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The RNA Genome of Hepatitis E Virus Robustly Triggers an Antiviral Interferon Response

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The outcomes of hepatitis E virus (HEV) infection are diverse, ranging from asymptomatic carrier, self-limiting acute infection, and fulminant hepatitis to persistent infection. This is closely associated with the immunological status of the host. This study aimed to understand the innate cellular immunity as the first-line defense mechanism in response to HEV infection. Phosphorylation of signal transducer and activator of transcription 1, a hallmark of the activation of antiviral interferon (IFN) response, was observed in the liver tissues of the majority of HEV-infected patients but not in the liver of uninfected individuals. In cultured cell lines and primary liver organoids, we found that HEV RNA genome potently induced IFN production and antiviral response. This mechanism is conserved among different HEV strains, including genotypes 1, 3, and 7 as tested. Interestingly, single-stranded HEV RNA is sufficient to trigger the antiviral response, without the requirement of viral RNA synthesis and the generation of an RNA replicative form or replicative intermediate. Surprisingly, the m^7G cap and poly A tail are not required, although both are key features of the HEV genome. Mechanistically, this antiviral response occurs in a retinoic acid–inducible gene-I–independent, melanoma differentiation–associated protein 5–independent, mitochondrial antiviral signaling protein–independent, and β-catenin-independent but IRF3-dependent and IRF7-dependent manner. Furthermore, the integrity of the Janus kinase–signal transducer and activator of transcription pathway is essentially required. *Conclusion:* HEV infection elicits an active IFN-related antiviral response *in vitro* and in patients, triggered by the viral RNA and mediated by IFN regulatory factors 3 and 7 and the Janus kinase–signal transducer and activator of transcription cascade; these findings have revealed new insights into HEV–host interactions and provided the basis for understanding the pathogenesis and outcome of HEV infection. (HEPATOLOGY 2018;67:2096-2112).

Over the last decade, hepatitis E virus (HEV) infection has emerged as a global health issue. It is one of the most common causes of acute viral hepatitis in the world. Although the infection is generally self-limiting, severe complications and high mortality rates have been reported in special populations, including pregnant women, immunocompromised patients, and patients with preexisting liver disease.[1-3] HEV outbreaks periodically occur throughout resource-limited countries, including the large ongoing outbreak in Niger, resulting in a heavy clinical burden with a high mortality rate in pregnant

Abbreviations: DDX, DEAD box; DHX, DEAH box; dsRNA, double-stranded RNA; Gt, genotype; HCV, hepatitis C virus; HEV, hepatitis E virus; IFN, interferon; ISG, IFN-stimulated gene; IRF, IFN regulatory factor; ISRE, IFN-stimulated response element; JAK, Janus kinase; MAVS, mitochondrial antiviral signaling; MDA5, melanoma differentiation-associated protein 5; MEF, mouse embryonic fibroblast; NOD, nucleotide-binding oligomerization domain; ORF, open reading frame; PRR, pattern recognition receptor; P-STAT1, phosphorylated STAT1; RIG-I, retinoic acid-inducible gene-I protein; RLR, RIG-I-like receptor; ssRNA, single-stranded RNA; STAT, signal transducer and activator of transcription; WT, wild type.

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women.[4,5] Unfortunately, there is no Food and Drug Administration–approved medication available, and its infection biology is poorly understood.

Viral infections universally evoke active interactions between the virus and host. Host cells are equipped with mechanisms that rapidly detect and respond to virus invasion. These defense mechanisms largely rely on receptors that monitor the cytosol for the presence of atypical nucleic acids from the virus. DExD/H-box RNA helicases of the retinoic acid–inducible gene-I (RIG-I)–like receptor (RLR) family have been identified as essential intracellular sensors of RNA viruses. Two of the RLR family members, RIG-I and melanoma differentiation–associated protein 5 (MDA5), are ubiquitously expressed, which enables the detection of viral infection in almost all cell types. Upon the detection of viral RNA ligand, RIG-I or MDA5 interacts with a mitochondrion-anchored adaptor protein, mitochondrial antiviral signaling (MAVS), to initiate downstream signaling that eventually leads to the transcription and production of interferons (IFNs). Once secreted, IFNs create a state of antiviral alertness by inducing the expression of hundreds of IFN-stimulated genes (ISGs). RIG-I has been reported to be essential for IFN production in the setting of Newcastle disease virus, vesicular stomatitis virus, influenza, and Japanese encephalitis virus infections.[6,7] IFN production is impaired in MDA5-deficient cells infected with Picornaviridae, murine norovirus 1, and murine hepatitis virus.[8-10] Some viruses such as West Nile virus and dengue virus are recognized by both RIG-I and MDA5.[11,12] Other intricate viral RNA sensor systems outside the RLR family have also been implicated in eliciting an IFN response to virus infection, including DEAD box 3 (DDX3),[13] DEAH box 9 (DHX9),[14] DDX1–DDX21–DHX36 complex,[15] nucleotide-binding oligomerization domain (NOD)–like receptor NOD2,[16] and LRRFIP1.[17] It is believed that these intricate RNA sensors act independently or cooperatively to mediate the innate immune response upon virus invasion.

HEV is a single-stranded positive-sense RNA virus within the family Hepeviridae. The genome contains short 5′ and 3′ noncoding regions, 5′-m^7G cap, 3′-poly A tail, and three open reading frames (ORF1, ORF2, and ORF3).[18] In patients, particularly in cases of acute infection with severe hepatitis, active virus–host interactions are likely the cause of pathogenesis but also the process of combating the infection.[19] In this study, we found the phosphorylation of signal transducer and activator of transcription 1 (STAT1, Y701), a hallmark of the IFN-related antiviral response, in the liver of HEV-infected patients. Because viral nucleic acid is the main pathogen-associated molecular pattern recognized by the host innate immune system, we delivered *in vitro* generated HEV genomic RNA into host cells to investigate the host response. Consistently, HEV RNA potently induces IFN production and antiviral response in both cell lines and three-dimensional cultured primary liver organoids. Surprisingly, the single-stranded RNA (ssRNA) of HEV is sufficient to trigger the host response. This occurs in a RIG-I-independent, MDA5-independent, MAVS-independent, and β-catenin-independent but IFN regulatory factor 3 (IRF3)–dependent and IRF7-dependent manner. Importantly, the integrity of the Janus kinase (JAK)–STAT cascade is required for the antiviral response triggered by HEV. These results have provided insights into HEV–host interactions.

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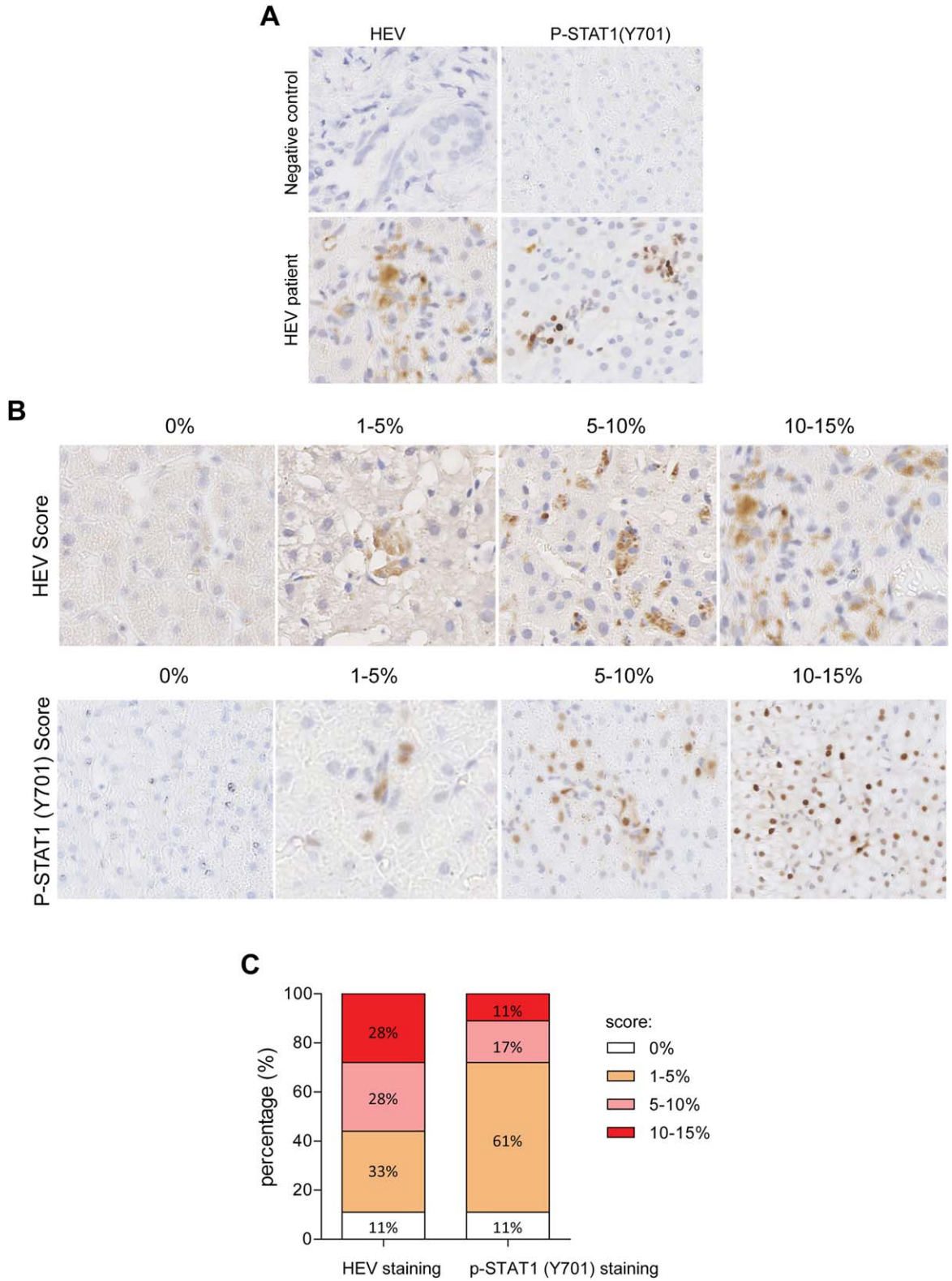
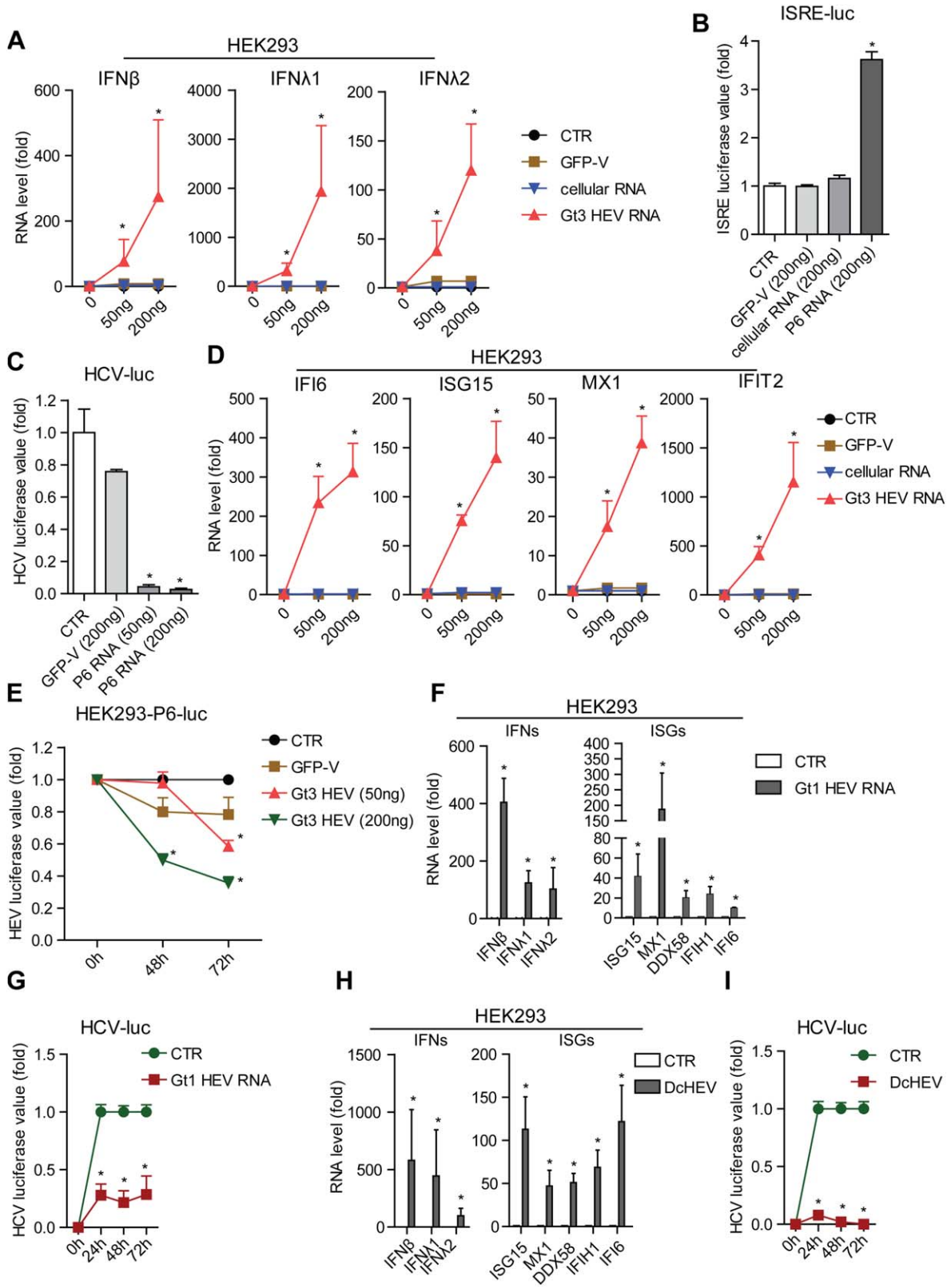


FIG. 1. Immunohistochemistry showed positive staining of P-STAT1 in liver tissues of HEV patients. (A) Representative staining of HEV and P-STAT1 in liver tissue of HEV patients or negative control individuals. (B) Representative staining indicating variable levels of HEV and P-STAT1 on liver tissues. (C) The distribution of HEV and P-STAT1 scores among liver tissues.


A
HEK293
IFNβ
IFNλ1
IFNλ2
RNA level (fold)
CTR
GFP-V
cellular RNA
Gt3 HEV RNA
0
50ng
200ng
B
ISRE-luc
ISRE luciferase value (fold)
CTR
GFP-V (200ng)
cellular RNA (200ng)
P6 RNA (200ng)
C
HCV-luc
HCV luciferase value (fold)
CTR
GFP-V (200ng)
P6 RNA (50ng)
P6 RNA (200ng)
D
HEK293
IFI6
ISG15
MX1
IFIT2
RNA level (fold)
CTR
GFP-V
cellular RNA
Gt3 HEV RNA
E
HEK293-P6-luc
HEV luciferase value (fold)
CTR
GFP-V
Gt3 HEV (50ng)
Gt3 HEV (200ng)
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48h
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IFNs
ISGs
RNA level (fold)
CTR
Gt1 HEV RNA
IFNβ
IFNλ1
IFNλ2
ISG15
MX1
DDX58
IFIH1
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HCV-luc
HCV luciferase value (fold)
CTR
Gt1 HEV RNA
0h
24h
48h
72h
H
HEK293
IFNs
ISGs
RNA level (fold)
CTR
DcHEV
I
HCV-luc
HCV luciferase value (fold)
CTR
DcHEV

FIG. 2

Materials and Methods

PATIENT MATERIALS

Eighteen liver biopsies from patients (2010-2017) diagnosed with acute or chronic hepatitis E were retrieved at Beijing 302 Hospital, China. The use of patient materials was approved by the medical ethics committee of Beijing 302 Hospital. Expression of phosphorylated STAT1 (P-STAT1, Y701) was stained. Five liver biopsies from hepatic hemangioma patients were collected as negative controls. Patient information is shown in Supporting Table S1.

IMMUNOHISTOCHEMICAL STAINING

Immunohistochemical staining of HEV ORF2 viral protein or P-STAT1 (Y701) was performed to validate HEV infection and visualize P-STAT1. In detail, liver biopsies were fixed in 10% formalin for 1.5 hours at room temperature, processed for paraffin embedding, and sectioned at a thickness of 4 μm. Sections were deparaffinized in xylene and rehydrated through graded ethanol treatment, followed by high pressure in citrate buffer (pH 6.0) for 3 minutes for antigen retrieval. Then they were blocked with 3% H_2O_2 in trishydroxymethylaminomethane-buffered saline for 15 minutes and further blocked with goat serum for 1 hour. Sections were incubated with anti-HEV ORF2 viral protein (Millipore, 1:600) or anti-P-STAT1 (Cell Signaling, 1:800) monoclonal antibody overnight at 4°C and incubated with goat antimouse/rabbit secondary antibody (ZSGB-BIO, KIT-5030) for 15 minutes at 37°C. Subsequently, sections were developed with diaminobenzidine (ZSGB-BIO, ZLI-9018), followed by counterstaining hematoxylin. Immunostained sections were scanned using a Leica DFC400 digital camera and Leica Application Suite software (Leica Microsystems).

PLASMIDS AND REAGENTS

Plasmid constructs containing the full-length Gt1 HEV genome (Sar55/S17; GenBank accession no. AF444002), Gt3 HEV genome (Kernow-C1 P6 clone; GenBank accession no. JQ679013), and HEV replication–defective genome (GAD) were kindly provided by Suzanne U. Emerson (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The plasmid containing the full-length dromedary camel HEV genome (GenBank accession no. KJ496144) was kindly provided by Tian-Cheng Li (National Institute of Infectious Diseases, Japan). Plasmid constructs containing the full-length HEV genome (Kernow-C1 P6 clone) with 1634R or 1634K mutations were generated accordingly.[20] Plasmid pLVX-ORF2-IRES-zsGrenn1 was kindly provided by Alexander Ploss (Princeton University).[21] Plasmid pEGFP-C1-ORF3 was constructed in our lab. pTRIP.CMV.IVSb.ISG.ires.TagRFP-based RIG-I and MDA5 expression vectors were a kind gift from Prof. Charles M. Rice (Rockefeller University).[22] Human IFN-α (Thermo Fisher Scientific, Life Sciences, The Netherlands) was dissolved in phosphate-buffered saline. The 5′ triphosphate double-stranded RNA (dsRNA) was purchased from InvivoGen (no. tlrl-3prna). FuGENE HD Transfection reagent (E2311) was purchased from Promega. Stocks of JAK inhibitor I were dissolved in dimethyl sulfoxide at a concentration of 10 mM. Antibodies including P-STAT1 (58D6; no. 9167); RIG-I (D14G6; no. 3743); MDA5 (D74E4; no. 5321); β-catenin (6B3; no. 9582); antirabbit immunoglobulin G(H+L), F(ab′) 2 fragment (Alexa Fluor 488 conjugate); and antimouse immunoglobulin G (H+L),

FIG. 2. Transfection of HEV RNA potently induces IFN production and IFN response in HEK293 cells. HEK293 cells were transfected with control (transfection reagent only), green fluorescence protein vector (plasmid), cellular RNA, or Gt3 HEV RNA. The expression levels of indicated IFNs (A) and ISGs (D) were quantified at 48 hours posttreatment by quantitative RT-PCR (n = 4). (B) The ISRE luciferase value was measured (at 48 hours) after the treatment of conditioned medium from HEK293 cells transfected with control, green fluorescence protein vector, cellular RNA, or Gt3 HEV RNA (n = 6). (C) HCV viral replication–related firefly luciferase activity was measured after the treatment of conditioned medium from HEK293 cells transfected with control, green fluorescence protein vector, or Gt3 HEV RNA (n = 4). (E) HEV viral replication–related firefly luciferase activity was measured after the transfection of control, green fluorescence protein vector, or Gt3 HEV RNA in HEK293-P6-luc cells (n = 4). HEK293 cells were transfected with control, Gt1 HEV RNA (F) or dromedary camel HEV RNA (H). Expression levels of indicated IFNs and ISGs were quantified at 48 hours posttreatment by quantitative RT-PCR (n = 4). HCV viral replication–related firefly luciferase activity was measured after the treatment of conditioned medium from HEK293 cells transfected with Gt1 HEV RNA (G) or dromedary camel HEV RNA (I) (n = 4). Abbreviations: CTR, control; Dc-HEV, dromedary camel HEV; GFP-V, green fluorescence protein vector; luc, luciferase.

F(ab′)2 fragment (Alexa Fluor 488 conjugate) were purchased from Cell Signaling Technology, The Netherlands. Hepatitis E monoclonal antibody was purchased from EMD Millipore Corporation. Anti-rabbit or antimouse IRDye-conjugated antibodies were used as secondary antibodies for western blotting (Stressgen, Victoria, BC, Canada).

Additional procedures are described in detail in the Supporting Information.

Results

ACTIVATION OF STAT1 PHOSPHORYLATION IN THE LIVER OF HEV-INFECTED PATIENTS

To investigate whether HEV infection activates host antiviral response in patients, expression of P-STAT1 (Y701), a hallmark of the IFN-related antiviral response, was stained in the liver biopsies (Fig. 1A). The staining of HEV viral protein ORF2 and P-STAT1 was scored independently based on the proportion of positive cells (Fig. 1B). Up to 89% of HEV-infected patients showed positive staining of P-STAT1 in the liver, whereas no staining was observed in the liver tissues from uninfected individuals (Fig. 1C; Supporting Table S1). These results indicated that HEV infection elicits an active IFN-related antiviral response in patients.

HEV GENOMIC RNA POTENTLY INDUCES AN ANTIVIRAL IFN RESPONSE

Upon HEV invasion, HEV-derived components, like viral capsid protein and viral genomic RNA, can be sensed as "non-itself" by host innate immunity. Therefore, the potential role of HEV proteins ORF2 and ORF3 in the IFN response was evaluated firstly. HEK293 cells were transfected with control, ORF2-expressing, or ORF3-expressing vectors. Expression of ORF2 or ORF3 protein (Supporting Fig. S1A,B) has no significant effect on IFN expression or on the subsequent ISG induction (Supporting Fig. S1C,D). To further investigate whether any functional IFNs are produced, we collected the conditioned medium from the transfected cells (supernatant) (Supporting Fig. S1E) and performed an IFN-stimulated response element (ISRE)–based IFN reporter assay and a highly IFN-sensitive hepatitis C virus (HCV) replicon–based bioassay. Consistently, no IFN production was detected in both models (Supporting Fig. S1F,G).

We next examined whether HEV viral RNA is the trigger of the host innate immune response. *In vitro* generated HEV genomic RNA was used for efficient delivery into host cells. Upon transfection of genotype 3 (Gt3) HEV RNA (Kernow-C1, P6) into human liver hepatoma Huh7.5 cells (named Huh7.5-P6), the viral protein ORF2 was subsequently detected by immunofluorescent assay (Supporting Fig. S2A). The anti-HEV effects of IFN-α and ribavirin were confirmed in these cells (Supporting Fig. S2B). After inoculation with conditioned cell culture medium derived from Huh7.5-P6 cells, viral protein was also detected in HEK293 cells (Supporting Fig. S2C). Therefore, Gt3 HEV RNA generated *in vitro* functions to initiate the essential steps of the HEV life cycle.

Importantly, transfection of Gt3 HEV RNA strongly induced an IFN response in a dose-dependent manner; while no response was observed in the negative controls, transfection of a green fluorescent protein vector or cellular RNA. Specifically, expression of IFNβ (type I IFN), IFNλ1, and IFNλ2 (type III IFN) was strongly induced (Fig. 2A), although there was no significant change in levels of IFNα (type I IFN) and IFNγ (type II IFN) (Supporting Fig. S2D). To examine whether functional IFNs are produced, we collected the conditioned medium from the transfected cells (supernatant) (Supporting Fig. S1E) and performed an ISRE-based IFN reporter assay and a highly IFN-sensitive HCV-replicon–based bioassay. Supernatant from HEK293 cells transfected with Gt3 HEV RNA strongly induced ISRE-coupled luciferase activity (Fig. 2B). Consistently, HCV replicon–related luciferase activity was decreased upon the same treatment (Fig. 2C). Because ISGs are the downstream antiviral effectors of IFN signaling, several well-known antiviral ISGs were quantified. Expression levels of these ISGs were significantly up-regulated upon transfection of Gt3 HEV RNA (Fig. 2D). Correspondingly, HEV replicon–related luciferase activity was decreased in HEK293 cells (Fig. 2E). In addition, an HEV-induced IFN response was further confirmed using *in vitro* generated Gt1 HEV and dromedary camel HEV (Gt7)[23] (Fig. 2F-I; Supporting Fig. S2F). Therefore, in HEK293 cells, HEV could potently induce an antiviral IFN response upon viral RNA entry into the cytoplasm.

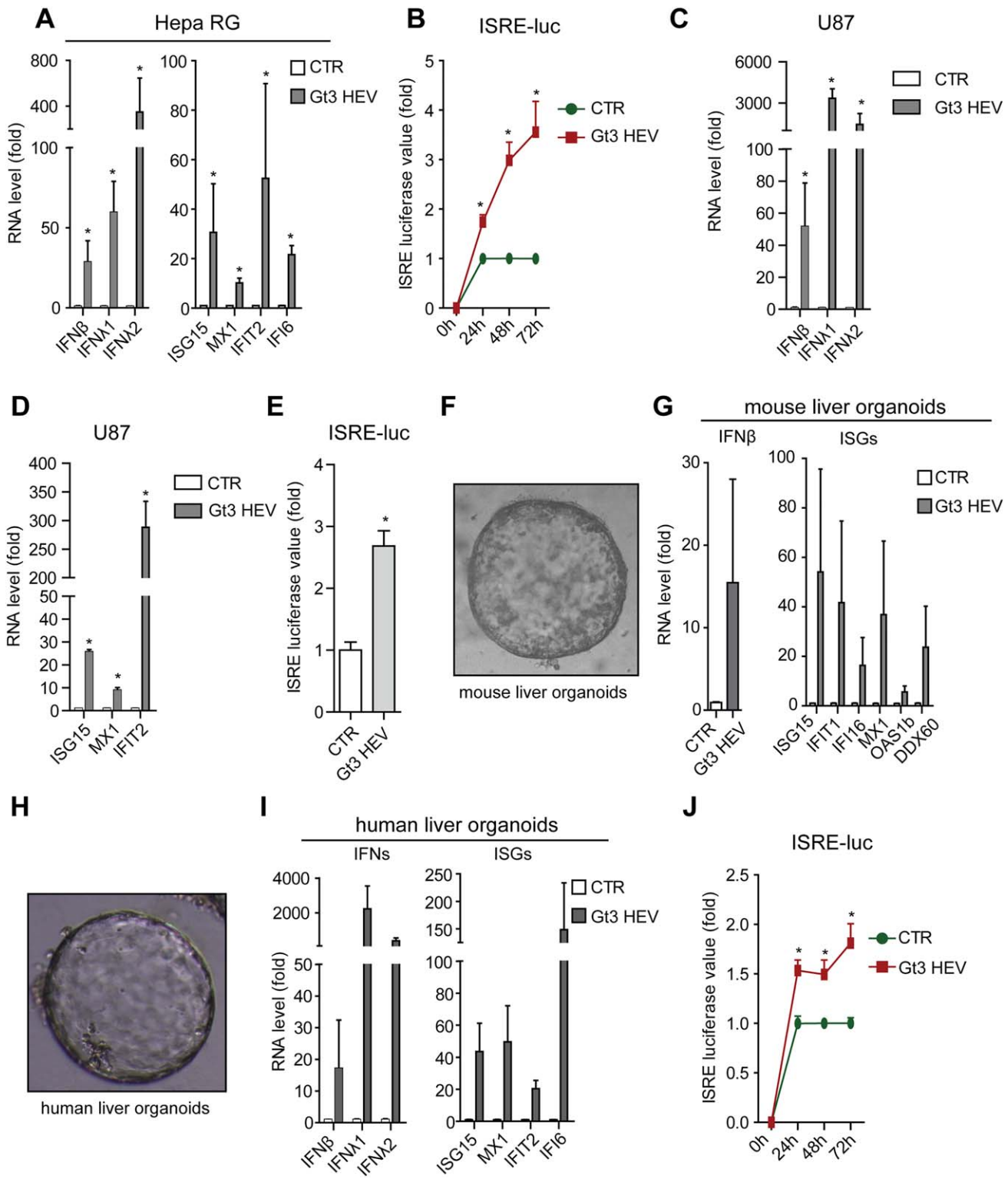
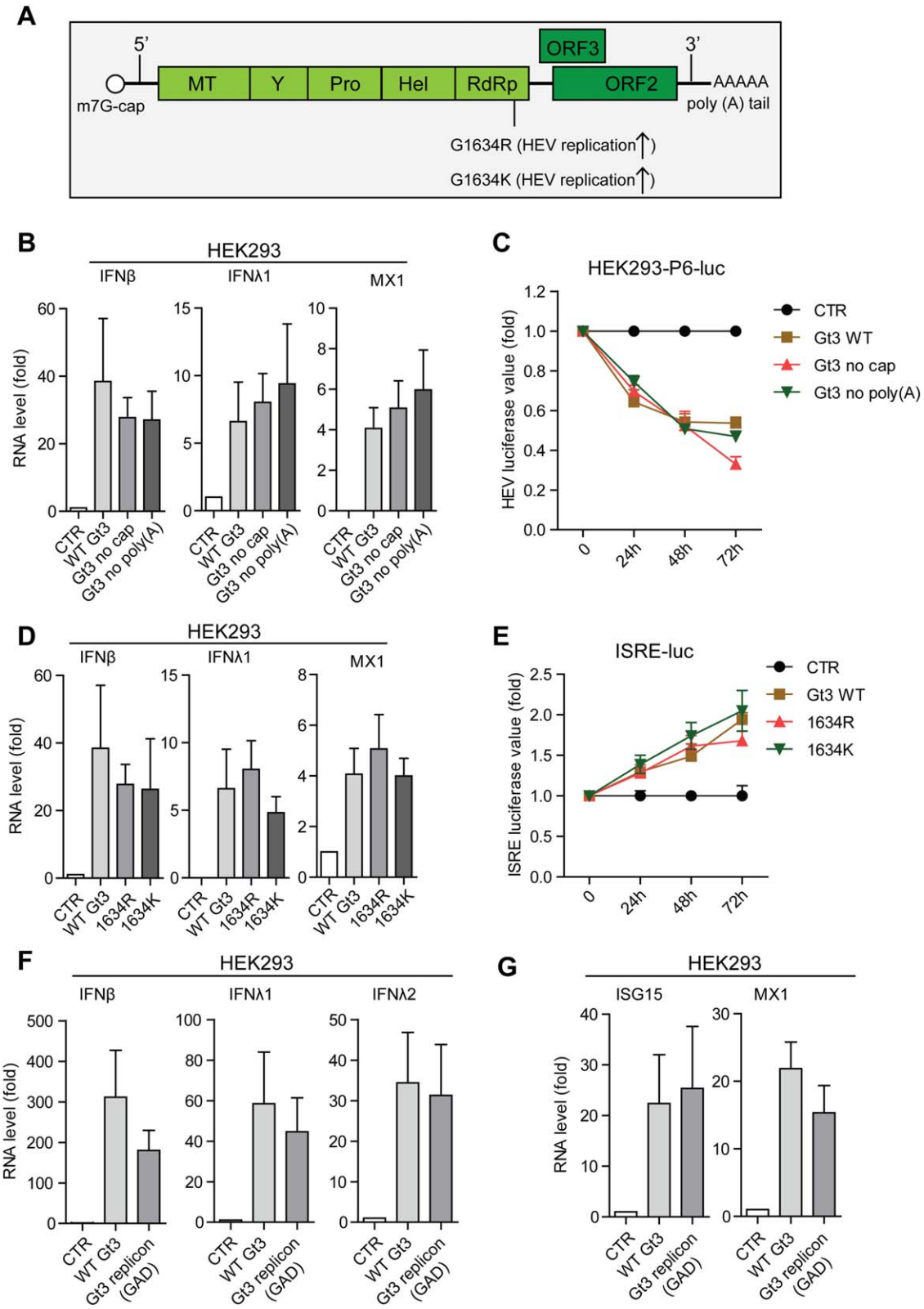


FIG. 3. Transfection of HEV RNA induces antiviral response in both two-dimensional and three-dimensional cell culture models. (A) HepaRG cells were transfected with control, green fluorescence protein vector, or Gt3 HEV RNA (200 ng/well) in 96-well plates. Expression levels of indicated IFNs and ISGs were quantified at 48 hours posttreatment by quantitative RT-PCR (n = 4). (B) The ISRE luciferase value was measured after the treatment of conditioned medium from HepaRG cells transfected with control, green fluorescence protein vector, or Gt3 HEV RNA (n = 5). (C,D) Same as (A) for U87 cells. (E) Same as (B) for U87 conditioned medium measured at 24 hours. Representative microscopy images of three-dimensional cultured mouse (F) and human (H) primary liver organoids. (G) After the transfection of Gt3 HEV RNA in mouse liver organoids, expression levels of IFNβ and indicated ISGs were quantified at 48 hours posttreatment by quantitative RT-PCR (n = 4). (I) Same as (G) for human liver organoids (n = 4). (H) Same as (B) for human liver organoids (n = 5). Abbreviations: CTR, control; luc, luciferase.


A
5'
3'
ORF3
MT
Y
Pro
Hel
RdRp
ORF2
AAAAA
poly (A) tail
m7G-cap
G1634R (HEV replication↑)
G1634K (HEV replication↑)
B
HEK293
IFNβ
IFNλ1
MX1
RNA level (fold)
CTR
WT Gt3
Gt3 no cap
Gt3 no poly(A)
C
HEK293-P6-luc
HEV luciferase value (fold)
CTR
Gt3 WT
Gt3 no cap
Gt3 no poly(A)
0
24h
48h
72h
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HEK293
IFNβ
IFNλ1
MX1
RNA level (fold)
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WT Gt3
1634R
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ISRE-luc
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WT Gt3
Gt3 replicon (GAD)
G
HEK293
ISG15
MX1
RNA level (fold)
CTR
WT Gt3
Gt3 replicon (GAD)

FIG. 4

HEV infection was reported to cause both hepatic and extrahepatic manifestations; thus, we extended our study to hepatic cell lines (Huh7.5 and HepaRG) as well as another extrahepatic cell line (U87, a neural cell line). Consistently, transfection of HEV RNA led to strong IFN production and IFN response in Huh7.5 (Supporting Fig. S3), HepaRG (Fig. 3A,B), and U87 (Fig. 3C-E) cells. Recently, three-dimensional cultured primary liver organoids have emerged as innovative models for studying liver physiology and pathology. They contain various types of cells and recapitulate most, if not all, aspects of *in vivo* liver tissue architecture.[24] We further validated that transfection of HEV RNA led to strong IFN expression and subsequent ISG induction in primary liver organoids cultured from mouse or human liver tissues (Fig. 3F-J).

THE HEV RNA–TRIGGERED HOST RESPONSE IS INDEPENDENT OF THE m^7G CAP AND POLY A TAIL

The HEV genome is an approximately 7.2-kb ssRNA with the 5′ terminus capped (m^7G cap) and the 3′ terminus polyadenylated (Fig. 4A). The m^7G cap is essential for HEV infectivity both *in vivo* and *in vitro*.[25,26] The poly A tail is crucial for viral RNA-dependent RNA polymerase binding to the 3′ untranslated region.[27] Because host cells specifically recognize certain features of viral nucleic acids, we investigated whether the m^7G cap and poly A tail are required for the HEV-triggered IFN response. Thus, Gt3 HEV RNA lacking the m^7G cap or poly A tail were generated *in vitro* and transfected in both HEK293 and Huh7.5 cells. Interestingly, compared with the wild-type (WT) form, HEV RNA lacking the m^7G cap or poly A tail retain a comparable potency to induce IFN response (Fig. 4B,C; Supporting Fig. S4A-C). These data indicated that HEV triggers an IFN response independent of the m^7G cap and poly A tail.

HEV ssRNA IS SUFFICIENT TO TRIGGER THE ANTIVIRAL RESPONSE

Upon transfection of the HEV RNA genome, the virus replicates and produces different RNA species including ssRNA, viral RNA replicative form (dsRNA), and replicative intermediate. To clarify whether the IFN response induced by HEV depends on viral RNA replication and which specific RNA species is involved, two mutant forms of Gt3 HEV RNA (G1634R, G1634K) (Fig. 4A) were transcribed *in vitro*. These two mutant forms have comparable ribavirin sensitivity to the WT HEV but possessed an enhanced replication fitness *in vitro*.[20,28] However, they exerted comparable activity in inducing an IFN response in both HEK293 and Huh7.5 cells when compared with the WT form (Fig. 4D,E; Supporting Fig. S5A-D). These results imply that the replication of HEV RNA may not be important in this process. To confirm this notion, a replication-defective Gt3 HEV replicon (GAD) carrying an alanine substitution in the polymerase active site was used.[29,30] Consistently, compared with the WT form, transfection of Gt3 HEV replicon (GAD) RNA induced strong and comparable IFN responses in both HEK293 and Huh7.5 cells (Fig. 4F,G; Supporting Fig. S5E-G). More convincingly, the IFN induction ability of the Gt3 HEV replicon (GAD) was also confirmed in other two-dimensional cultured cell models (e.g., HepaRG and U87) (Fig. 5A-E) as well as three-dimensional mouse and human primary liver organoids (Fig. 5F-H). Collectively, these results indicate that HEV ssRNA is the specific RNA species involved in triggering the IFN response and that this process is independent of viral replication.

FIG. 4. HEV ssRNA is sufficient to trigger an IFN response, while being independent of viral RNA m^7G cap and poly A tail. (A) Illustration of HEV RNA genome. (B) HEK293 cells were transfected with control, WT, no m^7G cap, or no poly A tail Gt3 HEV RNA (200 ng/well) in 96-well plates. Expression levels of indicated IFNs and ISGs were quantified at 48 hours posttreatment by quantitative RT-PCR (n = 3). (C) HEV viral replication–related firefly luciferase activity was measured after the same transfection indicated in (B) in HEK293-P6-luc cells (n = 4). (D) HEK293 cells were transfected with control, WT, 1634R mutant, or 1634K mutant Gt3 HEV RNA (200 ng/well) in 96-well plates. Expression levels of indicated IFNs and ISGs were quantified at 48 hours posttreatment by quantitative RT-PCR (n = 3). (E) ISRE luciferase value was measured after the treatment of conditioned medium from HEK293 cells transfected with control, WT, 1634R mutant, or 1634K mutant Gt3 HEV RNA (n = 4). HEK293 cells were transfected with control, WT, or replication defective Gt3 HEV replicon (GAD) RNA (n = 4). Expression levels of indicated IFNs (F) and ISGs (G) were quantified at 48 hours posttreatment by quantitative RT-PCR. Abbreviations: CTR, control; Hel, helicase; luc, luciferase; MT, methyltransferase; Pro, protease; RdRp, RNA-dependent RNA polymerase; Y, Y domain.

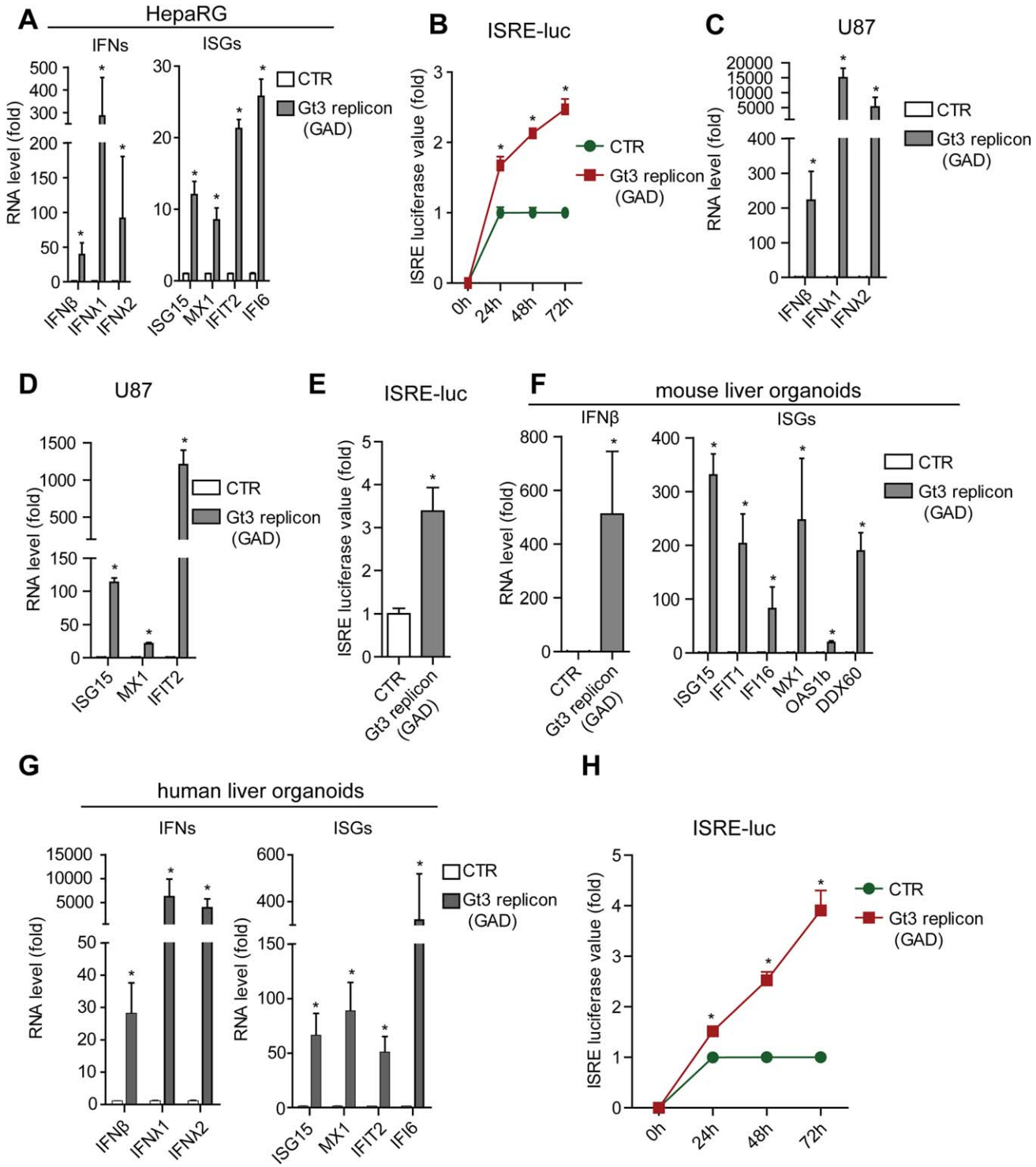

FIG. 5. HEV ssRNA triggers an IFN response in both two-dimensional and three-dimensional cell culture models. (A) HepaRG cells were transfected with Gt3 HEV replicon (GAD) RNA (200 ng/well) in 96-well plates. Expression levels of indicated IFNs and ISGs were quantified at 48 hours posttreatment by quantitative RT-PCR (n = 4). (B) The ISRE luciferase value was measured after the treatment of conditioned medium from HepaRG cells transfected with Gt3 HEV replicon (GAD) RNA (n = 5). (C,D) Same as (A) for U87 cells. (E) Same as (B) for conditioned medium from U87 cells. (F) Same as (A) for mouse liver organoids (n = 4). (G) Same as (A) for human liver organoids (n = 4). (H) Same as (B) for human liver organoids (n = 5). Abbreviations: CTR, control; luc, luciferase.

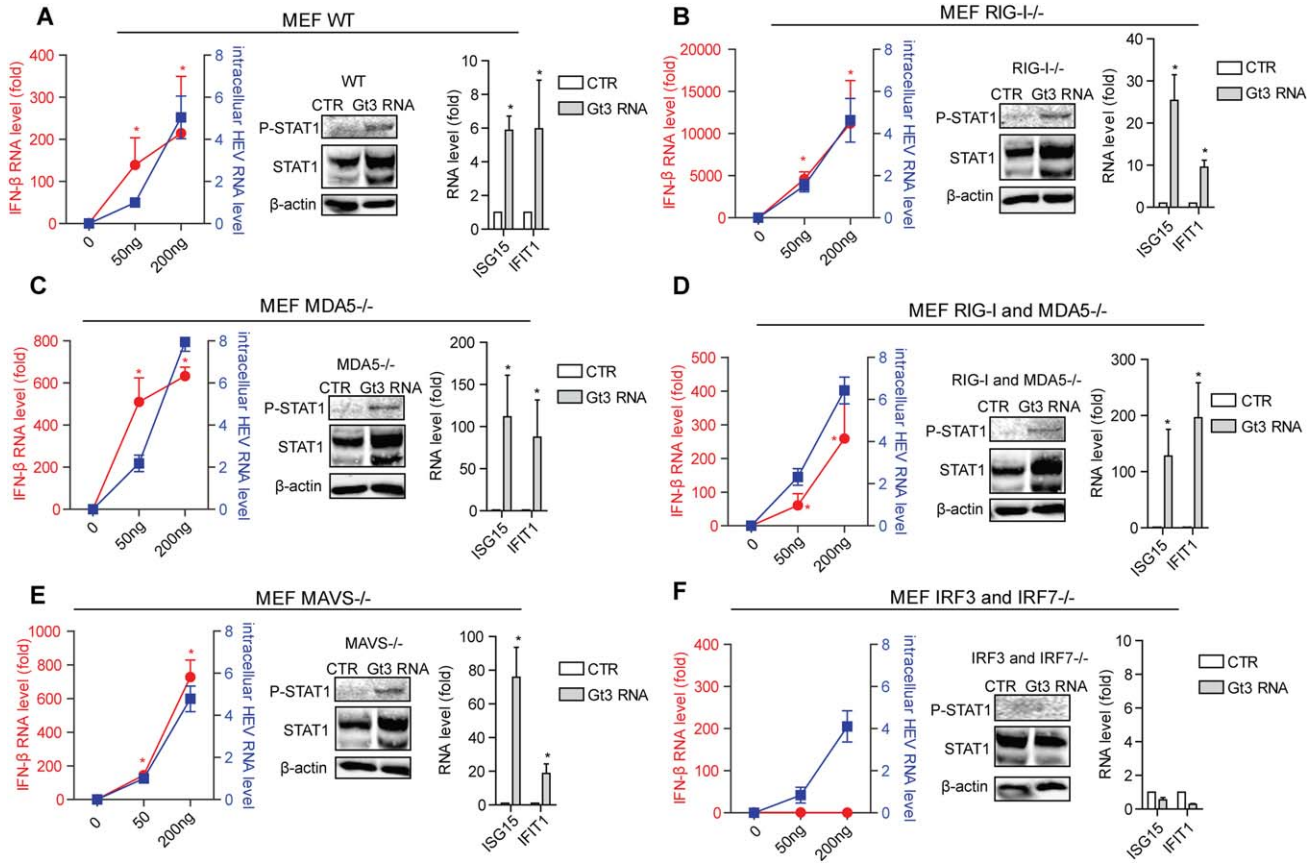

FIG. 6. HEV triggers an IFN response in a RIG-I-independent, MDA5-independent, and MAVS-independent but an IRF3/IRF7-dependent manner. The transfection of Gt3 HEV RNA (200 ng/well) was performed in WT (A), RIG-I$^{-/-}$ (B), MDA5$^{-/-}$ (C), RIG-I and MDA5$^{-/-}$ (D), MAVS$^{-/-}$ (E), and IRF3/IRF7$^{-/-}$ (F) MEFs in 96-well plates. Levels of IFNβ (red line) and the relative intracellular HEV RNA (blue line) were quantified at 48 hours posttreatment by quantitative RT-PCR (n = 4, left panel). MEF cells were treated with conditioned medium collected from indicated cells for 24 hours. Protein levels of total and phosphorylated (Y701) STAT1 were detected by western blotting (middle panel). Expression levels of representative ISGs (ISG15 and IFIT1) were quantified at 48 hours posttreatment by quantitative RT-PCR (n = 4, right panel). Abbreviation: CTR, control.

IT IS INDEPENDENT OF RIG-I AND MDA5

Pattern recognition receptors (PRRs) are the primary sensors detecting viral RNA and subsequently activate the antiviral IFN response. They are generally categorized into two major classes depending on their subcellular location, membrane-bound PRRs (e.g., Toll-like receptors) and intracellular PRRs (e.g., RLRs).[31] Membrane-bound PRRs are predominantly expressed in immune cells, such as macrophages and dendritic cells. Intracellular PRRs are ubiquitously expressed. Our previous study demonstrated that overexpression of the intracellular PRRs, MDA5, or RIG-I exerts anti-HEV effects.[32] Therefore, we investigated whether these two molecules mediate the HEV RNA–triggered IFN response.

Interestingly, overexpression of MDA5 triggered IFN production and subsequent IFN response in both HEK293 and Huh7.5 cells (Supporting Fig. S6A-L). A similar response was observed in HEK293 cells upon overexpression of RIG-1 (Supporting Fig. S7A-F), but Huh7.5 cells are defective in RIG-I.[32] Consistently, transfection of 5′ triphosphate RNA, a specific RIG-I agonist, was unable to induce any IFN responses in Huh7.5 cells (Supporting Fig. S8A-C). Thus, the fact that HEV triggers an IFN response in Huh7.5 cells upon transfection of viral RNA (Supporting Fig. S2C-H) indicates a RIG-I-independent mechanism. To further confirm this notion, WT and RIG-I knockout (RIG-I$^{-/-}$) mouse embryonic fibroblasts (MEFs) were employed. The efficient knockout of RIG-I was confirmed by both 5′ triphosphate RNA

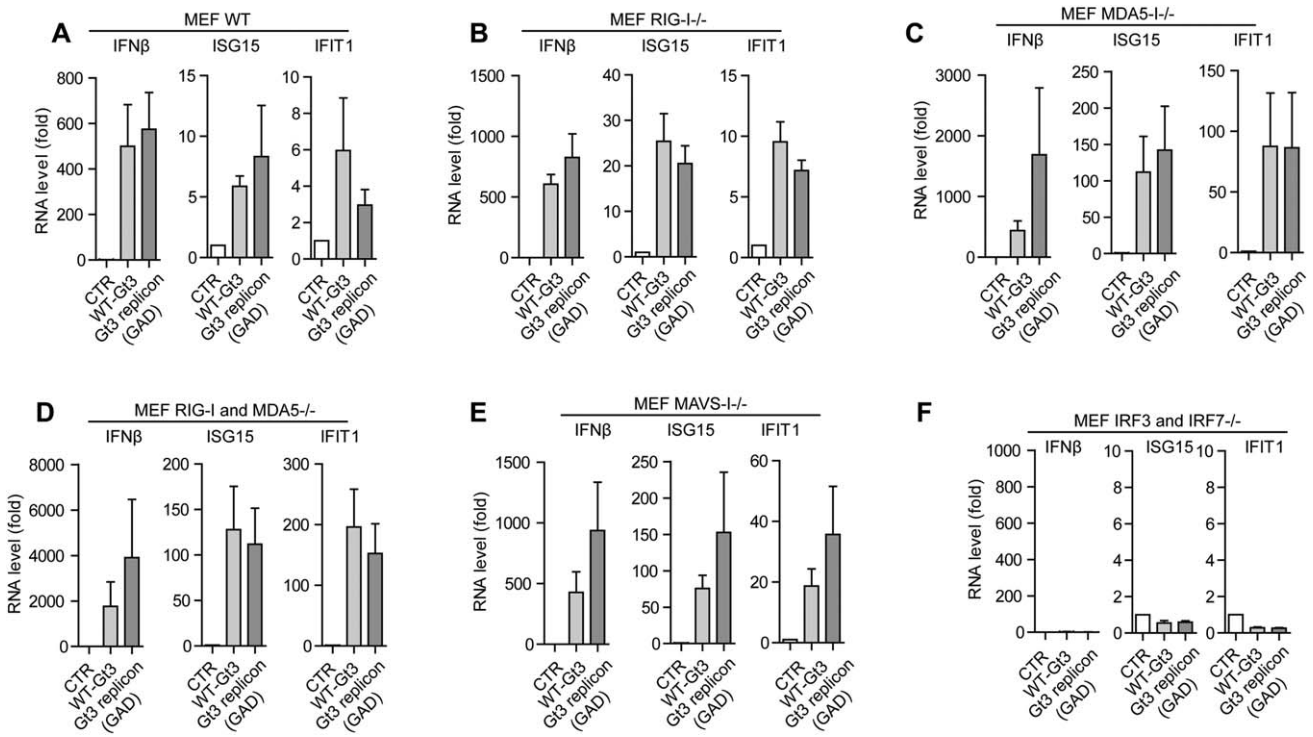

FIG. 7. HEV ssRNA triggers an IFN response in a MAVS-independent but IRF3/IRF7-dependent manner. Transfection of WT or Gt3 HEV replicon (GAD) RNA was performed in WT (A), RIG-I$^{-/-}$ (B), MDA5$^{-/-}$ (C), RIG-I and MDA5$^{-/-}$ (D), MAVS$^{-/-}$ (E), and IRF3/IRF7$^{-/-}$ (F) MEFs. Levels of IFNβ and the indicated ISGs were quantified at 48 hours posttreatment by quantitative RT-PCR (n = 4). Abbreviation: CTR, control.

transfection assay and western blot assay (Supporting Fig. S8D,E). Transfection of Gt3 HEV RNA strongly induced IFNβ and representative ISG expression in RIG-I$^{-/-}$ MEF cells (Fig. 6A,B, left and right panels). In addition, the conditioned medium was collected from RIG-I$^{-/-}$ MEF cells transfected with Gt3 HEV RNA. Incubation of conditioned medium efficiently induced STAT1 phosphorylation, a key feature of IFN signaling pathway activation. Collectively, HEV initiates IFN production and the IFN response independently of RIG-I.

Next, we examined the involvement of MDA5. The efficient knockout of MDA5 (MDA5$^{-/-}$) was confirmed by western blot assay (Supporting Fig. S8F). Strikingly, transfection of HEV RNA in MDA5$^{-/-}$ MEF cells can also strongly induce IFN production and the IFN response (Fig. 6C), indicating an MDA5-independent mechanism. Some viruses such as West Nile virus and dengue virus are recognized by both MDA5 and RIG-I to initiate the IFN response.[11,12] This forced us to clarify whether HEV-induced IFN responses require both MDA5 and RIG-I. Therefore, HEV RNA was transfected in RIG-I and MDA5 double knockout (RIG-I$^{-/-}$ and MDA5$^{-/-}$) MEF cells (Supporting Fig. S8E,F). However, IFN production and IFN responses were still efficiently initiated (Fig. 6D). On the contrary, transfection of a commonly used RIG-I/MDA5 agonist, poly (I;C), induced IFNβ and ISG expression only in WT and not in RIG-I$^{-/-}$ and MDA5$^{-/-}$ MEF cells (Supporting Fig. S8G). Thus, the HEV RNA-triggered IFN response is independent of both MDA5 and RIG-I.

THE HEV RNA–TRIGGERED ANTIVIRAL RESPONSE IS INDEPENDENT OF MAVS AND β-CATENIN BUT REQUIRES IRF3 AND IRF7

In addition to RIG-I and MDA5, other proteins have been implicated in cytosolic sensing of viral RNA to trigger IFN response. They include DDX3,[13]

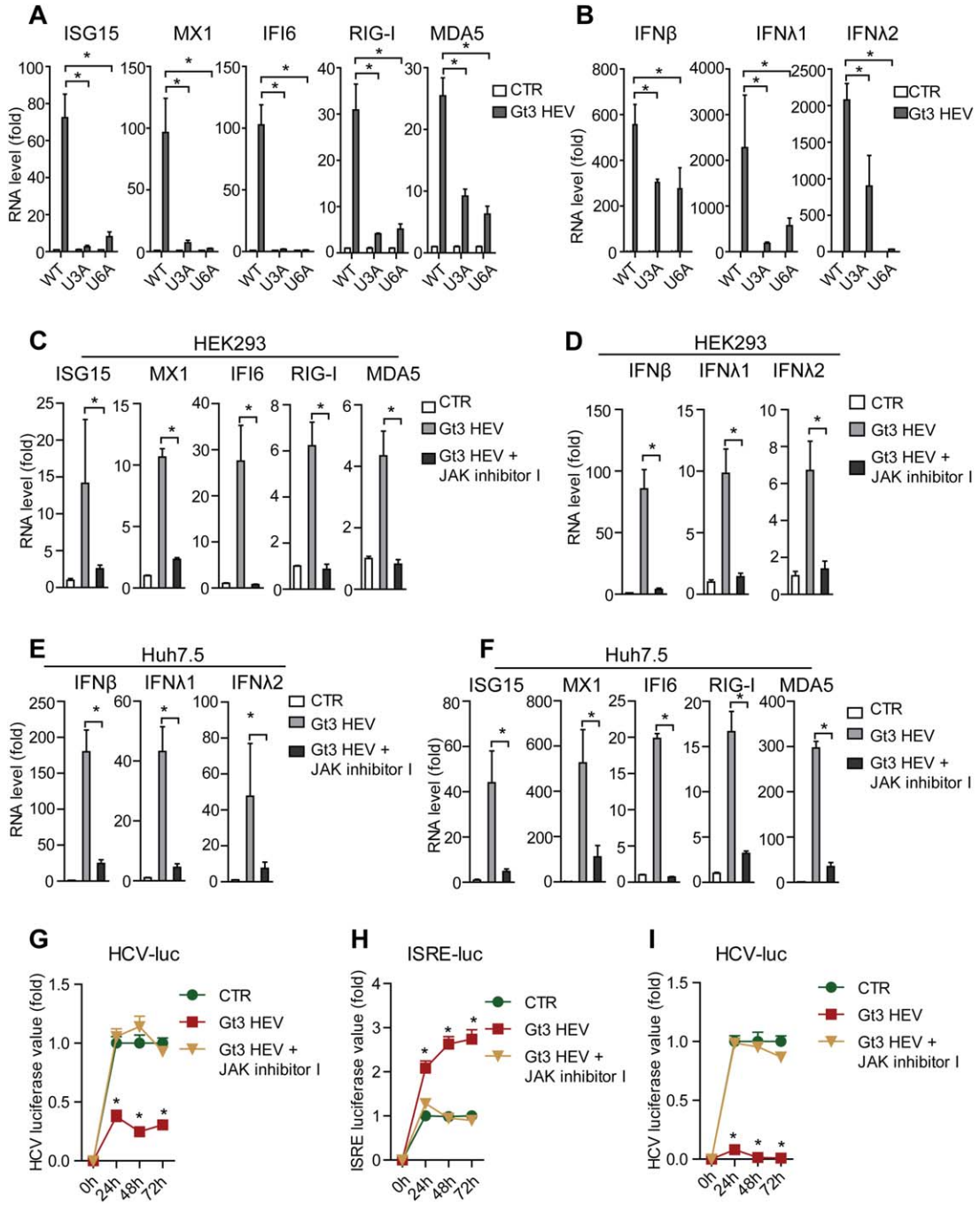

FIG. 8. The integrity of the JAK–STAT pathway is essential for HEV-induced antiviral response. Gt3 HEV RNA was transfected in WT, U3A (STAT1-deficient), and U6A (STAT2-deficient) cells. Levels of ISGs (A) and IFNs (B) were quantified at 48 hours posttreatment by quantitative RT-PCR (n = 4). Gt3 HEV RNA was transfected in HEK293 cells with or without JAK inhibitor I (10 μm). Levels of ISGs (C) and IFNs (D) were quantified at 48 hours posttreatment by quantitative RT-PCR (n = 4). (E) Same as (C) for Huh7.5 cells. (F) Same as (D) for Huh7.5 cells. (G) HCV viral replication–related firefly luciferase activity was measured after the treatment of conditioned medium from HEK293 cells transfected with Gt3 HEV RNA with or without JAK inhibitor I (n = 4). (H) The ISRE luciferase value was measured after the treatment of conditioned medium from Huh7.5 cells transfected with Gt3 HEV RNA with or without JAK inhibitor I (n = 4). (I) Same as (G) for Huh7.5 cells. Abbreviations: CTR, control; luc, luciferase.

DHX9,[14] DDX1–DDX21–DHX36 complex,[15] and NOD-like receptor NOD2.[16] Importantly, similar to RIG-I and MDA5, these proteins are thought to sense viral RNA and induce IFN expression in a MAVS-dependent manner. Surprisingly, in MAVS knockout ($MAVS^{-/-}$) MEFs, the IFN response was strongly induced after transfection of HEV RNA (Fig. 6E). Notably, the cytosolic nucleic acid sensor LRRFIP1 has been reported to sense DNA and RNA viruses, thus mediating the IFN response through a MAVS-independent but β-catenin-dependent pathway.[17] Therefore, the involvement of β-catenin was investigated by employing $\beta\text{-catenin}^{-/-}$ MEF cells (Supporting Fig. S8H). Remarkably, transfection of HEV RNA initiated a strong IFN response in $\beta\text{-catenin}^{-/-}$ MEF cells (Supporting Fig. S8I). These results indicated that HEV RNA is most likely recognized by a currently undefined or unknown cytosolic nucleic acid sensor to activate antiviral IFN responses through MAVS-independent and β-catenin-independent mechanisms.

Given the essential role of two transcription factors, IRF3 and IRF7, in the production of IFNs, we investigated whether these two factors are required. Transfection of HEV RNA failed to stimulate any IFNβ and ISG expression in IRF3 and IRF7 knockout MEFs (Fig. 6F, left and right panel). Correspondingly, incubation of conditioned medium (supernatant, $IRF3^{-/-}$ and $IRF7^{-/-}$ MEFs transfected with HEV RNA) had no effect on STAT1 phosphorylation (Fig. 6F, middle panel). Therefore, HEV induces IFN responses in an IRF3-dependent and IRF7-dependent manner. Because HEV ssRNA is the specific RNA species involved in IFN responses, the replication-defective Gt3 HEV replicon (GAD) RNA was tested as well. Consistent with WT, Gt3 HEV replicon (GAD) RNA induces the IFN response in a RIG-I-independent, MDA5-independent, MAVS-independent, and β-catenin-independent but IRF3-dependent and IRF7-dependent manner (Fig. 7A-F; Supporting Fig. S8J).

THE INTEGRITY OF THE JAK–STAT CASCADE IS ESSENTIAL FOR THE ANTIVIRAL RESPONSE

Upon IFN production, the released IFN molecules bind to cell-surface receptors and initiate signal transduction prominently through the JAK–STAT pathway. This activates the transcription of hundreds of ISGs that are the effectors of cell-autonomous antiviral defense.[33] Transfection of HEV RNA in U3A cells (which are STAT1-deficient) or U6A cells (which are STAT2-deficient) achieved much lower expression levels of ISGs compared with WT (Fig. 8A). Strikingly, expression of IFNs was also largely demolished (Fig. 8B). This was further supported by the use of JAK inhibitor I, a pharmacological JAK inhibitor to block JAK–STAT signal transduction (Supporting Fig. S8K). JAK inhibitor I sufficiently blocked HEV RNA-induced ISG and IFN expression in both HEK293 and Huh7.5 cells (Fig. 8C-I). Therefore, the integrity of the JAK–STAT pathway is essential for HEV-induced IFN responses and (in turn) IFN production.

Discussion

The innate immune system is a major host defense mechanism triggered by viral infections. One prominent characteristic is the rapid and efficient detection of invading pathogens through recognition of the pathogen-associated molecular patterns by host PRRs. After specific ligand recognition, host PRRs initiate distinct signaling transduction that leads to the production and secretion of IFNs. IFNs transcriptionally stimulate hundreds of ISGs through the JAK–STAT pathway, thus creating an antiviral state. IFNs, in particular IFN-α, have been approved for treating viral infections in the clinic for decades, including chronic hepatitis B virus and HCV infections. IFN-α has also been used as an off-label drug to treat chronic HEV infection.[2] Therefore, identifying the cellular innate immune response during virus infection has attracted much attention in recent years. In this study, we found that HEV infection could elicit an active IFN-related antiviral response in most patients. Mechanistically, we found that HEV RNA could potently induce IFN production and antiviral response upon entry into the cytoplasm. This observation was captured in two-dimensional culture of hepatic and extrahepatic cell lines as well as three-dimensional culture of mouse and human primary liver organoids.

The HEV genome is a positive-stranded RNA with the 5′ terminus capped (m^7G cap) and the 3′ terminus polyadenylated. The m^7G cap structure was critical for efficient infectivity in cell culture models.[26] Furthermore, the intrahepatic inoculation of uncapped transcripts failed to initiate HEV infection in chimpanzees followed for 20 weeks.[25] The 3′ end of the HEV genome could bind specifically to the viral RNA-dependent RNA polymerase, directing the synthesis of

complementary-strand RNA.[27] However, these key features are not essential for the HEV-triggered IFN response.

After the HEV RNA genome enters host cells, viral replication is initiated. Therefore, different viral RNA species are produced and coexist, including ssRNA, dsRNA, and replicative intermediate. With respect to different viruses, host PRRs recognize different specific pathogen-associated molecular patterns to initiate IFN production. For most viruses, like dengue virus, Japanese encephalitis virus or picornavirus, the viral replicative form (dsRNA) serves as an IFN inducer.[8,34] However, for some viruses, like respiratory syncytial virus and influenza A virus, ssRNA could induce IFN production.[16,31] In our study, we found that HEV ssRNA is sufficient to induce an IFN response, independent of viral replication. This suggests that host cells are capable of immediately sensing HEV invasion before the start of viral replication.

In the cytosol, the RLR helicase subfamily members (e.g., RIG-I and MDA5) serve as essential immune sensors to detect viral nucleic acids. Upon ligand binding and recognition, RIG-I and MDA5 undergo conformational changes that activate the signaling partner MAVS on the mitochondrial and peroxisomal membranes. MAVS can signal to downstream signaling pathways by activating the serine/threonine-protein kinase IKK and TBK-1 kinases, leading to the induction of IFNs. In our study, overexpression of either RIG-I or MDA5 could efficiently initiate IFN production and subsequent IFN response in particular cell lines. In addition to RLRs, other RNA sensor-related pathways have been implicated in the IFN response to viruses. They include DDX3,[13] DHX9,[14] DDX1–DDX21–DHX36 complex,[15] NOD-like receptor NOD2,[16] and LRRFIP1–β-catenin pathways.[17] With respect to the fact that host cells may encounter a wide variety of intracellular virus infections, these diversified RNA sensors may act independently and/or cooperatively with the classical RLRs to more efficiently mediate antiviral responses. Strikingly, we found that the HEV ssRNA-induced IFN response is largely independent of the classical RLRs as well as the other RNA sensing pathways mentioned. However, it is in an IRF3-dependent and IRF7-dependent manner, which is consistent with the essential role of these two transcription factors in IFN production. Therefore, our present study strongly indicates that HEV RNA is likely recognized by an undefined or unknown cytosolic RNA-sensing system, which deserves further investigation. An unbiased biochemical screen or a genome-wide CRISPR-based screen represents the state of the art in identifying and investigating uncharacterized host factors possessing PRR function.

The IFN-mediated innate immune response forms a first line of cell-autonomous defense against pathogens. IFN activates the JAK–STAT pathway, leading to the induction of a wide array of ISGs. Functionally, they are divided into three groups: antiviral effector, negative regulator, and positive regulator. ISGs, such as MX1 and ISG15, are antiviral effectors. They control infection by directly targeting pathways and functions essential for pathogen life cycles. Some ISGs (e.g., SOCS, USP18) are negative regulators. They help resolve the IFN-induced state and the return to cellular homeostasis. ISGs, including RIG-I, MDA5, IRFs, and STAT1/2, serve as positive regulators to reinforce IFN responses. In our study, when the integrity of the JAK–STAT pathway was compromised, expression levels of ISGs induced by HEV were largely blocked. They include antiviral effectors (e.g., MX1 and ISG15) as well as positive regulators (e.g., RIG-I and MDA5) (Fig. 7). This in turn led to the attenuation of IFN expression and production. Therefore, the integrity of the JAK–STAT cascade is required for an HEV-triggered antiviral IFN response.

Our findings that the host cells can rapidly recognize the incoming HEV genomic ssRNA and evolve potent antiviral responses may explain the asymptomatic infection in the general population. A subset of patients with acute hepatitis eventually clears the infection through active virus–host interactions, although pregnant women bear high risk of developing fulminant hepatitis, with a mortality rate reaching 25%.[18] In immunocompromised patients, chronic infection has been widely reported, which is conceivably attributed to compromised innate and adaptive immunity.[35,36] Consistently, a recent study reported that persistent HEV infection in cell culture does not activate type I IFN, although it was accompanied by a type III IFN response.[37] In our study, we found that HEV RNA activates both type I and type III IFN responses, resulting in potent antiviral effects, which more likely reflected the infection phase with active virus–host interactions in HEV patients. Of note, other elements of the virus, in particular the HEV viral proteins, are also capable of modulating antiviral responses[38-41] and thus collectively determine the eventual infection course and clinical outcome.

In summary, we have demonstrated that HEV infection elicits an active antiviral IFN response

triggered by the viral genome. The incoming genomic ssRNA is the specific viral RNA species to trigger the response. This occurs in a RLR-independent, MAVS-independent, and β-catenin-independent but an IRF3-dependent and IRF7-dependent manner. Importantly, the integrity of the JAK–STAT pathway is required for the host antiviral response. These findings have revealed insights into HEV–host interactions and may provide new avenues for antiviral drug development.

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Supporting Information

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