Supplementary information to:

Hepatitis E virus infection activates NLRP3 inflammasome antagonizing interferon response but therapeutically targetable (Yang Li, et al.)

Supplementary materials and methods:

Plasmids and reagents

The plasmids containing the full-length GT1 HEV genome (Sar55/S17, GenBank Accession Number: AF444002), GT3 HEV genome (Kernow-C1 P6 clone, GenBank Accession Number: JQ679013), and GT7 dromedary camel HEV genome (GenBank Accession: KJ496144) were used.

FuGENE® HD Transfection reagent (E2311) was purchased from Promega, USA. Human IL-1 beta/IL-1F2 Quantikine ELISA Kit (DLB50, Bio-Techne and E4818-100, Biovision). Rabbit IL-1 beta/IL-1F2 Quantikine ELISA Kit (ml027836, MLBIO), BioLux® Gaussia Luciferase Assay Kit from Bioké. Lipopolysaccharide (LPS; L6529, Sigma) and adenosine 5-triphosphate disodium salt hydrate (ATP; A3377, Sigma) were dissolved in PBS. 12-O-Tetradecanoylphorbol 13-acetate (PMA, P1585, Sigma), JAK inhibitor I (sc-204021, Santa cruz), BAY11 7085 (sc-202490, Santa Cruz Biotech, CA), MCC950 (inh-mcc, Invivogen) and Belnacasan (VX-765) (S2228, Bio-Connect BV) were dissolved in DMSO. Recombinant Viral 136R/Y136 Protein (8976-BR-025, Biotechne) was dissolved in PBS. VECTASHIELD® Antifade Mounting Medium with DAPI (13285184) obtained from Fisher Scientific. Antibodies including Phospho-STAT1 (Tyr701) (58D6, Rabbit mAb, 9167), STAT1 (Rabbit mAb, 9172), IL-1β (D3U3E) (Rabbit mAb,12703), Cleaved IL-1beta (Asp116) (D3A3Z) (Rabbit mAb, 83186s), Cleaved Caspase-1 (Asp297) (D57A2) (Rabbit mAb, 4199s), NF-κB p65 (C22B4) (Rabbit mAb, 4764), anti-rabbit IgG(H+L),F(ab') 2 Fragment (4412s, Alexa Fluor 488 conjugate) were obtained

from Cell Signaling. Caspase-1 Antibody (14F468) (Mouse mAb, sc-56036), β-actin antibody (mouse mAb, sc-47778) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NLRP3 antibody (PA5-20838) (Rabbit, pAb) was obtained from Fisher Scientific. Anti-rabbit and anti-mouse IRDye-conjugated secondary antibodies (Li-Cor Biosciences, Lincoln, NE, USA) were also used. Hepatitis E Monoclonal Antibody was purchased from EMD Millipore Corporation, USA. CD14-eFluor450 (eBioscience, 48-0149-42), CD11b-PE-Cy7 (BD Pharmingen, 557743), CD206-APC (BD Pharmingen, 550889), CD163-PE (BD Pharmingen, 556018), CD68-FITC (eBioscience, 11-0689-73) and Aqua live/dead-AmCyan (Life Technologies, L34957).

qRT-PCR

Total RNA was isolated with a Macherey NucleoSpin RNA II Kit (Bioke, Leiden, The Netherlands) and quantified with a Nanodrop ND1000 (Wilmington, DE, USA). During RNA isolation, DNase was added to remove genomic DNA according to the manufacturer's instructions. cDNA was synthesized from 500 ng of RNA using a cDNA synthesis kit (TaKaRa Bio, Inc., Shiga, Japan). The cDNA of all targeted genes transcript were quantified by SYBR-Green-based (Applied Biosystems) real-time PCR on the StepOnePlus™ System (Thermo Fisher Scientific Life Sciences) according to the manufacturer's instructions. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene to normalize gene expression. The relative expression of targeted gene was calculated as $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT$ sample - ΔCT control (ΔCT = CT[targeted gene] - CT[GAPDH]).

MTT assay

Cells were seeded in 96-well plates, and 10mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) was added. The plate was incubated at 37°C with 5% CO2 for 3 hours, then the medium was removed, and 100 µL of DMSO was added to each well. The plate was incubated at 37°C

for 1 hour. The absorbance was read on the microplate absorbance reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 490 nm.

H&E staining

The liver biopsies were stained with Harris' hematoxylin solution for 6 hours at a temperature of 60–70 °C and were then rinsed in tap water until the water was colorless. Next, 10% acetic acid and 85% ethanol in water were used to differentiate the tissue 2 times for 2 hours and 10 hours, and the biopsies were rinsed with tap water. In the bluing step, we soaked the tissue in saturated lithium carbonate solution for 12 hours and then rinsed it with tap water. Subsequently, staining was performed with eosin Y ethanol solution for 48 hours.

Immunohistochemistry (IHC) staining

Liver biopsies were fixed in 10% formalin for 1.5 hour at room temperature, processed for paraffin embedding, and sectioned at a thickness of 4 μm. The sections were deparaffinized in xylene and rehydrated through graded ethanol treatment, followed by high pressure in citrate buffer (pH 6.0) for 3 min for antigen retrieval. Then they were blocked with 3% H_2O_2 in TBS for 15 min and further blocked with goat serum for 1 hour. The sections were incubated with anti-HEV ORF2 viral protein monoclonal antibody overnight at 4 °C, and incubated with goat anti-mouse for 15 min at 37°C. Subsequently, the sections were developed with diaminobenzidine (DAB), followed by counterstaining hematoxylin. Immunostained sections were scanned using Leica DFC400 digital camera and Leica Application Suite software (Leica Microsystems).

Confocal microscopy

PMA-differentiated THP-1 cells was cultured or infected with HEV for 24 hours. Cells were fixed in 4% paraformaldehyde at room temperature for 15 min. After washing three times with PBS, the cells were permeabilized with PBS

containing 0.1% Triton X-100 for 10 min, washed three times with PBS, and finally blocked with blocking buffer (Li-Cor Biosciences) for 1 hour. The cells were then incubated with the anti-NF-κB p65 (C22B4) (Rabbit mAb, 4764) antibody (1:100) or NLRP3 antibody (PA5-20838) overnight at 4°C, followed by incubation with anti-rabbit IgG(H+L),F(ab') 2 Fragment (4412s, Alexa Fluor 488 conjugate) for 1 hour. After washing three times, cells were incubated with VECTASHIELD® Antifade Mounting Medium with DAPI (13285184) for 5 min and then washed three times with PBS. Finally, the cells were analyzed using a confocal laser scanning microscope (Fluo View FV1000; Olympus, Tokyo, Japan).

Immunoblot analysis

Concentrated supernatant or lysates were heated at 95°C for 5 min. Proteins were subjected to a 15% or 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), separated at 100 V for 100 min, and electrophoretically transferred onto a PVDF membrane (pore size: 0.45 μm; Thermo Fisher Scientific Life Sciences) for 100 min with an electric current of 230 mA. Subsequently, the membrane was blocked with blocking buffer (Li-Cor Biosciences) in PBS containing 0.05% Tween-20. Membranes were incubated with primary antibodies overnight at 4°C. The membrane was washed 3 times, followed by incubation for 1 hour with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (1:5000; Li-Cor Biosciences) at room temperature. β-actin served as the loading standard. The membrane was scanned by Odyssey Infrared Imaging System (Li-Cor Biosciences). Results were visualized with Odyssey 3.0 software. Band intensity data of each immunoblot was also quantified by Odyssey Software.

HEV inoculation

Primary macrophages, THP-1 and HL60 macrophages were inoculated with cell culture-derived infectious HEV particles, inactivated HEV by 40 minutes

exposure to UV-light or heat inactivation at 90 °C for 30 minute. Supernatant and cell lysates were collected for ELISA, Western blot or qRT-PCR assays at indicated time points.

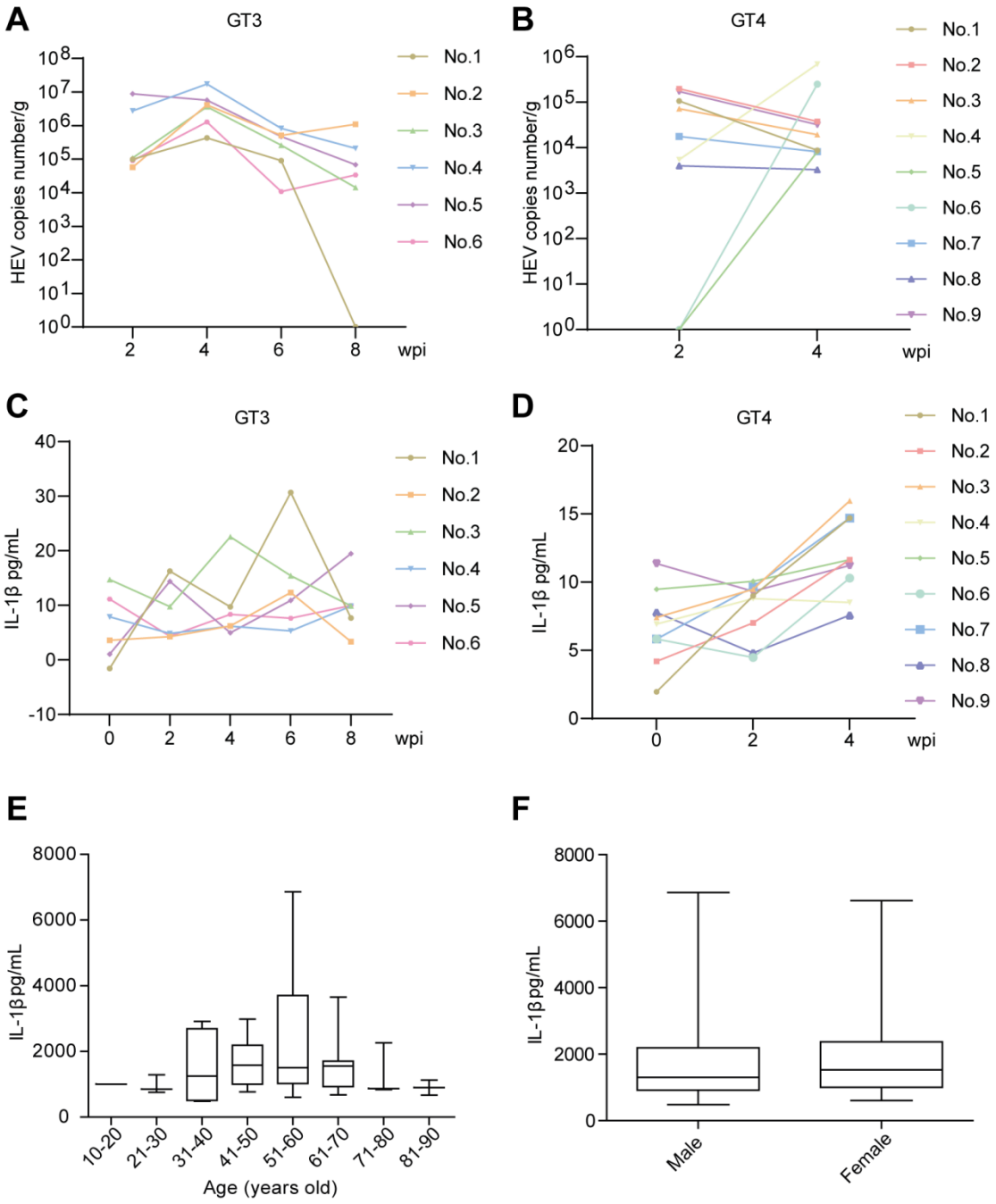
Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-1β in cell culture supernatant were measured by the ELISA Kit (BD Biosciences, San Jose, CA, USA). The concentrations of IL-1β in the serum of patients and rabbits were measured by Human IL-1 beta/IL-1F2 Quantikine ELISA Kit (E4818-100, Biovision) and Rabbit IL-1 beta/IL-1F2 Quantikine ELISA Kit (ml027836, MLBIO), respectively.

IFN response bioassay

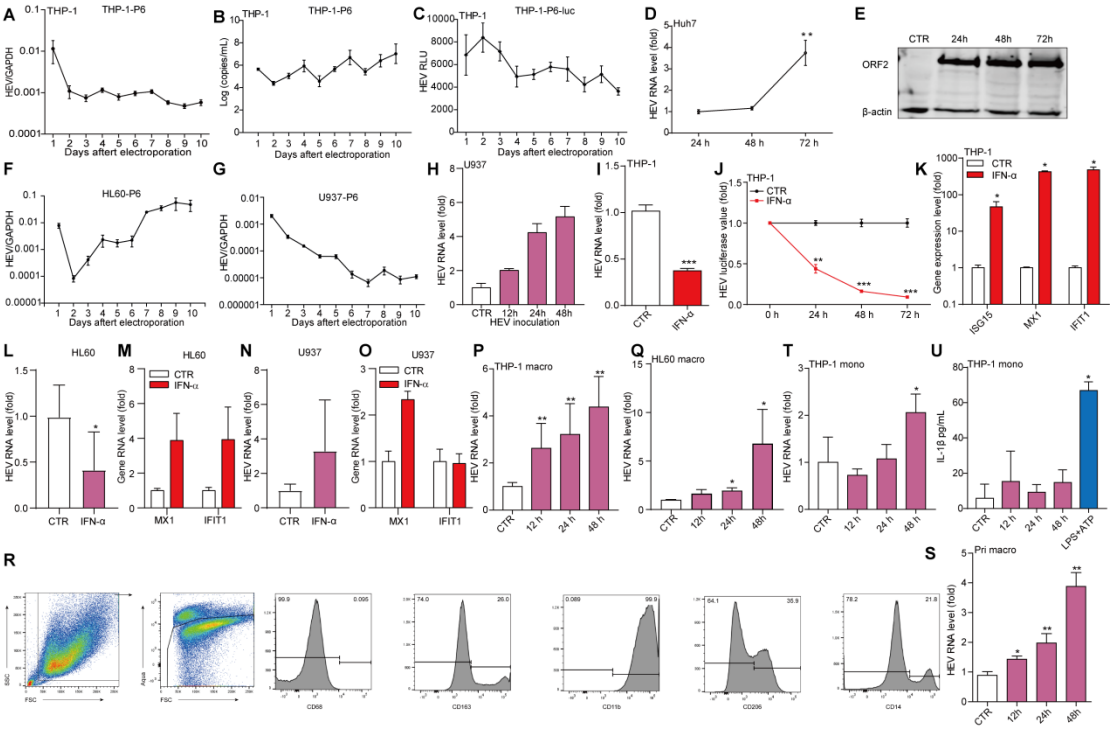
Differentiated THP-1 cells in 6-well plates were transfected with HEV RNA. After 4 hours, medium was removed, and cells were washed 3 times with PBS and cultured with fresh medium for 48 hours. The cultured supernatant was subsequently collected and filtered through a 0.45 μm pore size membrane and added to Huh7-ISRE-luciferase and hepatitis C virus (HCV)-luciferase reporter cells, as both models are sensitive to IFNs.

Supplementary figures



Supporting Fig. S1 Dynamics of different markers in HEV-infected rabbits and patients (A, B) Dynamics of genotype 3 HEV and genotype 4 HEV viral shedding into feces in each animal. (C, D) IL-1β level alteration after Genotype 3 HEV and genotype 4 HEV infection over time in each animal. (E) IL-1β levels in the serum of different age groups of patients (IgM positive) (n = 70) were

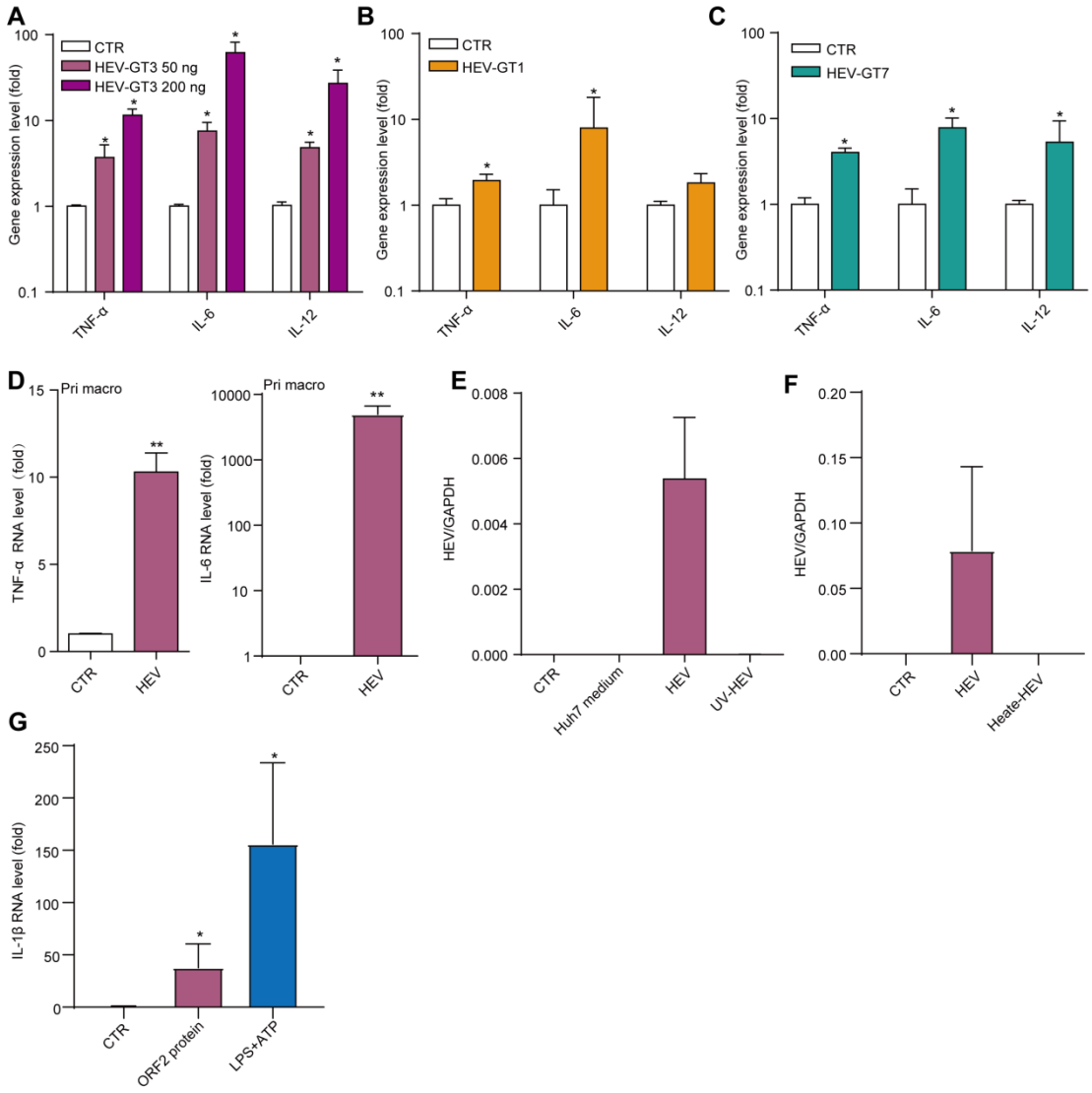
determined by ELISA. (F) IL-1β levels in the serum of different genders of patients (IgM positive) (n = 70). Data are means ± SD.



Supporting Fig. S2 Human monocytes or macrophages support the full life cycle of HEV infection, but monocytes failed to induce IL-1β secretion

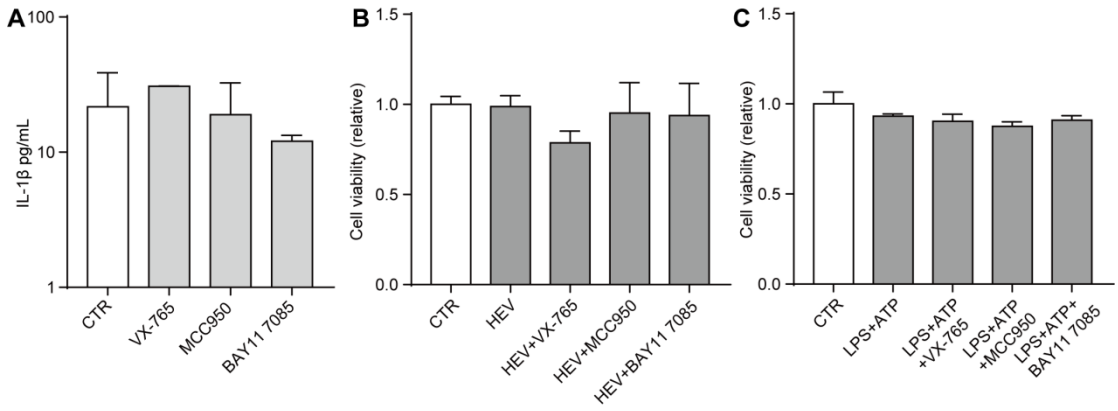
Quantitative RT-PCR analysis of HEV RNA level in lysates (A) or in supernatant (B) after full-length GT3 HEV genome RNA electroporation in THP-1 monocytes (n=6). (C) Analysis of HEV-related Gaussia luciferase activity after subgenomic HEV replicon RNA electroporation in THP-1 monocytes (n=6). (D) Quantitative RT-PCR analysis HEV RNA level in Huh7 cells inoculated with HEV produced from THP-1-P6 cells (n=6). (E) Immunoblot analysis of HEV ORF2 expression in Huh7 cells inoculated with HEV particles produced from THP-1-P6 cells. (F) Quantitative RT-PCR analysis of HEV RNA level after full-length GT3 HEV genome RNA electroporation in HL60 (n=4). (G, H) Quantitative RT-PCR analysis of HEV RNA level after full-length GT3 HEV genome RNA electroporation (n=5) or inoculation of infectious HEV particles in U937 monocytes (n=3). (I) Analysis of HEV mRNA level in THP-1 cells after IFN-α (1000 IU/mL) treatment for 48 hours (n=4). (J) Analysis of HEV-related Gaussia luciferase activity after IFN-α (1000 IU/mL) treatment for 48 hours (n=6). (K) Quantitative RT-PCR analysis of ISG15, MX1 and IFIT1 level in THP-1

monocytes cells after IFN-α (1000 IU/mL) treatment for 48 hours (n=4). (L) Analysis of HEV mRNA level in HL60 cells after IFN-α (1000 IU/mL) treatment for 48 hours (n=8). (M) Analysis of MX1 and IFIT1 mRNA level in HL60 cells after IFN-α (1000 IU/mL) treatment for 48 hours (n=3). (N) Analysis of HEV mRNA level in U937 cells after IFN-α (1000 IU/mL) treatment for 48 hours (n=7). (O) Analysis of MX1 and IFIT1 mRNA level in U937 cells after IFN-α (1000 IU/mL) treatment for 48 hours (n=3). (P, Q) Quantitative RT-PCR analysis of HEV mRNA level in THP-1 macrophages or HL60 macrophages after HEV infection for 12, 24 and 48 hours (n=6). (R) Flow cytometry-based phenotyping of primary monocyte derived macrophages. PBMCs from healthy donors were differentiated into macrophages by culturing monocytes with 50 ng/mL M-CSF for seven days. On day 7, macrophages were harvested and labeled for commonly used monocyte/macrophage markers CD68, CD163, CD11b, CD206 and CD14 to assess their phenotype. Cells were first gated based on their size and granularity (FSC vs. SSC) and viability (aqua live/dead viability dye). Marker expression was plotted in histograms. Histogram gates were set on unstained controls. (S) Quantitative RT-PCR analysis of HEV mRNA level in primary macrophages after HEV infection for 12, 24 and 48 hours (n=6). (T) Quantitative RT-PCR analysis of HEV mRNA level in THP-1 monocytes after HEV infection for 12, 24 and 48 hours (n=4). (U) IL-1β levels in the supernatant of THP-1 monocytes was determined by ELISA (n=4). Data were normalized to the control (CTR, set as 1). Data are means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. Abbreviation: CTR, control.

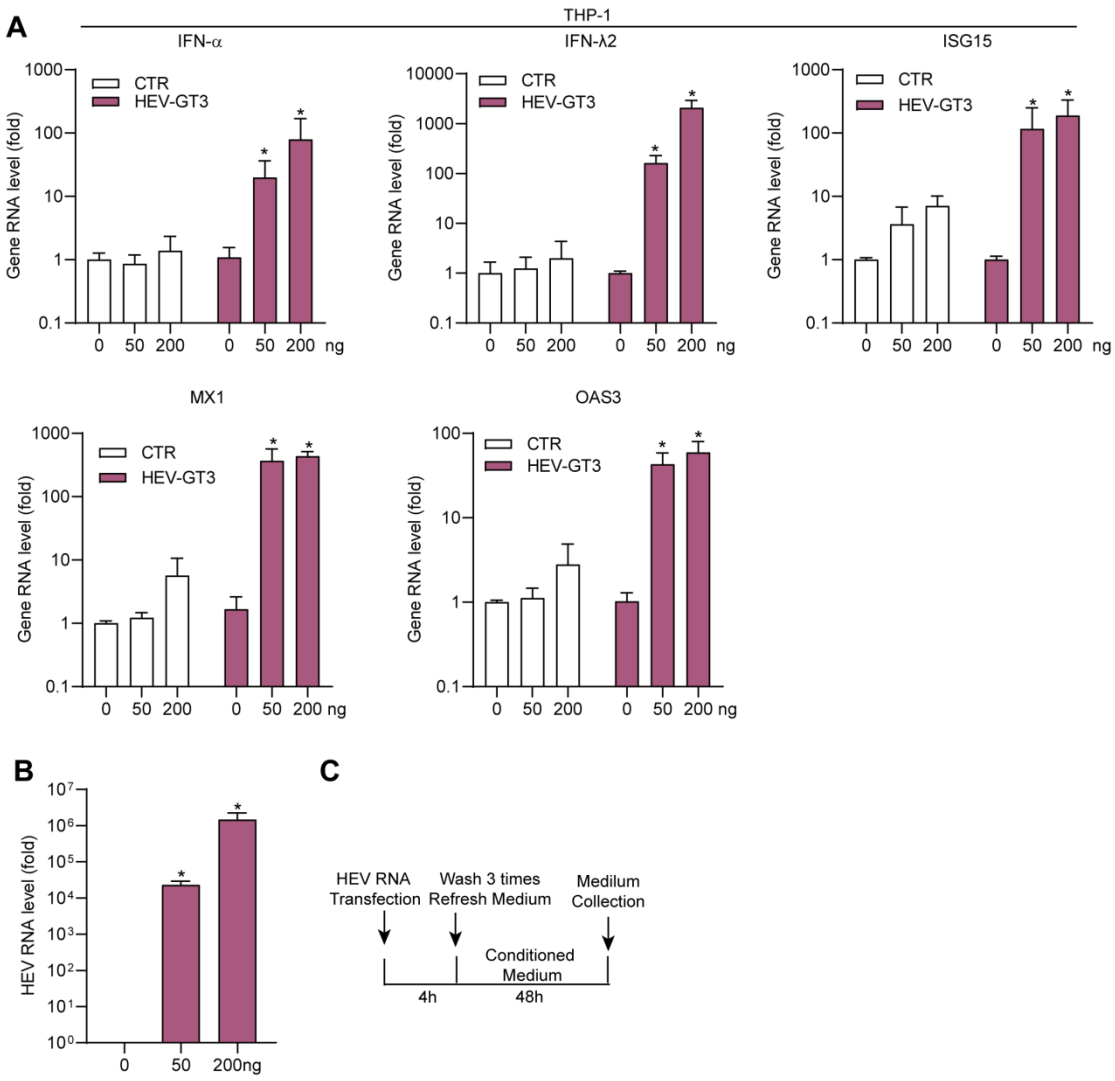


Supporting Fig. S3 HEV triggered expression of pro-inflammatory cytokines in macrophages. Quantitative RT-PCR analysis of mRNA level of cytokines in THP-1 macrophages after GT3 (A), GT1 (B) and GT7 (C) HEV transfection (n=4). (D) Quantitative RT-PCR analysis of mRNA level of cytokines in primary macrophages upon exposure to HEV particles (n=6). (E, F) Quantitative RT-PCR analysis of mRNA level of HEV upon exposure to live HEV, UV-inactivated HEV and heat-inactivated HEV particles in THP-1 macrophages (n=6). (G) Quantitative RT-PCR analysis of mRNA level of IL-1β in THP-1 macrophages after incubation of purified recombinant ORF2 protein for 24 hours (n=6). Data were normalized to the control (CTR, set as 1). Data

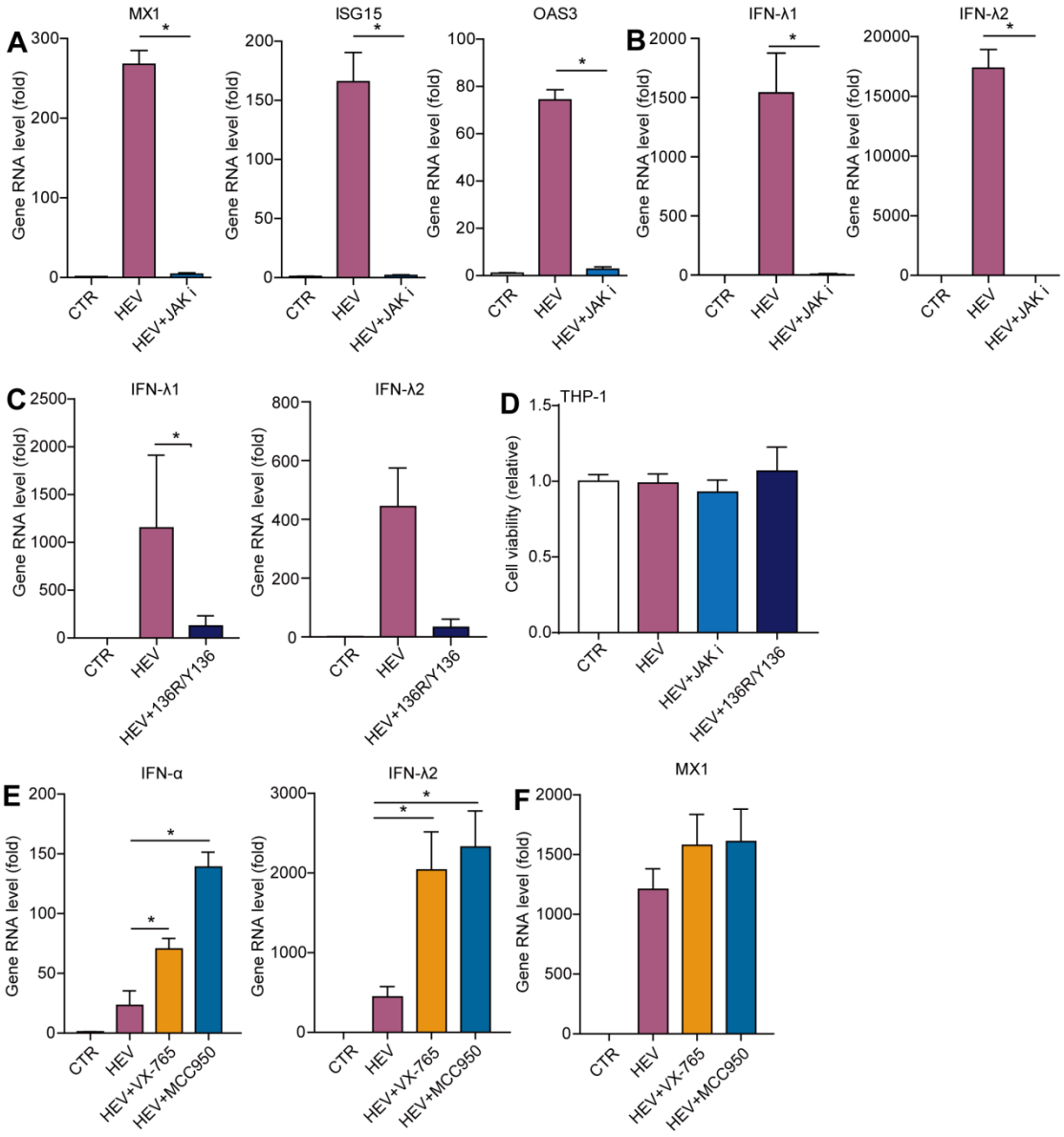
are means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. Abbreviation: CTR, control.



Supporting Fig. S4 VX-765, MCC950 and BAY11 7085 treatment on basal level of IL-1β secretion and cell viability (A) Quantitative analysis of IL-1β secretion by ELISA after 50 μM VX-765, 10 μM MCC950 or 10 μM BAY11 7085 treatment THP-1 macrophages for 24 hours (n=4). (B) MTT assay analysis of cell viability in THP-1 macrophages infected with HEV or HEV plus 50 μM VX-765, 10 μM MCC950 or 10 μM BAY11 7085 for 24 hours (C) MTT assay analysis of cell viability in THP-1 macrophages treated with LPS or LPS plus 50 μM VX-765, 10 μM MCC950 or 10 μM BAY11 7085 for 6 hours (n=4-6). Data were normalized to the control (CTR, set as 1). Data are means ± SD. Abbreviation: CTR, control.

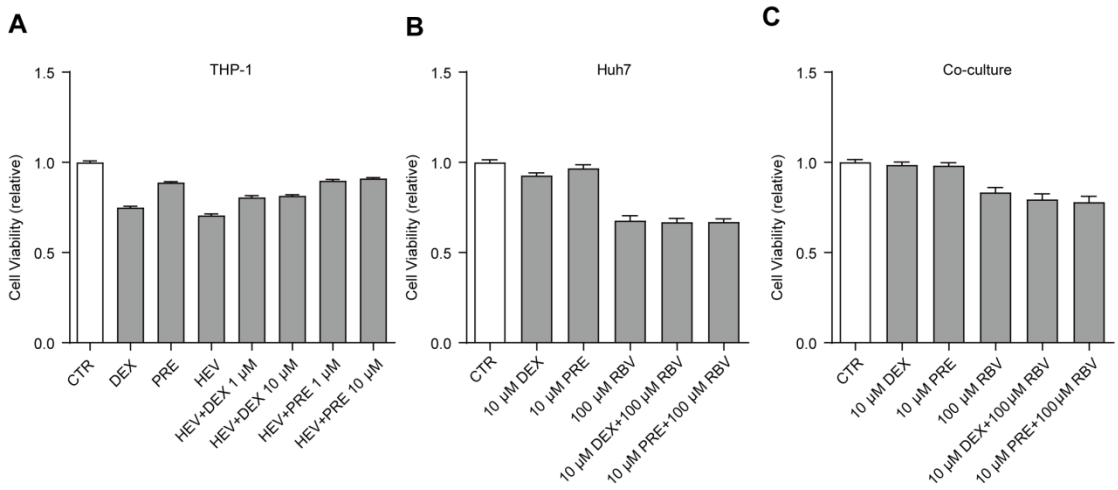


Supporting Fig. S5 transfection of HEV genomic RNA triggered IFN response in human macrophages (A) Quantitative analysis of IFNs and ISGs mRNA expression after HEV transfection in THP-1 macrophages (n=4-6). (B) Quantitative analysis of HEV mRNA expression after HEV transfection in THP-1 macrophages (n=4). (C) Schematic illustration of production of conditioned medium (supernatant). Cells were transfected with HEV RNA for 4 hours. The cells were then washed three times, and medium was refreshed. Cells were cultured for another 48 hours, and the supernatant was collected as conditioned medium. Data were normalized to control (CTR, set as 1). Data are means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. Abbreviation: CTR, control.



Supporting Fig. S6 Crosstalk between IFN response and inflammasome response in HEV-infected macrophages THP-1 macrophage cells were transfected with GT3 HEV RNA or GT3 HEV RNA plus JAK inhibitor 1 (10 μM) for 48 hours. ISGs (A) and IFNs (B) were quantified by qRT-PCR. THP-1 macrophages were transfected with GT3 HEV RNA or GT3 HEV RNA plus recombinant viral 136R/Y136 (1000 ng/mL) for 48 hours, and the expression levels of IFNs (C) were quantified at 48 hours by qRT-PCR (n=4). (D) MTT assay analysis of cell viability in THP-1 macrophage cells infected with HEV, and HEV plus JAK inhibitor 1 (10 μM) or recombinant viral 136R/Y136 (1000 ng/mL) for 48 hours. THP-1 macrophages were infected with HEV or HEV with

50 µM Casp-1 inhibitor (VX-765) or 10 µM NLRP3 inhibitor (MCC950) for 24 hours. The expression levels of IFNs (E) and ISGs (F) were quantified by qRT-PCR (n=4). Data were normalized to the control (CTR, set as 1). Data are means ± SD. Abbreviation: CTR, control.



Supporting Fig. S7 Cell viability Cell viability were measured in THP-1 macrophages, Huh7 and the co-culture system with 10 µM dexamethasone, 10 µM prednisone, 100 µM ribavirin, 10 µM dexamethasone plus 100 µM ribavirinand or 10 µM prednisone plus 100 µM ribavirin treatment for 24 hours (n=6-8). Data were normalized to the control (CTR, set as 1). Data are means ± SD. Abbreviation: CTR, control.

Supplementary tables

Supplementary table 1. Clinical characteristics of HEV-infected patients and healthy individuals

	HEV-infected patients (IgM^+/IgG^+) (N)	Healthy donors (IgM^-/IgG^-)	Healthy donors (IgM^-/IgG^+)
Total (N)	70	51	19
Gender (male)	56	32	17
Age (years)	53.4 ±12.9	42.4 ± 14.9	37.7 ± 8.9
IL-1β (pg/mL)	1733.1 ± 1234.3	715.1 ± 736.3	774.0 ± 595.1
ALP (IU/L ± SD)	129.7 ± 55.3 (60)		
Prealbumin (IU/L ± SD)	135.7 ± 74.8 (60)		
ALT (IU/L ± SD)	107.4 ± 300.4 (61)		
AST (IU/L ± SD)	74.6 ± 143.7 (60)		
AST/ALT	3.0 ± 13.0 (60)		
GGT (IU/L ± SD)	118.2 ± 123.8 (60)		
Total protein (g/L ± SD)	61.9 ± 9.0 (59)		
Albumin (g/L ± SD)	34.9 ± 5.8 (59)		
globulin(g/L ± SD)	27.1 ± 6.0 (59)		
Albumin/globulin (g/L ± SD)	1.34 ± 0.4 (60)		
Direct bilirubin (mcmole/L ± SD)	80.4 ± 104.1 (61)		
Total bilirubin (mcmole/L ± SD)	104.4 ± 141.1 (61)		
Direct bilirubin/Total bilirubin	0.8 ± 1.3 (61)		
Total bile acid (μmol/L ± SD)	134.6 ± 352.2 (60)		

Cholinesterase (IU/L ± SD)	4162.2 ± 2263.8 (60)		
Lactate dehydrogenase (IU/L ± SD)	201.8 ± 92.6 (60)		
Adenosine Deaminase (IU/L ± SD)	32.3 ± 11.0 (59)		
Urea (µmol/L)	4.57 ± 1.95 (47)		
Creatinine (µmol/L)	69.8 ± 22.9 (45)		
Estimate the glomerular filtration rate (mL/(min/1.73 m^2))	100.6 ± 22.4 (45)		
Uric acid (µmol/L)	277.7 ± 109.0 (44)		
Glucose Urea (µmol/L)	16.7 ± 61.9 (31)		
Potassium chlorine (µmol/L)	4.0 ± 1 (42)		
Chlorine (µmol/L)	104.6 ± 4.0 (42)		
Carbon dioxide binding capacity	26.1 ± 2.6 (42)		

Number, N; ALP, Alkaline phosphatase; AST, Aspartate transaminase; ALT, alanine aminotransferase; GGT, Gamma-glutamyl transferase. Mean ± standard deviation

Supplementary table 2. Primer sequences

Name	Sequence	Supplier
HEV-F	ATCGGCCAGAAGTTGGTTTTTAC	Sigma
HEV-R	CCGTGGCTATAACTGTGGTCT	Sigma
GAPDH-F	GTCTCCTCTGACTTCAACAGCG	Sigma
GAPDH-R	ACCACCCTGTTGCTGTAGCCAA	Sigma
ISG15-F	CTCTGAGCATCCTGGTGAGGAA	Sigma
ISG15-R	AAGGTCAGCCAGAACAGGTCGT	Sigma
MX1-F	GGCTGTTTACCAGACTCCGACA	Sigma
MX1-R	CACAAAGCCTGGCAGCTCTCTA	Sigma
STAT1-F	ATGGCAGTCTGGCGGCTGAATT	Sigma
STAT1-R	CCAAACCAGGCTGGCACAATTG	Sigma
OAS3-F	CCTGATTCTGCTGGTGAAGCAC	Sigma
OAS3-R	TCCCAGGCAAAGATGGTGAGGA	Sigma
OASL-F	GTGCCTGAAACAGGACTGTTGC	Sigma
OASL-R	CCTCTGCTCCACTGTCAAGTGG	Sigma
IFN-α-F	TGGGCTGTGATCTGCCTCAAAC	Sigma
IFN-α-R	CAGCCTTTTGGAACTGGTTGCC	Sigma
IFN-β-F	CTTGGATTCCTACAAAGAAGCAGC	Sigma
IFN-β-R	TCCTCCTTCTGGAACTGCTGCA	Sigma
IFN-λ1-F	GGAAGACAGGAGAGCTGCAACT	Sigma
IFN-λ1-R	AACTGGGAAGGGCTGCCACATT	Sigma
IFN-λ2-F	TCGCTTCTGCTGAAGGACTGCA	Sigma
IFN-λ2-R	CCTCCAGAACCTTCAGCGTCAG	Sigma
IFN-γ-F	GAGTGTGGAGACCATCAAGGAAG	Sigma
IFN-γ-R	TGCTTTGCGTTGGACATTCAAGTC	Sigma
IL-1β-F	CCACAGACCTTCCAGGAGAATG	Sigma
IL-1β-F	GTGCAGTTCAGTGATCGTACAGG	Sigma
TNF-α -F	CTCTTCTGCCTGCTGCACTTTG	Sigma

TNF-α -R	ATGGGCTACAGGCTTGTCACTC	Sigma
IL-6-F	AGACAGCCACTCACCTCTTCAG	Sigma
IL-6-R	TTCTGCCAGTGCCTCTTTGCTG	Sigma
IL-12-F	GACATTCTGCGTTCAGGTCCAG	Sigma
IL-12-R	CATTTTTGCGGCAGATGACCGTG	Sigma