate immune response in the context of infection with full-length genomes (Fig. 3B) on Huh7.5 cells stably expressing individual PRRs (Fig. S2A and B). HAV and HCV replicated with similar efficiency in all (Fig. 3E), including

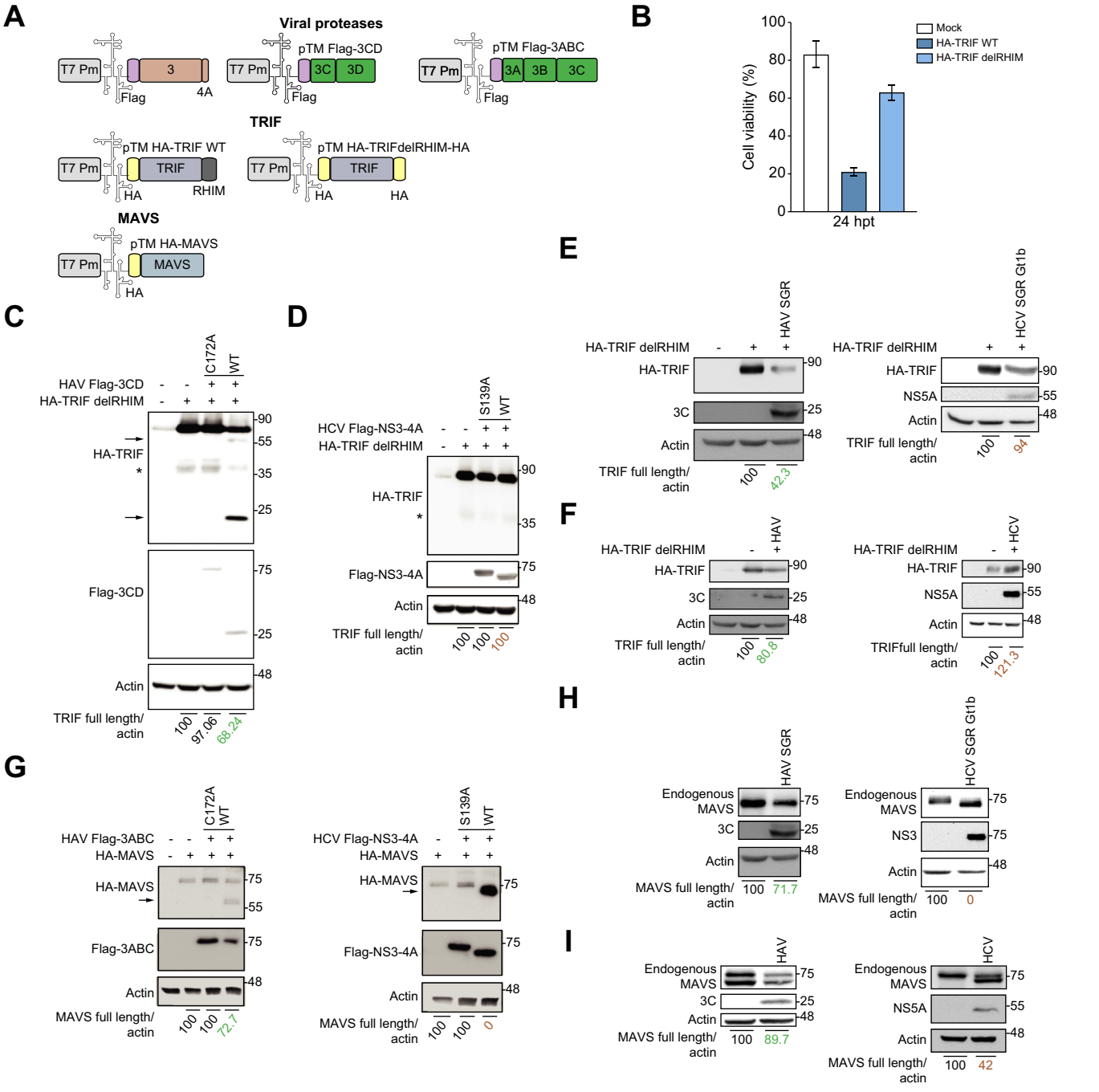

Fig. 4. Analysis of TRIF and MAVS cleavage by HCV and HAV proteases. (A) Schematic of the expression vectors used for transfection experiments. (B) Huh7-Lunet T7 cells were transfected with a pTM vector encoding TRIF WT or TRIF delRHIM. WST-1 assay was used to determine cell viability after 24 h. Shown are mean values and SD of triplicates from 1 experiment. (C, D, G) Huh7-Lunet T7 cells were co-transfected with plasmids encoding the indicated WT or mutant proteases and adaptor proteins and harvested 16 h after transfection. Arrows indicate specific cleavage products. Caspase-mediated TRIF cleavage products are indicated by an asterisk. (E) HAV (left) or HCV (right) persistent subgenomic replicon cells expressing T7 RNA polymerase were transfected with a plasmid encoding HA-TRIF under transcriptional control of a T7 promoter and harvested 16 h after transfection. (H) Cleavage of endogenous MAVS in HAV (left) or HCV (right) persistent subgenomic replicon cells. (F, I) Huh7-Lunet T7 CD81h cells were infected with HAV orHCV as indicated. Three days after infection, the cells were transfected with a plasmid encoding HA-TRIF for detection of TRIF cleavage (F), or directly harvested for detection of endogenous MAVS cleavage (I). 10 μg of protein were analysed by immunoblotting, using specific antibodies against HA, FLAG, HAV 3C, HCV NS5A, MAVS, and β-actin (Actin), as indicated. Sizes of protein markers are indicated on the right (kDa). Band intensities of TRIF or MAVS were normalised to the respective β-actin band intensities and quantified using Fiji. Note that 3ABC and 3CD cleavage products were not detectable in infected or in replicon cells. MAVS, mitochondrial antiviral-signalling protein; TRIF, TIR-domain-containing adapter-inducing interferon-β; WT, wild-type. (This figure appears in color on the web.)

empty-vector-transduced cells (Fig. S2C). However, although HCV triggered TLR3 robustly and RIG-I transiently, concordantly with previous studies,[5,8] HAV again did not induce a detectable ISG response in any of the cell lines (Fig. 3F and Fig. S2D).

The HAV 3C protease precursors cleave the adaptor proteins TRIF and MAVS only partially; HCV NS3-4A does not cleave TRIF, but fully degrades MAVS

Our work so far demonstrated that HAV induced innate immunity in human hepatocytes *in vivo*, but not in Huh7 cells. HAV replication in the latter was particularly robust, potentially resulting in a high expression of the viral protease 3C and the precursors 3CD and 3ABC, reported to cleave TLR3 and RLRs adaptors TRIF and MAVS, respectively.[9,23] We first cloned TRIF, MAVS, the HAV and HCV proteases in individual expression vectors (Fig. 4A). As we observed strong cell death upon overexpression of TRIF, and were unable to detect TRIF WT even with cells expressing lower levels of T7 RNA polymerase (Fig. S3E), we switched to a TRIF variant devoid of the RIP homotypic interaction motif (RHIM) domain[24] which indeed rescued cell viability (Fig. 4B). Upon co-transfection of TRIF and the HAV 3CD protease we detected the two expected cleavage products, concomitantly with a partial reduction of the full-length TRIF molecule upon 3CD WT, but not for a cleavage-deficient protease mutant (Fig. 4C, black arrows). In contrast to literature, but in line with TLR3 activation by HCV replication,[7,8] we could not detect any TRIF cleavage by HCV NS3-4A, neither for gt1b (Fig. 4D), nor for gt2a (Fig. S3A). TRIF-cleavage was enhanced upon increasing amounts of 3CD expression, but not for NS3-4A (Fig. S3B). To ensure expression of viral proteases in all cells, we also transfected TRIF in stable subgenomic replicon cells, detecting around 50% reduction of full length TRIF by HAV (Fig. 4E left), almost none for HCV Gt1b (Fig. 4E right, longer exposure Fig. S3G) and none for HCV Gt2a (Fig. S3C). Treatment of replicon cells with the NS3-4A inhibitor simeprevir also had no impact on TRIF abundance (Fig. S3F). In infected cells, TRIF was cleaved incompletely by HAV 3CD (Fig. 4F left) and not detectably cleaved by HCV NS34A (Fig. 4F right).

For MAVS we also detected only partial cleavage by HAV 3ABC, with full-length MAVS still being the most prominent species (Fig. 4G, left). On the contrary, HCV NS3-4A fully cleaved MAVS, in line with literature[3] (Fig. 4G, right), also with gt2a NS3-4A (Fig. S4C). Indeed, minimal HCV NS3-4A concentrations already cleaved MAVS at high efficiency, in contrast to HAV 3ABC (Fig. S4D).

In replicon cells endogenous MAVS was again partly cleaved by HAV and fully by HCV NS3-4A (Fig. 4H). In HCV- and HAV-infected cells MAVS cleavage efficiency was reduced for both viruses, likely as a result of the presence of uninfected cells (Fig. 4I). Therefore, we aimed at validating MAVS cleavage in a microscopy-based approach using green fluorescent protein (GFP) with a nuclear translocation signal, fused to the C-terminal membrane anchor of MAVS[25] encoding the canonical protease cleavage sites of both viruses. Both proteases were able to induce nuclear GFP translocation upon infection, yet to a higher degree for HCV (Fig. S4A and B). Instead, upon transfection of the proteases, MAVS translocation upon 3ABC cleavage seemed impaired (Fig. S4C and D), despite similar expression levels and localisation of HAV 3ABC and HCV NS3-4A (Fig. S4E and F), hinting at the cleavage upon infection being the most physiological of the three approaches we used.

In summary, these data showed that the HAV protease 3CD and 3ABC cleave TRIF and MAVS incompletely, and corroborated that HCV NS3-4A fully degrades MAVS, whereas no TRIF cleavage was detectable.

Partial TRIF and MAVS cleavage by HAV protease precursors does not result in efficient interference with innate immune pathways

We next aimed to study the impact of TRIF and MAVS cleavage on functional counteraction of innate immunity by generating stable cell lines expressing either the HAV or HCV protease, WT or mutant, along with the individual dsRNA sensor. Because of the cytotoxicity of HAV 3CD (Fig. S5A), we needed to rely on transient lentiviral transduction (Fig. S5B). Next, we stimulated the cells with increasing concentrations of p(I:C) and quantified *IFIT1* mRNA levels as a measure of ISG induction. Huh7.5 TLR3 cells were able to mount a full response to p(I:C), regardless of the presence of any viral protease (Fig. 5A). RIG-I and MDA5 responses were in contrast fully abrogated by HCV NS3-4A WT, but functional upon HAV 3ABC expression (Fig. 5B and C), despite comparable protease expression levels (Fig. S5C). A small but significant reduction upon TLR3 stimulation in NS3-4A expressing cells was not reproducible in independent cell populations even modulating the levels of HCV NS3-4A (Fig. S6). To include possible contributions of other viral proteins to the interference activity reported for HAV towards TLR3, RIG-I and MDA5, we further assessed ISG induction upon p(I:C) stimulation in subgenomic replicon cell lines reconstituted with each PRR, confirming that HAV replicons did not abrogate ISG induction, while HCV was blocking the RLRs (Fig. S7).

We next established a single-cell analysis, infecting Huh7.5 cells reconstituted with individual PRRs and successively stimulating them with p(I:C) (Fig. S8A). Here, we did not detect a significant difference in *IFIT1* levels upon poly(I:C) stimulation between mock- and HAV-infected cells for all PRRs (Fig. 5D–F and Fig. S8B–D). In striking contrast, HCV-infected cells completely abrogated RIG-I and MDA5 activation (Fig. 5E and F and Fig. S8C and D). TLR3-reconstituted cells infected with HCV showed a higher *IFIT1* expression even in absence of p(I:C), confirming the TLR3-specific sensing of HCV and the lack of strong counteraction by the virus (Fig. 5D and Fig. S8B).

Altogether, we showed that TRIF and MAVS cleavages do not represent an efficient strategy of innate immune interference for HAV, as opposed to the strong HCV functional interference with the RLR pathways. Furthermore, we demonstrated that HCV did not counteract the TLR3 pathway through NS3-4A.

HAV triggers innate immunity in cell culture models with an intact MDA5 pathway, essentially requiring LGP2

Having observed upregulation of ISGs in the HAV-infected humanised mice, but not in Huh7 with reconstituted dsRNA receptors, we assumed that Huh7 cells might be defective for

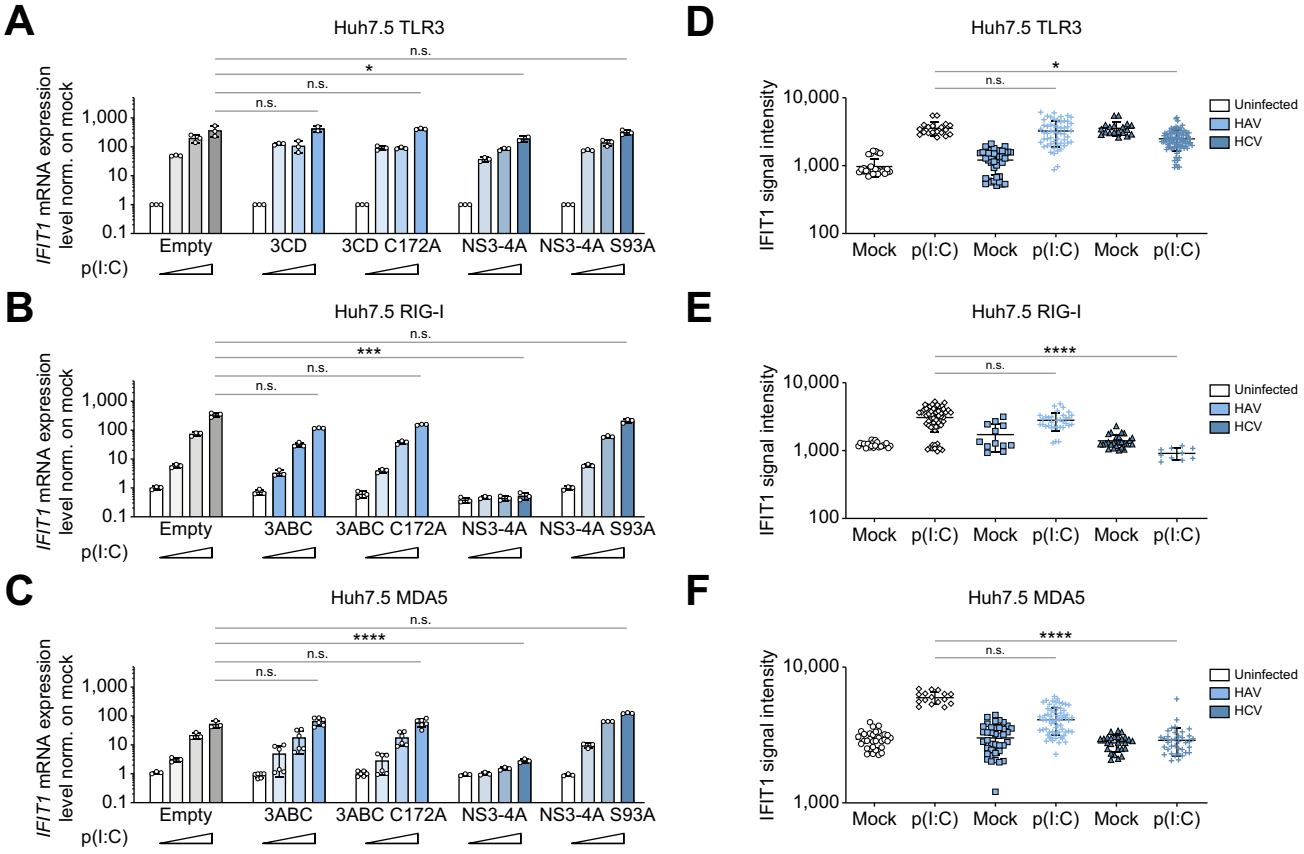

Fig. 5. Impact of HAV and HCV protease mediated cleavage of TRIF and MAVS on cell intrinsic innate immune response. (A, B, C) Huh7.5 cells stably expressing the indicated PRR were transduced with lentiviral vectors encoding WT or mutant HAV (3CD, (3ABC) or HCV protease (NS3-4A) or with empty vectors. Cells were transfected with increasing amounts of poly(I:C) (0, 0.01 μg/ml; 0.1 μg/ml; 1 μg/ml) and harvested 6 h after stimulation to quantify *IFIT1* mRNA by RT-qPCR. Data are normalised to *GAPDH* and shown as fold expression relative to untransfected cells. Mean values with SD from biological replicates (n = 3). Statistical significance was assessed by Welch's unpaired *t* test. (D, E, F) Huh7.5 cells stably expressing the indicated PRR were infected with HAV or HCV. Three days after infection, cells were transfected with 0.5 μg poly(I:C) or mock treated (unstim) and immunofluorescence staining was performed 6 h later using HAV Vp3-, HCV NS5A-, and IFIT1-specific antibodies. The signal intensity for virus and innate immune response was quantified using Fiji. Each dot represents a single cell. Statistical analysis was performed using Welch's unpaired *t*-test. MAVS, mitochondrial antiviral-signalling protein; MDA5, melanoma differentiation-associated protein 5; PRR, pattern recognition receptor; RIG-I, Retinoic acid-inducible gene I; TLR3, Toll-like-receptor 3; TRIF, TIR-domain-containing adapter-inducing interferon-β.

HAV sensing. LGP2 was shown to be an essential co-factor of MDA5 in mounting an IFN response[26,27] and MDA5 was reported to be the main sensor for HAV.[6] Thus, we assessed *LGP2* mRNA expression in liver-based cell lines and indeed found that Huh7.5 cells lacked *LGP2* expression (Fig. 6A). However, stable reconstitution of *LGP2* expression in Huh7.5 MDA5 cells (Fig. S9A) increased ISG induction upon HAV replication (Fig. S9B and C), but also induced higher baseline ISG expression in absence of infection, rendering these data difficult to interpret (Fig. S9C).

We alternatively chose HepG2 and HepaRG because of their high *LGP2* expression (Fig. 6A). HepG2 were more permissive compared with HepaRG (Fig. 6B), but both cell lines showed a clear upregulation of *IFIT1* upon HAV infection (Fig. 6C). Neither HepaRG nor HepG2 were permissive for HCV in our work, the latter not even after restoration of miR122 and CD81 expression,[28] therefore we could not compare these data to HCV infection.

As HepG2 cells expressed TLR3 at undetectable levels, we restored this signalling pathway by ectopic expression (Fig. S9D and E), but found similar degrees of *IFIT1* induction upon HAV replication in presence or absence of TLR3, suggesting its limited role in HAV sensing (Fig. S9F and G). Next, we used the same HepG2 TLR3 cells to generate knockout (KO) pools of *RIG-I* or *MDA5* (Fig. S10A). Upon robust HAV replication (Fig. S10B and C) the only PRR involved in detecting HAV was MDA5 (Fig. S10D), whereas lack of RIG-I expression did not impact on *IFIT1* induction (Fig. S10E).

To further examine the role of LGP2 in HAV sensing, we infected *LGP2* KO HepaRG cell pools[29] with HAV, allowing similar replication levels (Fig. 6F), and found a significant reduction of *CXCL10* (Fig. 6D) and *IFIT1* (Fig. 6E).

In conclusion, our results indicate that HAV triggers an innate immune response in human hepatocytes *in vivo* and *in vitro*, in permissive models with intact signalling pathways. HAV replication was sensed by MDA5 and essentially required LGP2 expression. The moderate proteolytic activity observed by HAV towards MAVS obviously did not abolish sensing in HepG2 and HepaRG cells. We further excluded sensing of HAV by TLR3, questioning the functional significance of partial TRIF cleavage.

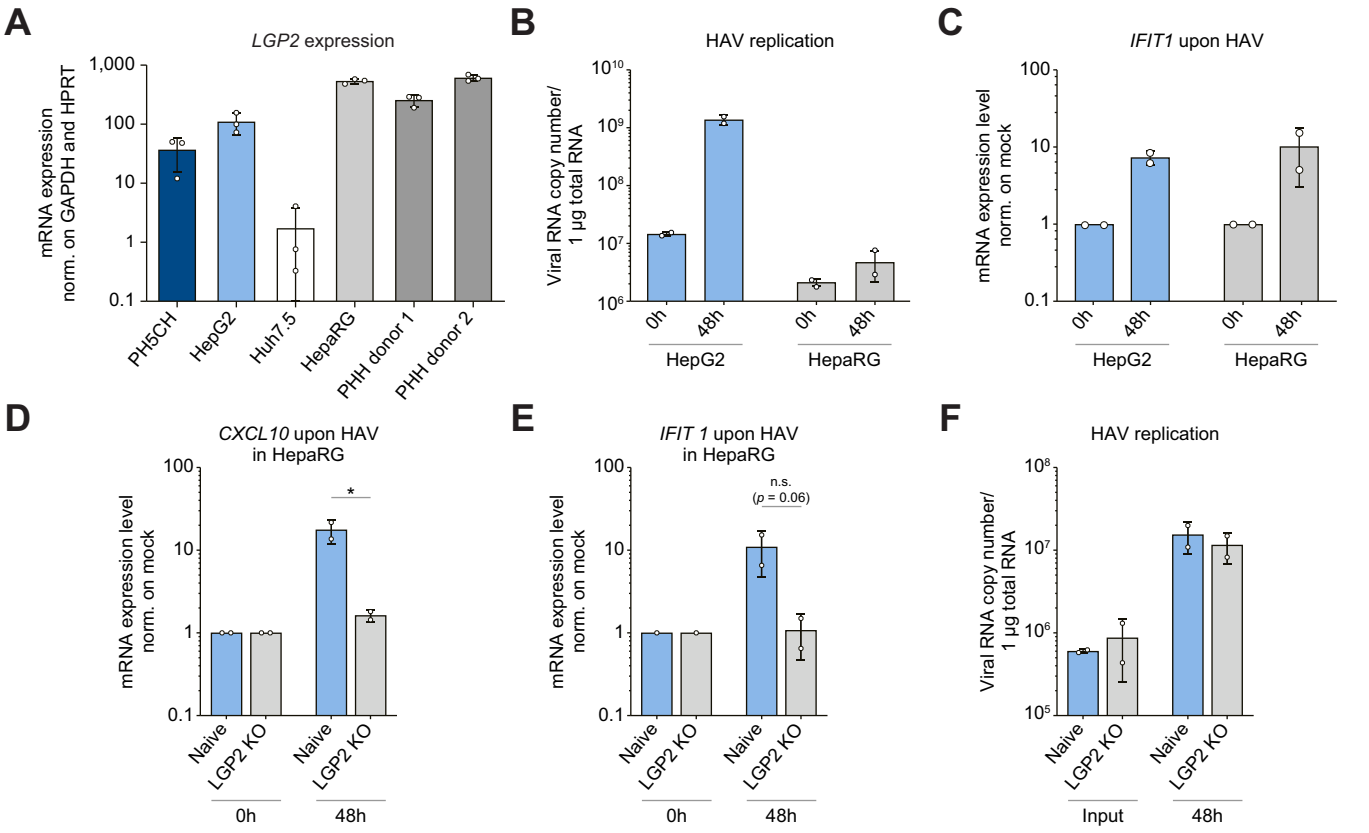

Fig. 6. The sensing of HAV in hepatocytes requires LGP2 expression. (A) *LGP2* mRNA levels were quantified by RT qPCR and normalised to *GAPDH* and *HPRT* expression in four different hepatocyte cell lines and PHH. (B) HepG2 and HepaRG cells were infected with HAV and *IFIT1* mRNA (C), and viral RNA (B), were quantified. (D–F) HepaRG cell pools with knockout for *LGP2*, or mock, were infected with HAV. *CXCL10* (D) and *IFIT1* (E) mRNA and viral RNA (F) were quantified at the indicated time points. (C–E) *IFIT1* and *CXCL10* mRNA expression levels were normalised to *GAPDH* expression relative to infected cells at time point 0. Statistical significance was assessed by Welch's unpaired *t* test. All values shown are mean values with SD from biological replicates (n = 2). LGP2, laboratory of genetics and physiology 2.

Discussion

This study provides the first side-by-side analysis of innate immune induction and counteraction by HAV and HCV in hepatocytes *in vivo* and in cell culture. So far, both viruses have been shown to cleave MAVS and TRIF by virus-encoded proteases, abrogating IFN responses. However, although HCV in most *in-vivo* studies induced a detectable ISG expression, HAV was regarded as not inducing IFN in infected hepatocytes, based on data from infected chimpanzees.[12,30] However, our data demonstrate that both viruses induce similar levels of ISG expression in infected human hepatocytes *in vivo*. Our comprehensive cell culture studies suggest that HCV mainly triggers TLR3, because of the lack of TRIF cleavage, in contrast to control of RLR signalling by cleavage of MAVS. We further show that HAV is exclusively sensed by MDA5 with support by LGP2, triggering an ISG response that is insufficiently blocked.

Huh7 cells are so far the only cell culture model allowing robust, comparable replication levels of HAV and HCV. In particular, the subclone Huh7.5 lacks expression of RLRs and TLR3, allowing individual reconstitution and analysis of RIG-I, MDA5, and TLR3. However, all PRRs are ISGs, induced by Interferons or dsRNA stimulation. Constitutive expression cannot mimic the inducibility of PRR expression, but in our experience rather represents expression levels of an induced state[8] and such increased basal expression might impact on ISG induction. Although reconstitution of PRRs in general did not induce innate immunity in absence of stimuli, this was observed for ectopic LGP2 expression in Huh7 cells. As all available hepatoma cell lines permissive for hepatotropic viruses differ substantially from PHH,[31] all data obtained with these models need to be carefully interpreted.

The innate immune response in the context of HAV is controversially discussed in literature. Some studies indicate that HAV barely induces innate immune activation in infected cells, because of the efficient disruption of PRR sensing pathways upon proteolytic cleavage of MAVS and TRIF by HAV proteases 3ABC and 3CD.[3,9,23] This seemed plausible because of the limited ISG induction observed in the livers of HAV infected chimpanzees.[12] In contrast, an innate immune response upon HAV infection was described in PHH and HepG2 cells,[6] and knockout of *mavsmavs* in mice is sufficient to allow HAV productive infection, suggesting that induction of innate immunity in hepatocytes is a clear restriction factor in this model.[16] Although this was initially attributed to the incapability of HAV proteases to cleave murine MAVS, a very recent study showed that humanisation of MAVS by restoration of the protease cleavage site in this model was not sufficient to restore permissivity.[17] We used a comprehensive set of approaches, including expression of the HAV proteases, persistent replicons and HAV infection in hepatic cells with all data

agreeing in detecting only a partial cleavage of MAVS and TRIF, insufficiently counteracting induction by p(I:C). Indeed, HAV replication induced ISGs in HepG2 and HepaRG, but not in Huh7 cells. This was attributed to the absence of LGP2, which we found essential for sensing of HAV by MDA5, underpinning the importance of choosing appropriate models. We excluded a role of TLR3 in HAV sensing, therefore questioning the biological significance of partial TRIF cleavage. Although we cannot exclude the possibility that partial TRIF cleavage might contribute to the observed lack of TLR3 induction, we assume that HAV dsRNA does not reach the endosome, in contrast to HCV,[8,32] for example because of the different organisation of the replication organelles of picornaviruses.[33]

Importantly, HAV infection of Alb/uPA-SCID mice with humanised liver clearly showed, in absence of adaptive immune responses, ISG and chemokines produced to a similar extent as for HCV. In chimpanzees, ISG induction was also observed but interpreted as induced by cytokine secretion of T cells.[12] In fact, cytokine expression was quite variable among all the chimpanzees analysed, suggesting that overlapping innate and adaptive immune responses might have masked the cell intrinsic induction by HAV replication in hepatocytes. However, we cannot exclude that HAV cleavage of MAVS, or other counteraction mechanisms,[3] might attenuate the innate immune response, but to a lower extent than previously described.

For HCV, efficiency of MAVS cleavage by NS3-4A completely blocks RLR-mediated ISG induction.[3] Although efficient TRIF cleavage has been reported, mainly based on *in-vitro* translation models or using non-hepatic cells,[10] other studies could not reproduce it[11] and indicated that TLR3 was still active upon HCV infection.[7,8] In agreement, no detectable cleavage was observed in the experimental systems used by us. Studies on TRIF are generally hampered by low expression levels and high cytotoxicity upon ectopic expression.[24] Therefore, we included a TRIF mutant lacking the RHIM domain.[24] Although we cannot formally exclude an impact of this deletion on TRIF folding, potentially hampering protease cleavage, this appears an unlikely scenario based on *in silico* models (Fig. S11). Other mechanisms interfering with TLR3 responses upon HCV infection, such as secretion of dsRNA[8] or NS4B-mediated degradation of TRIF[34] also do not block TLR3 induction, but might rather weaken it, given the robust, TLR3-specific ISG response induced by HCV infection in cell culture. Sensing of HCV by TLR3 therefore might be the primary candidate for the ISG induction observed by us and others in Alb/uPA-SCID mice with humanised liver.[35,36] Although here ISG induction was mainly found in infected hepatocytes, this is different in chronic HCV patients[14] and HCV-infected chimpanzees,[12,30] likely caused by cytokines secreted by activated pDCs-[37] and T cells.[38] Whether the differences in innate immune response among the three GLT1-infected animals can be attributed to the virus strain remains to be addressed in future studies with larger group sizes.

Altogether, our data suggest similar levels of innate immune induction in HCV- and HAV-infected hepatocytes, originating from very divergent sensing and counteraction schemes. Limited induction of TLR3 accompanied by complete control of the RLR pathway might contribute balancing HCV replication and innate immune response, with potential support of persistence. For HAV, an inefficient counteraction of the MDA5/LGP2-mediated sensing might contribute to expose HAV to pDCs- and T-cell-mediated immunity.[38–40] Overall, adaptive immune responses remain the key for clearance of both viral infections.[38–40] A comprehensive understanding of the determinants of persistence *vs.* clearance of HCV and HAV infections will therefore require fully immunocompetent animal models, which are currently not available.

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Abbreviations

AHA, acute hepatitis A; Alb/uPA-SCID, albumin urokinase-type plasminogen activator/severe combined immunodeficiency; ds, double-stranded; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; GFP, green fluorescent protein; HMW, high molecular weight; HPRT, Hypoxanthine-guanine phosphoribosyltransferase; IFN, interferon; IHC, immunohistochemistry; ISGs, IFN-stimulated genes; KO, knock-out; LGP2, laboratory of genetics and physiology 2; MAVS, mitochondrial antiviral-signalling protein; MDA5, melanoma differentiation-associated protein 5; PRR, pattern recognition receptor; RHIM, RIP homotypic interaction motif; RIG-I, retinoic acid-inducible gene I; RLRs, RIG-I-like receptors; RT-qPCR, Real-Time quantitative PCR; TLR3, Toll-like receptor 3; TRIF, TIR-domain-containing adapter-inducing interferon-β; WT, wild-type.

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Conflicts of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study conception: VL, OC; Experimental design: OC, VL, KEN, OG, JT; Performed experiments: OC, RBu, HEH, DH, NG, LC; Provided essential reagents and protocols: MC, MB, FFWV, UM, JYL; Analysed and interpreted the data: OC, VL, RBu, HEH, TR, SFD, TFL, CST, RB, PM, MH; Wrote the manuscript: VL, OC; Responsible for the overall content of this work: VL; All authors reviewed and approved the final version of the manuscript.

Data availability statement

Details related to materials and methods are provided in the Supplementary section and CTAT methods table. Further data supporting the findings of this study is available upon request. Please contact the corresponding author.

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Supplementary data

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Author names in bold designate shared co-first authorship.

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Update

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Corrigendum

JOURNAL OF HEPATOLOGY

Corrigendum to "Comparison of HAV and HCV infections *in vivo* and *in vitro* reveals distinct patterns of innate immune evasion and activation" [J Hepatol (2023) 645–656]

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It has come to our attention that there was an error in Fig. 4G (left panel) of our manuscript. The size of the protein Flag-3ABC was incorrectly labelled as '75 kDa' instead of its correct size '38 kDa'. This is corrected in the updated figure below. We apologise for any inconvenience caused.

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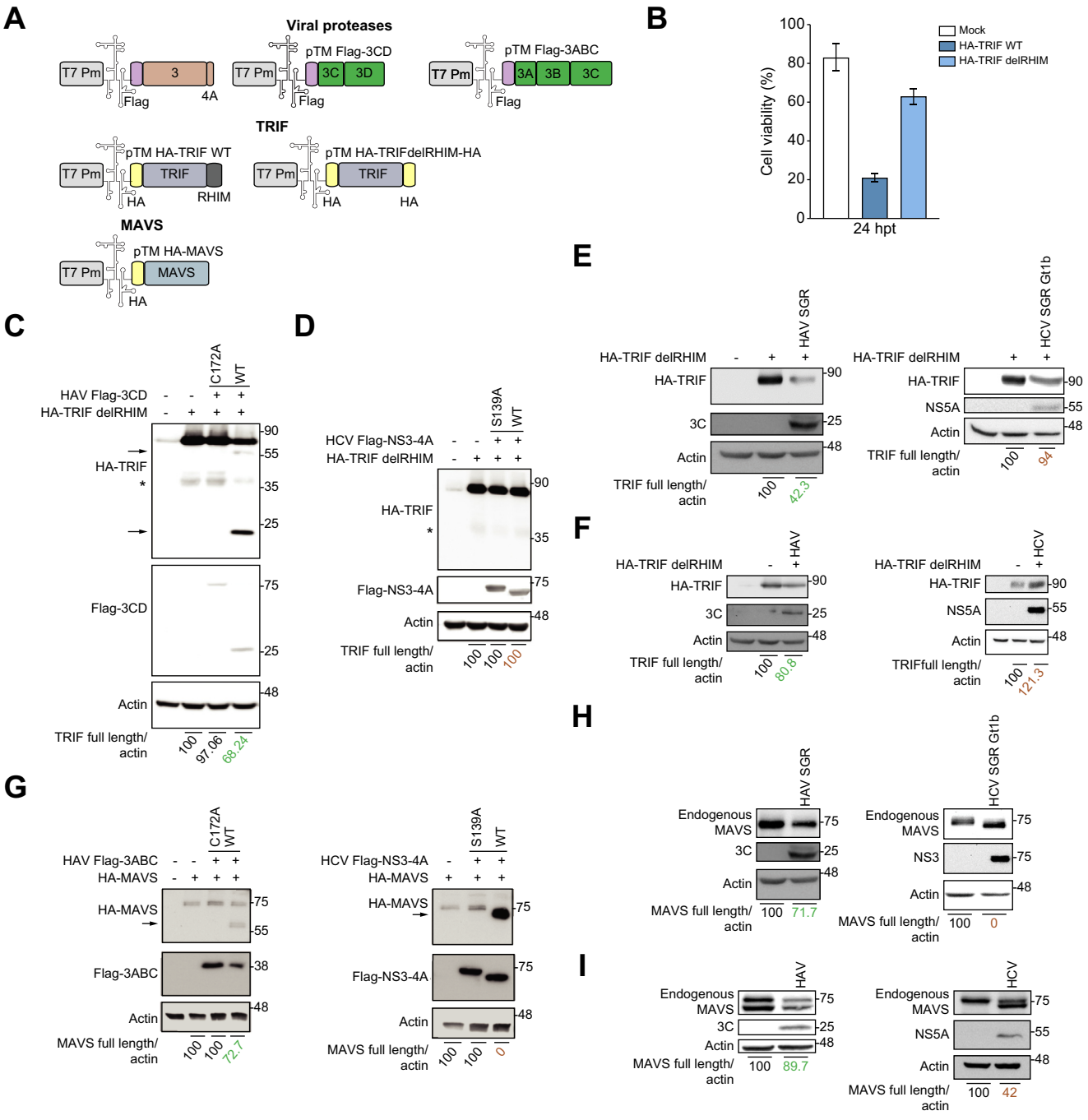
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TRIF
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RHIM
pTM HA-TRIFdelRHIM-HA
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MAVS
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Mock
HA-TRIF WT
HA-TRIF delRHIM
Cell viability (%)
24 hpt
C
C172A
WT
HAV Flag-3CD
HA-TRIF delRHIM
HA-TRIF
Flag-3CD
Actin
TRIF full length/ actin
D
S139A
HCV Flag-NS3-4A
Flag-NS3-4A
E
HAV SGR
HCV SGR Gt1b
3C
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F
HAV
HCV
TRIFfull length/ actin
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