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ORIGINAL ARTICLE

Immunocompromised rabbit model of chronic HEV reveals liver fibrosis and distinct efficacy of different vaccination strategies

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Abstract

Background and Aims: HEV infection can lead to chronicity and rapid progression to liver fibrosis and cirrhosis in immunocompromised organ transplant recipients. Robust animal models are urgently needed to study the pathogenesis and test the efficacy of vaccines and antiviral drugs in immunosuppressed settings.

Approach and Results: Cyclosporin A was used to induce immunosuppression. Rabbits were challenged with genotype 3 or 4 HEV (i.e., the rabbit-derived HEV3 and human-derived HEV3 or HEV4). We assessed HEV markers within 13 weeks post inoculation (wpi) and pathological changes by hematoxylin and eosin and Masson staining at 4, 8, or 13 wpi. Chronic HEV infection was successfully established in immunocompromised rabbits. HEV RNA and/or antigens were detected in the liver, kidney, intestine, urine, and cerebrospinal fluid samples. Chronically infected animals exhibited typical characteristics of liver fibrosis development. Intrahepatic transcriptomic analysis indicated activation of both innate and adaptive immunity. Establishment of HEV chronicity likely contributed to the inhibited T-cell immune response. Ribavirin is effective in clearing HEV infection in immunocompromised rabbits. Most interestingly, vaccination completed before immunosuppression conferred full protection against both HEV3 and HEV4 infections, but vaccination during immunosuppression was only partially protective, and the efficacy did not improve with increased or additional vaccine doses.

Conclusions: The immunocompromised rabbit model of both chronic HEV3 and HEV4 infection that was established captured the key features of chronic HEV infection in transplant patients, including liver fibrogenesis, and revealed the distinct effectiveness of vaccination administered before or under

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; COL, collagen; CsA, cyclosporin A; CSF, cerebrospinal fluid; DEG, differentially expressed gene; GGT, gamma-glutamyltransferase; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MMP, matrix metallopeptidase; SNV, single nucleotide variant; Wpi, weeks post inoculation.

immunosuppression. This rabbit model is valuable for understanding the pathogenesis of chronic hepatitis E, as well as for evaluating antiviral agents and vaccines.

INTRODUCTION

HEV has become the leading cause of acute viral hepatitis in humans worldwide with genotype 1–4 (HEV1-4) contributing to most cases in humans.[1,2] Although most HEV infections are acute and self-limiting, chronic HEV infection (defined as persistent infection for > 3 months) frequently occurs in immunocompromised individuals, such as organ transplant recipients, and can rapidly result in liver fibrosis and even fatal cirrhosis.[3–5] Most of the chronic cases are caused by HEV3,[3–7] while those caused by HEV4 have recently been described in China.[8,9] The underlying mechanisms leading to the progression of chronic hepatitis E, especially the progression of liver fibrosis, in immunocompromised patients are largely unknown.

Current treatment options for chronic hepatitis E are limited to reducing the dose of immunosuppressants or using the broad-spectrum antiviral agent ribavirin.[6,10] Of note, HEV polymerase mutations occur during ribavirin treatment. The influence of these mutations in ribavirin treatment remains controversial.[11–15] Reports have described treatment failure or relapse associated with these mutations,[11–13] but a recent large retrospective multicenter study showed that these mutations did not have a negative impact on HEV clearance.[15] The lack of a specific anti-HEV drug for treating chronic hepatitis E necessitates a prevention strategy for this high-risk group. Hecolin, the only licensed HEV vaccine worldwide, can effectively protect the general population from HEV1 and HEV4 infection.[16,17] However, the vaccine efficacy against HEV3 and immunocompromised individuals remains unknown.

Given the burden and the unmet medical need for chronic hepatitis E, a robust and clinically relevant animal model is urgently needed to study the pathogenesis and disease progression of HEV, for preclinical drug testing, and for vaccine evaluation. This study aimed to establish rabbit-based animal models to recapitulate both HEV3 and HEV4 chronic infections. To mimic the clinical setting, cyclosporin A (CsA), a classic and commonly used immunosuppressant for organ transplant recipients, was used to induce immunosuppression in rabbits. Subsequent inoculation with HEV3 and HEV4 successfully established a chronic infection. The pathogenic profile was determined. Furthermore, the efficacies of a vaccine and antiviral treatment were assessed.

METHODS

Induction of immunosuppression in rabbits

Immunocompromised rabbits were prepared using CsA (Figure S1A). Trough blood concentrations were monitored using the high-performance liquid chromatography–mass spectrometry method. The animal experiments were approved by the Committee of Laboratory Animal Welfare and Ethics, Peking University Health Science Center and the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Virus challenge

Rabbits were inoculated with 1 ml (about 5×10^6 copies/ml) of rabbit-derived HEV-3ra, human-derived HEV3, or human-derived HEV4 strain (GenBank accession Nos. JX109834, MF996356, and MT993748). The subgenotypes of the two human-derived strains are 3b and 4d, respectively.

Sample collection and measurements

Feces and serum were collected weekly. Feces were processed into fecal suspensions and tested for HEV RNA. Serum was tested for alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), anti-HEV antibodies, HEV antigens, and HEV RNA. Urine, bile, cerebrospinal fluid (CSF), and tissue specimens were collected for HEV RNA and/or antigen detection, and liver tissues for transcriptomics analysis.

More details were provided in the Supporting Information.

RESULTS

Induction of immunosuppression in rabbits by CsA treatment

Each rabbit was treated with CsA for 7 consecutive days with 15 mg/kg daily in the loading phase, which was subsequently reduced to 10 mg/kg every 2 days as

the maintenance phase. Rabbits were challenged with HEV strains between these two phases (Figure S1A). Trough blood concentration was maintained at therapeutic concentration according to clinical practice[18] (100–200 ng/ml; Figure S1B).

Chronic infection of rabbit-derived HEV-3ra in immunocompromised rabbits

CsA-treated and untreated rabbits were challenged with rabbit-derived HEV-3ra strain or mock (CsA-HEV-3ra group, *n* = 15; CsA-mock group, HEV-3ra group, and mock group, *n* = 12; Figure 1A). According to the European Association for the Study of the Liver guidelines,[10] chronic HEV infection is defined as HEV-RNA persistence for more than 3 months. Therefore, rabbits were monitored until 13 weeks post inoculation (wpi).

The duration of fecal virus shedding was 4–8 weeks in the HEV-3ra group, indicating an acute self-limiting infection. Viremia was low and was occasionally detectable. Antigenemia was detected at 3–5 wpi in relatively low amounts in some rabbits (Figure 1B,C and Figure S2). In the CsA-HEV-3ra group, all rabbits showed stable and robust fecal virus shedding during the entire monitoring period. Low viremia was detected for 13 weeks. Antigenemia was detected early at 1 wpi and gradually increased over time after inoculation (Figure 1B,D and Figure S3). At 1 and 2 wpi, GGT levels were significantly higher in rabbits in the CsA-HEV-3ra group than those in the HEV-3ra group. In general, the two HEV-inoculated groups appeared to have higher levels of serum ALT or AST, but the difference was not statistically significant (Figure S4).

In the HEV-3ra group, anti-HEV seroconversion occurred in two rabbits at 2 wpi and in an additional six rabbits at 4 wpi. All rabbits eventually showed seroconversion before 9 wpi and remained anti-HEV-positive once seroconverted. In contrast, only two rabbits in the CsA-HEV-3ra group were seroconverted at 6 and 7 wpi, respectively, and subsequently remained anti-HEV positive. Interestingly, another two rabbits showed a weak and transient anti-HEV positivity: one at 5 wpi and the other at 6 and 8 wpi. The others remained negative for anti-HEV during the entire monitoring period (Figure 1E).

At 4 and 8 wpi, all sacrificed rabbits from the HEV-3ra or CsA-HEV-3ra groups were positive for HEV RNA in bile and liver tissues. The CsA-HEV-3ra group displayed a significantly higher viral load than the HEV-3ra group in the liver at 4 and 8 wpi, and in the bile at 8 wpi. At 13 wpi, all sacrificed rabbits in the CsA-HEV-3ra group were still positive for HEV RNA in the bile and liver, while none were positive in the HEV-3ra group. Hepatic HEV antigen was only detected at 4 wpi in two of three (66.7%) sacrificed rabbits in the HEV-3ra group, whereas all sacrificed rabbits in the CsA-HEV-3ra group were positive for HEV antigen and had a significantly higher amount of HEV antigen at each time point (Figure 1F). HEV infection was not observed in the CsA-mock and mock groups. The collective results indicate the successful establishment of a stable chronic infection in the CsA-induced immunocompromised rabbits.

Evidence of extrahepatic infection in HEV-3ra inoculated rabbits

Several types of extrahepatic manifestations have been reported in association with HEV infection, particularly neurological and renal manifestations.[19] Therefore, we screened for HEV RNA and antigens in urine and CSF in these rabbits. All rabbits from the HEV-3ra group tested negative for HEV RNA in the available urine and CSF samples at 4, 8, and 13 wpi. In contrast, HEV RNA was detected in the urine and CSF samples from the CsA-HEV-3ra group (Figure 2A). As for HEV antigen, only two rabbits from the HEV-3ra group sacrificed at 4 wpi were positive for urinary HEV antigen. Strikingly, all but one rabbit sacrificed at 13 wpi in the CsA-HEV-3ra group was positive for urinary HEV antigen. The CsA-HEV-3ra group had a significantly higher HEV antigen burden in urine at each time point. HEV antigen was only detected in two CSF samples from the CsA-HEV-3ra group (Figure 2B).

We also measured HEV viral load in the intestinal and kidney tissues of chronically infected rabbits. HEV RNA is frequently detected in different parts of the intestine, including the duodenum, jejunum, cecum, and colon. The viral load in the intestine was stable during infection, ranging from 10^6 to 10^8 copies/g. In kidney tissues, HEV RNA was detected in 25% (1 of 4), 50% (2 of 4), and 100% (7 of 7) sacrificed rabbits at 4, 8, and 13 wpi, respectively (Figure 2C).

Intrahepatic host response to acute and chronic HEV infection mapped by transcriptome analysis

Liver transcriptome analysis was performed in rabbits without CsA treatment at 4 wpi and 13 wpi using three rabbits at each time point in each group. Compared with the mock group, 373 differentially expressed genes (DEGs) were evident in the HEV-3ra group at 4 wpi. Of these, 236 DEGs were up-regulated (Figure S5A,B), including many interferon (IFN)–related genes (IFN regulatory factor 7 [*IFR7*], IFN-stimulated gene 15 [*ISG15*], IFN-induced protein with tetratricopeptide repeats 3/5 [*IFIT3/5*], IFN-induced protein 44 [*IFI44*], interferon induced with helicase C domain 1 [*IFIH1*], radical S-adenosyl methionine domain containing 2 [*RSAD2*], and DExD/H-box helicase 58 [*DDX58*]).

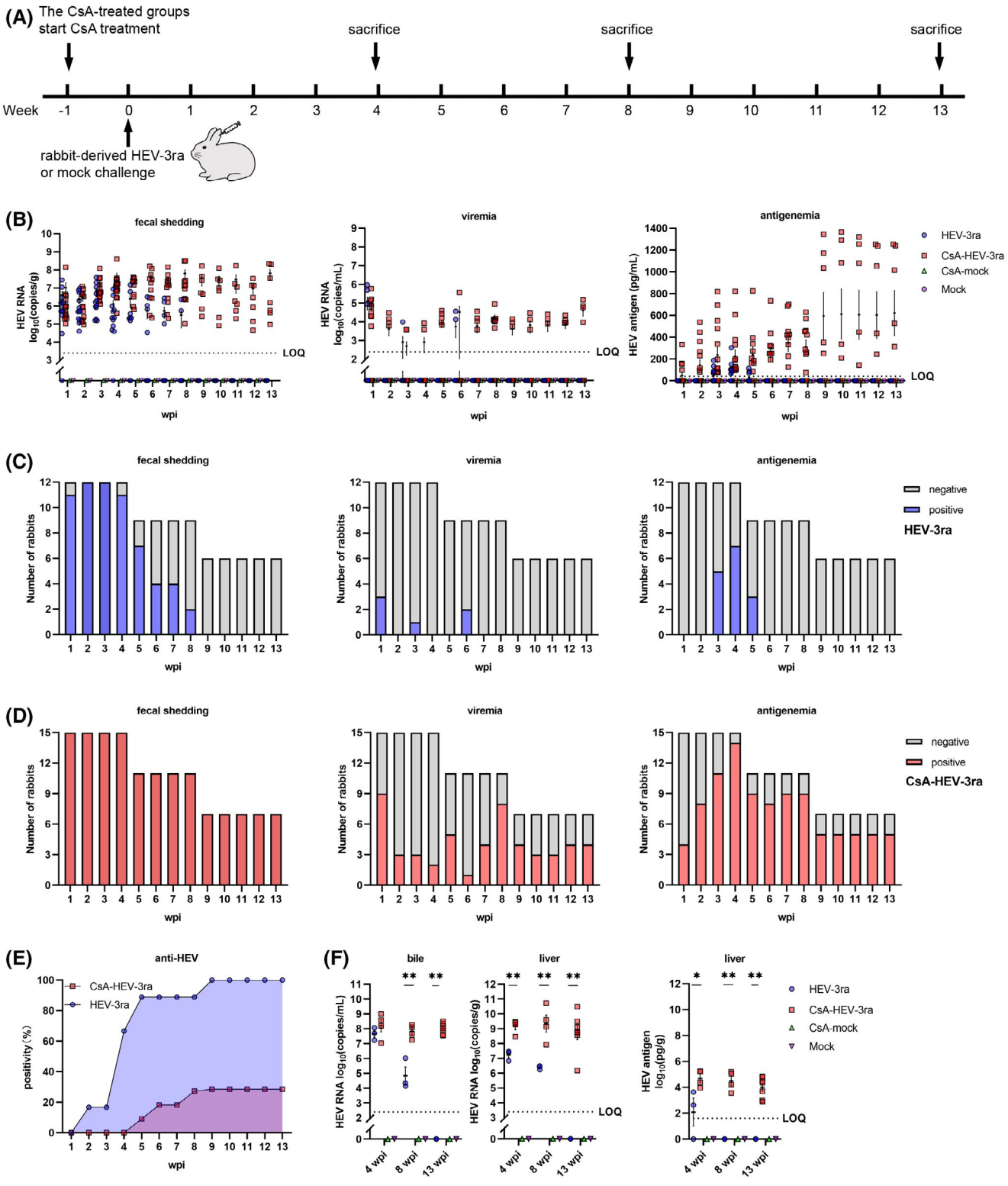


FIGURE 1 Infection of rabbit-derived HEV3 (HEV-3ra) strain in rabbits treated with or without cyclosporin A (CsA). (A) Experimental scheme. CsA-treated rabbits were challenged with HEV-3ra strain or mock (CsA-HEV-3ra group, $n = 15$; CsA-mock group, $n = 12$). Meanwhile, CsA-untreated rabbits were also challenged with HEV-3ra strain or mock (HEV-3ra group and mock group, $n = 12$/group). (B) Quantification of fecal HEV RNA, serum HEV RNA, and serum HEV antigen over time after inoculation. (C,D) Number of rabbits with HEV fecal shedding, viremia, or antigenemia from HEV-3ra group (C) and CsA-HEV-3ra group (D) over time after inoculation. (E) Weekly anti-HEV positive rates. (F) Quantification of HEV RNA in bile and liver, and HEV antigen in liver. Error bars indicate mean ± SEM. Comparison of HEV-3ra group and CsA-HEV-3ra group, with significance, $^{*}p < 0.05$; $^{**}p < 0.01$. LOQ, limit of quantification

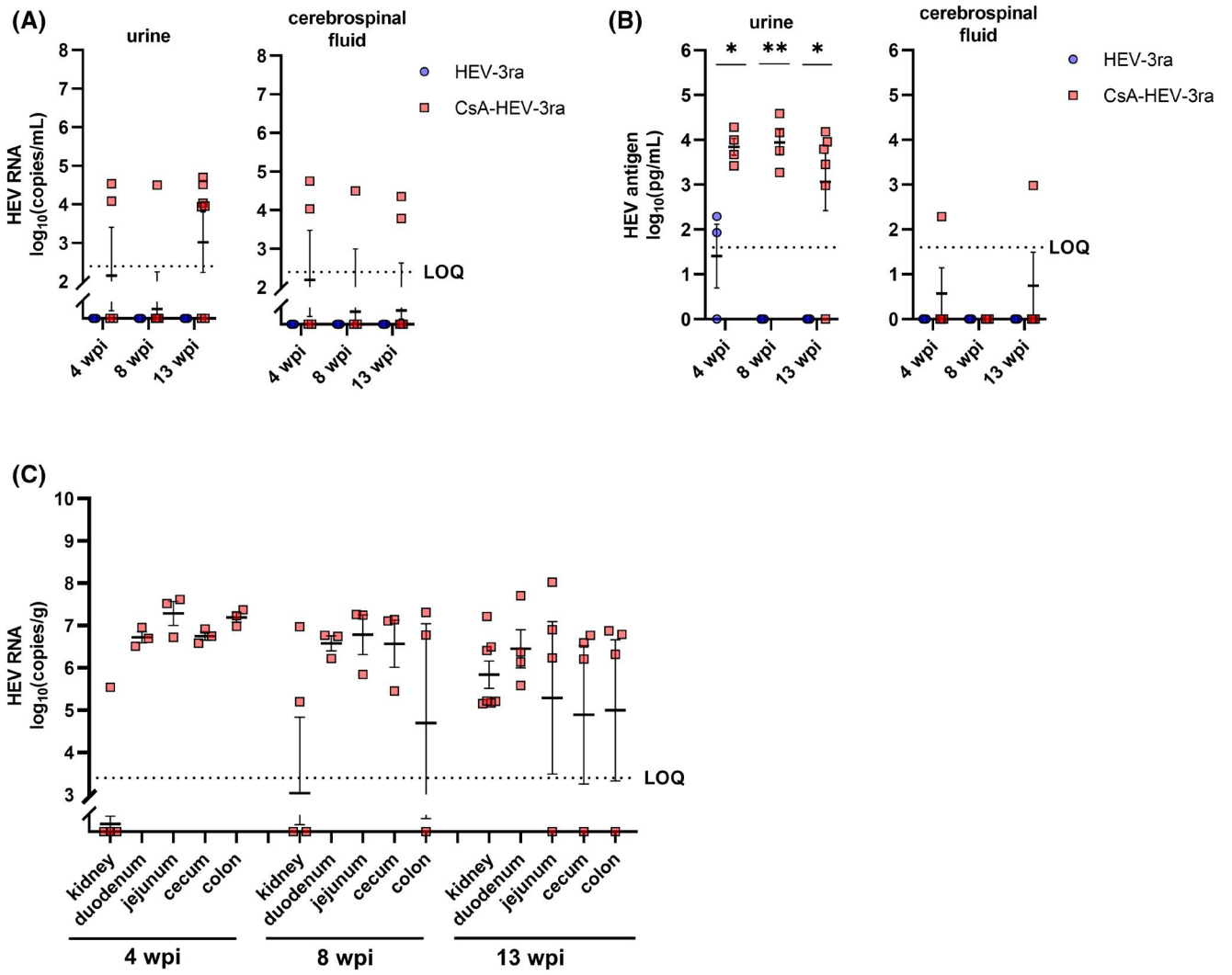
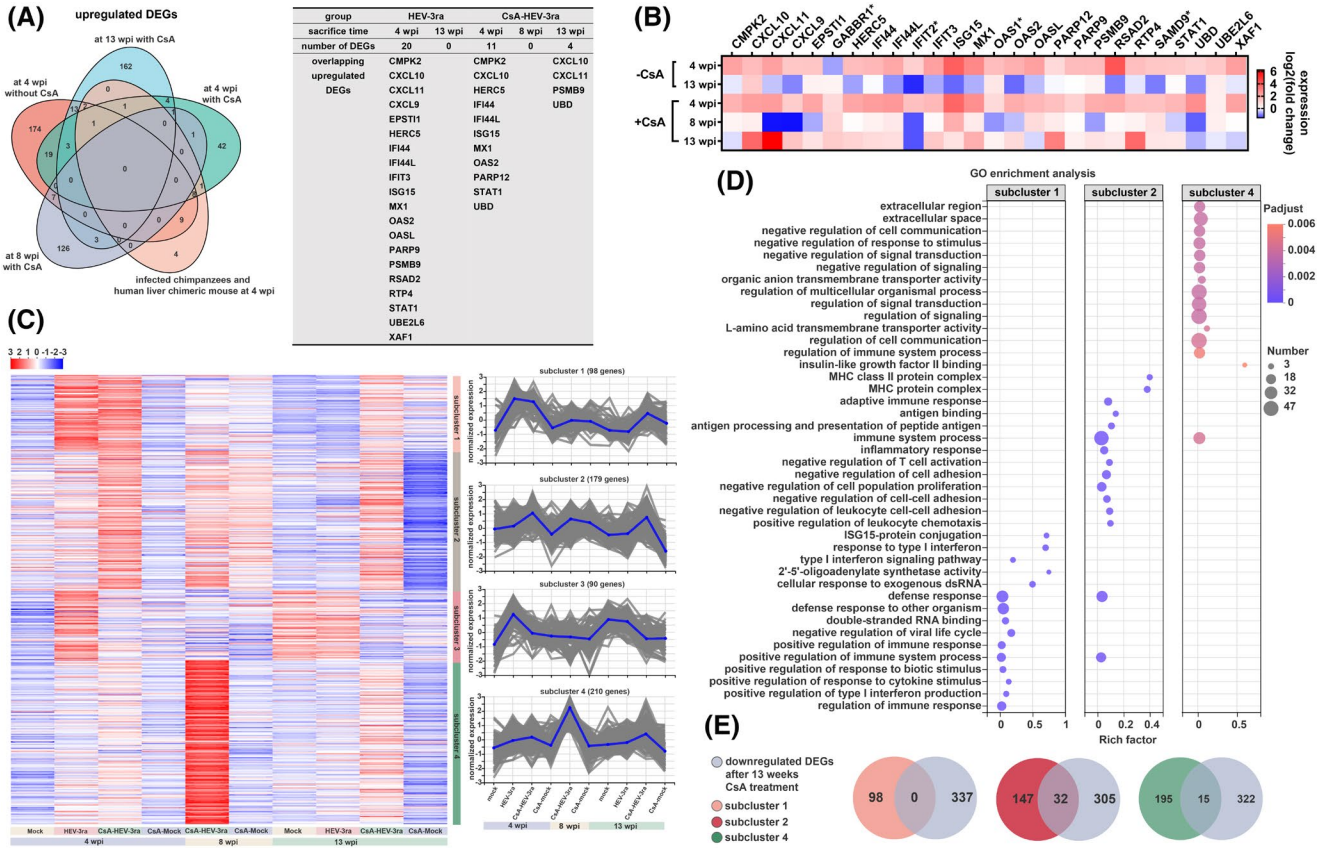


FIGURE 2 Extrahepatic HEV markers in rabbits inoculated with rabbit-derived HEV3 (HEV-3ra). (A,B) Quantification of HEV RNA (A) and HEV antigen (B) in urine and cerebrospinal fluid (CSF). We failed to collect some CSF samples in some rabbits, and detection of HEV RNA was prioritized when the sample did not have enough volume to test both HEV RNA and HEV antigen. Urine and CSF samples in the HEV-3ra group tested for both HEV RNA and antigen, (n = 3 at each timepoint). Urine samples in the CsA-HEV-3ra group tested for HEV RNA at 4, 8, and 13 weeks post inoculation (wpi; n = 4, 4, and 7, respectively) and for HEV antigen (n = 4, 4, and 6, respectively). CSF samples in the CsA-HEV-3ra group tested for HEV RNA at 4, 8, and 13 wpi (n = 4, 3, and 5, respectively) and for HEV antigen (n = 4, 3, and 4, respectively). Comparison of HEV-3ra group and CsA-HEV-3ra group, with significance: $^{*}p < 0.05$; $^{**}p < 0.01$. (C) Quantification of HEV RNA in kidney and intestine (duodenum, jejunum, cecum, and colon) tissues of CsA-HEV-3ra group rabbits. Kidney tissues at 4, 8, and 13 wpi (n = 4, 4, and 7, respectively). Intestine tissues at 4, 8, and 13 wpi (n = 3, 3, and 4, respectively). Each symbol represents one tested sample. Error bars indicate mean ± SEM

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that these up-regulated DEGs were significantly enriched in pathways related to viral infections or antiviral responses, such as influenza A, HCV, and RIG-I-like receptor signaling pathways (Figure S5C). At 13 wpi, when rabbits of the HEV-3ra group were recovered, only 45 DEGs remained, and no significantly enriched KEGG pathways were identified, which was consistent with viral clearance at this time point (Figure S5A). For the CsA-induced immunocompromised rabbits, liver transcriptome analysis was performed at 4, 8, and 13 wpi using three rabbits at each time point in each group. The numbers of up-regulated DEGs in the CsA-HEV-3ra group versus the CsA-mock group increased over time, with 113 at 4 wpi, 138 at 8 wpi, and 226 at 13 wpi (Figure S5A,B). KEGG analysis revealed that the up-regulated DEGs at 4 and 13 wpi were both enriched in pathways related to viral infections (Figure S5D). No significantly enriched KEGG pathways of the up-regulated DEGs at 8 wpi were identified. Previous studies have identified a total of 35 up-regulated DEGs in infected chimpanzees (34 genes) using microarray chips[20] and in humanized mice at 4 wpi (10 genes) by quantitative PCR.[21] In this rabbit model, 26 DEGs were detected by RNA sequencing. Most (84.6%, 22 of 26) were also

significantly up-regulated, mostly in the acute phase (4 wpi) of immunocompetent rabbits (Figure 3A,B).

These up-regulated DEGs identified in infected rabbits were pooled into one gene set containing 577 genes in total. *K*-means cluster analysis (K = 4) using this gene set (Figure 3C) was followed by Gene Ontology (GO) enrichment analysis for each of the four subclusters. Three of the subclusters showed distinct and unique gene-expression profiles, except for subcluster 3 (Figure 3C,D). Subcluster 1 contained 98 genes up-regulated during HEV infection regardless of CsA treatment, and primarily enriched in innate immunity and antiviral defense response. Subcluster 2 consisted of 179 genes characterized by up-regulation in the immunocompromised rabbits with HEV-3ra infection at all three timepoints and was primarily enriched in adaptive immunity, especially T-cell immune response. Subcluster 3 contained 90 genes but was not



group	HEV-3ra		CsA-HEV-3ra		
sacrifice time	4 wpi	13 wpi	4 wpi	8 wpi	13 wpi
number of DEGs	20	0	11	0	4
overlapping upregulated DEGs	CMPK2		CMPK2		CXCL10
	CXCL10		CXCL10		CXCL11
	CXCL11		HERC5		PSMB9
	CXCL9		IFI44		UBD
	EPSTI1		IFI44L		
	HERC5		ISG15		
	IFI44		MX1		
	IFI44L		OAS2		
	IFIT3		PARP12		
	ISG15		STAT1		
	MX1		UBD		
	OAS2				
	OASL				
	PARP9				
	PSMB9				
	RSAD2				
	RTP4				
	STAT1				
	UBE2L6				
	XAF1				

FIGURE 3 Intrahepatic host response to acute and chronic HEV infection mapped by transcriptome analysis. (A) Venn diagram displaying overlapping up-regulated differentially expressed genes (DEGs) in infected rabbits and two previous studies of infected chimpanzees and human liver chimeric mouse at 4 wpi. Gene names of the overlapping up-regulated DEGs are listed in the table on the right. (B) All 26 up-regulated DEGs from the two previous studies were plotted in alphabetical order. Color code of the heatmap represents fold change of the expression in the HEV-3ra group versus mock group (−CsA) or in the CsA-HEV-3ra group versus CsA-mock group (+CsA) at different time points, respectively. The four genes marked with asterisks were not significantly up-regulated in infected rabbits in either case. (C) Cluster analysis. The up-regulated DEGs identified in infected rabbits were pooled into one gene set containing 577 genes in total and were clustered using *K*-means clustering algorithm (K = 4). Color code of the heatmap represents the values of TPM (transcripts per million reads). The line graphs on the right represent the normalized expressions of the genes from the four subclusters, respectively. In each line graph, each gray line represents one gene, and the blue line represents the mean normalized expressions of all genes in this subcluster. (D) Dot plot visualization of enriched GO terms in subclusters 1, 2, and 4. The color of the dots represents the Benjamini-Hochberg–corrected p value (p adjust) for each enriched Gene Ontology (GO) term, and the size represents the number of enriched genes. Rich factor represents the ratio of the number of genes enriched in the GO term to the annotation genes in the background. p adjust < 0.05 was considered significant. Top 15 significantly enriched pathways are shown and ordered according to significance. (E) Venn diagram displaying overlapping down-regulated DEGs in CsA-mock group versus mock group at 13 wpi and up-regulated DEGs in subclusters 1, 2 and 4, respectively (n = 3 for each group at each time point). Abbreviations: CMPK, cytidine/uridine monophosphate kinase 2; CXCL, C-X-C motif chemokine ligand; EPSTI1, epithelial stromal interaction 1; GABBR1, gamma-aminobutyric acid type B receptor subunit 1; HERC5, HECT and RLD domain containing E3 ubiquitin protein ligase 5; IFI, interferon-induced protein; IFIT, interferon-induced protein with tetratricopeptide; ISG, interferon-stimulated gene; MHC, major histocompatibility complex; MX1, MX dynamin like GTPase 1; OAS, 2'-5'-oligoadenylate synthetase; PARP, poly(adenosine diphosphate ribose) polymerase; PSMB, proteasome subunit beta; RSAD2, radical S-adenosyl methionine domain containing 2; RTP4, receptor transporter protein 4; SAMD9, sterile alpha motif domain containing 9; STAT1, signal transducer and activator of transcription 1; UBD, ubiquitin D; UBE2L6, ubiquitin conjugating enzyme E2 L6; XAF1, X-linked inhibitor of apoptosis associated factor 1

significantly enriched in any GO pathways. Subcluster 4 contained 210 genes characterized by up-regulation in immunocompromised rabbits with HEV-3ra infection at 8 and 13 wpi. These genes were enriched in pathways related to collagen deposition, cell damage and repair, or some pro-fibrogenic phenotypes, such as collagen-containing extracellular matrix, regulation of apoptosis, and regulation of wound healing. There were 337 down-regulated DEGs in the CsA-mock group versus the mock group at 13 wpi. Of these down-regulated DEGs, 32 genes were in subcluster 2 (Figure 3E), the subcluster that is enriched in T-cell immune response, the target of the CsA immunosuppressant.

Viral adaptation in HEV-3ra-infected rabbits

To explore the viral adaptation of the HEV-3ra strain in this model, we further analyzed the HEV variants using the transcriptome data. Taking the sequence of HEV-3ra inoculum as the reference, many single nucleotide variants (SNVs) were found. Of these, 336 mutations of SNVs were synonymous (data not shown), and 41 were nonsynonymous (Table S1). Of these nonsynonymous SNVs, four nonsynonymous mutations (L190M, P271H, and S634P in ORF1; V581I in ORF2) were commonly seen in most of the infected rabbits. It should be noted that 34 exclusive nonsynonymous mutations were found in one rabbit from HEV-3ra group sacrificed at 4 wpi. Three nonsynonymous mutations were only found in one rabbit each from the HEV-3ra and CsA-HEV-3ra groups sacrificed at 4 wpi, respectively. In general, HEV-3ra exhibited more SNVs of both synonymous and nonsynonymous in rabbits without CsA treatment than immunocompromised rabbits at 4 wpi (Figure 4A). In immunocompromised rabbits, both synonymous and nonsynonymous SNVs of HEV-3ra were decreased over time (Figure 4B), but the differences were all not statistically significant.

Chronic infection of human-derived HEV3 and HEV4 strains in immunocompromised rabbits

To better mimic HEV infection in transplant patients, we next challenged the immunocompromised rabbits with human-derived HEV3 (CsA-hHEV3 group, n = 6) or HEV4 strain (CsA-hHEV4 group, n = 6) (Figure 5A).

All rabbits in the CsA-hHEV3 group showed stable fecal virus shedding for at least 8 weeks during the 13-week-long monitoring period. Four of six rabbits (66.7%) showed fecal virus shedding until 13 wpi, except for one with a transient negative fecal virus shedding between 11 and 12 wpi. Serum HEV RNA was occasionally detectable but much less frequent than in

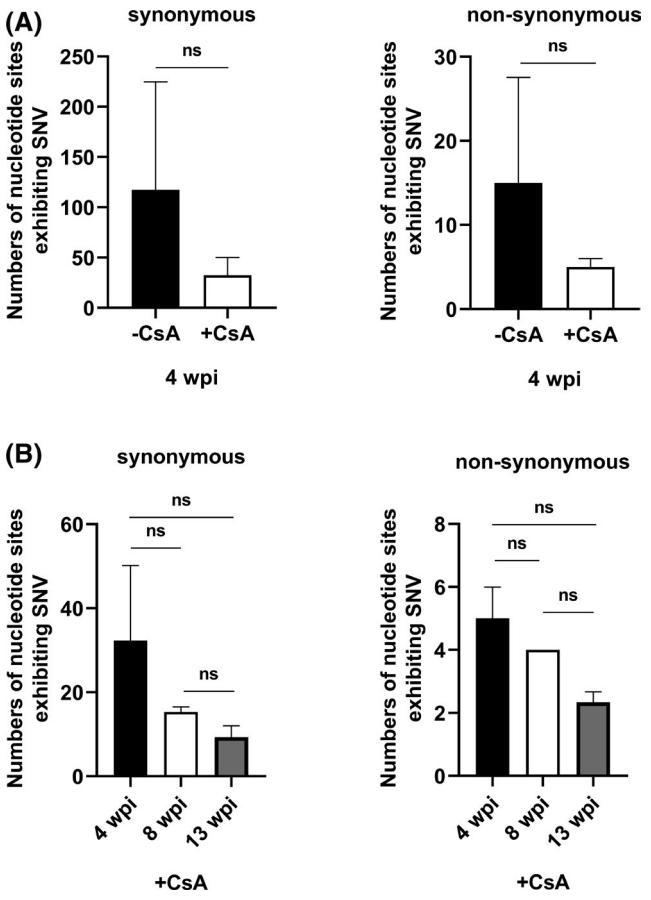


FIGURE 4 Total number of sites exhibiting variations in the HEV-3ra genome. (A) Number of sites exhibiting synonymous (left) and nonsynonymous (right) single nucleotide variants (SNVs) identified in rabbits sacrificed at 4 wpi from the HEV-3ra and CsA-HEV-3ra groups (n = 3/group). (B) Number of sites exhibiting synonymous (left) and nonsynonymous (right) SNVs identified in rabbits from the CsA-HEV-3ra group over time (n = 3/time point). ns, not significant

the CsA-HEV-3ra group. Antigenemia was only present in one rabbit at 2 and 3 wpi (Figure 5B,C and Figure S6). No rabbit seroconverted to anti-HEV (Figure 5E) during monitoring. We did not observe abnormal elevation of serum ALT, AST, or GGT levels (Figure S7). Four rabbits were sacrificed at 13 wpi. Of these, 75% (3 of 4) were positive for HEV RNA in the bile and liver, consistent with fecal virus shedding (Figure 5F). Only one rabbit tested positive for HEV RNA in both urine and CSF samples. All urine and CSF samples tested negative for HEV antigen (Figure S8A). Except for one rabbit that was positive for HEV RNA in the duodenum, cecum and colon, all other rabbits were negative for HEV RNA in the kidney and intestinal tissues (Figure S8B).

In rabbits inoculated with the HEV4 strain, 66.7% (4 of 6) showed stable infection for 13 consecutive weeks. Transient viremia and antigenemia were observed in some rabbits (Figure 5B,D and Figure S6). Transient seroconversion to anti-HEV was observed in 50% (3

of 6) of the rabbits (Figure 5E). No obvious elevation of serum ALT, AST, or GGT (Figure S7) was observed, except for one rabbit that had elevated ALT and AST levels at 4 and 5 wpi. Five rabbits were sacrificed at 13 wpi. Four were positive for HEV RNA in the liver and bile (Figure 5F). One urine sample was positive for HEV RNA, and two urine samples were positive for HEV antigen. All CSF samples were negative for HEV RNA and antigens (Figure S8A). No HEV RNA was detected in the kidney, but could be detected in the duodenum, cecum, and colon (Figure S8B).

HEV-induced liver inflammation and fibrosis

Mild but active inflammation was observed in the livers of sacrificed rabbits in the HEV-3ra group at 4 wpi. The inflammation was reduced at 8 wpi and not evident at 13 wpi. In the CsA-HEV-3ra group, more severe liver damage with disordered liver tissue structure, lymphocytic portal inflammation, and interface hepatitis were observed, indicating a pattern of chronic hepatitis, because of the persistent inflammatory damage and disease progression accompanied with continued HEV infection (Figure 6A). Pathological changes, including increased collagenous accumulation, fiber hyperplasia, and multifocal bile duct hyperplasia, were observed in some rabbits in the CsA-HEV-3ra group at 8 and 13 wpi (Figure 6B). Obvious liver fibrosis was observed in 0% (0 of 4), 50% (2 of 4), and 28.6% (2 of 7) of rabbits in the CsA-HEV-3ra group sacrificed at 4, 8, and 13 wpi, respectively. Liver fibrosis was not observed in the HEV-3ra group (n = 3, each time point) with acute HEV infection (Figure S9). Consistently, chronic hepatitis and liver fibrosis were also observed in human HEV3 or HEV4 infected immunocompromised rabbits sacrificed at 13 wpi (Figure 6A,B). Twenty-five percent (1 of 4) and 40% (2 of 5) of rabbits displayed pathological changes of obvious fibrosis in the CsA-hHEV3 and CsA-hHEV4 group, respectively (Figure S9C). No obvious pathological changes in the liver were observed in the mock and CsA-mock groups (n = 3, at each time point in each group; Figure 5A,B and Figure S8).

We next explored the possible molecular and cellular mechanisms underlying HEV-induced liver fibrosis in immunocompromised rabbits by intrahepatic transcriptome analysis. Compared with mock-infected immunocompromised rabbits, 563 up-regulated and 772 down-regulated DEGs were identified in infected immunocompromised rabbits with obvious liver fibrosis (Figure S10A) sacrificed at 8 wpi. Up-regulated DEGs included genes coding for the profibrotic cytokine *TGFβ1*, classical myofibroblast markers (Vimentin [*Vim*] and actin alpha 2 [*Acta2*]), inflammatory chemokines (C-C motif chemokine receptor 1 [*CCR1*], C-C motif chemokine receptor like 2 [*CCRL2*], motif chemokine receptor 4 [*CXCR4*], and C-X-C motif chemokine ligand 8 [*CXCL8*]), genes associated with matrix remodeling (tissue inhibitor of metalloproteinases 1/2 [*TIMP1/2*] and matrix metallopeptidase 9/14 [*MMP9/14*]), and genes associated with collagen deposition (*COL6A6*, *COL4A2*, *COL5A1*, *COL5A3*, *COL8A1*, and *COL1A1*; Figure 6C). Accordingly, GO enrichment analysis of cellular components of up-regulated DEGs identified pathways involved in extracellular space and collagen trimer (Figure S10B).

Ribavirin monotherapy clears HEV infection in immunocompromised rabbits

Next, we treated HEV-3ra-infected immunocompromised rabbits with 30 mg/kg ribavirin or distilled water orally every day (n = 3, each group) from 2 to 15 wpi. Fecal viral shedding was reduced 1–2 weeks after ribavirin treatment, and viral load remained until complete clearance within the 13-week treatment (Figure S11).

Vaccination before immunosuppression fully protects against HEV-3ra, hHEV3, or hHEV4 infection

The efficacy of the Hecolin vaccine in immunocompromised individuals has not yet been tested. We first evaluated the vaccination strategies before immunosuppression. Rabbits were vaccinated with two 10-μg doses of Hecolin on days 0 and 28. In the third week after the final dose was completed, we started to treat the vaccinated rabbits with CsA and then challenged them with HEV-3ra, hHEV3, or hHEV4 strains (vaccination-before-immunosuppression group, n = 6 in each subgroup; Figure 7A). The geometric mean titer of total anti-HEV antibodies during the vaccination process increased gradually to 1:51 on day 28 after the first dose and increased rapidly to 1:1340 in the third week after the final dose (Figure 7B). No rabbits from the three subgroups showed sign of infection when sacrificed at 6 wpi (Figure 7C), indicating full protection.

Four rabbits naturally positive for anti-HEV were also enrolled in a rechallenge experiment. After establishing immunosuppression, the rabbits were challenged with the HEV-3ra strain (Figure 7D). Three still showed fecal virus shedding for 2–4 weeks, indicating an acute infection. However, the fecal viral load was relatively low, ranging from to 10^4 to 10^5 copies/g (Figure 7E). This observation indicated that immunity against HEV acquired from natural infection could

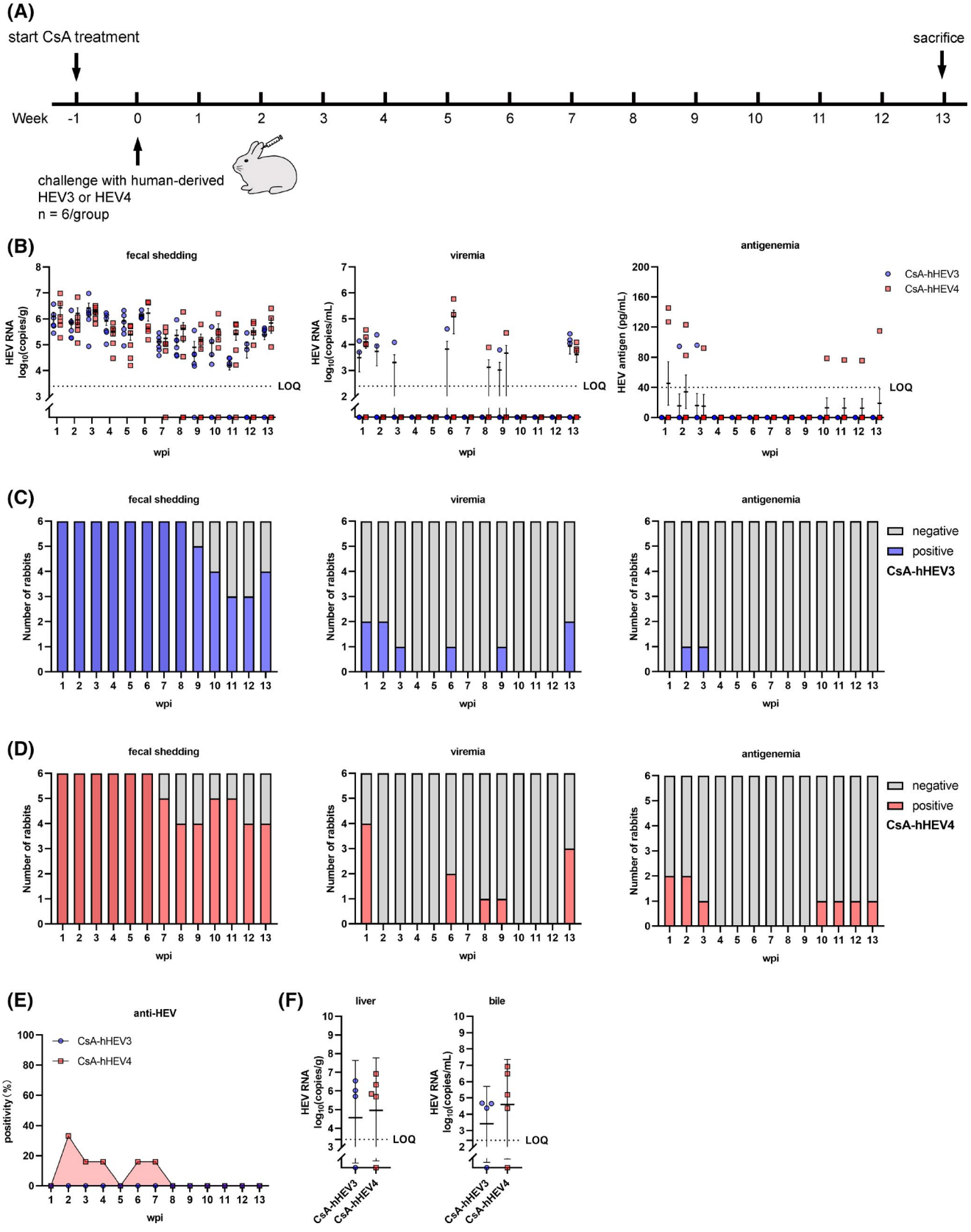


FIGURE 5 Infection of human-derived HEV3 and HEV4 (hHEV3 and hHEV4) strains in rabbits treated with CsA. (A) Experimental scheme. (B) Quantification of fecal HEV RNA, serum HEV RNA, and serum HEV antigen over time after inoculation. (C,D) Number of rabbits with HEV fecal shedding, viremia, or antigenemia from the CsA-hHEV3 group (C) and CsA-hHEV4 group (D) over time after inoculation. (E) Weekly anti-HEV positive rates. (F) Quantification of HEV RNA in liver and bile. Each symbol represents one tested sample. Error bars indicate mean ± SEM

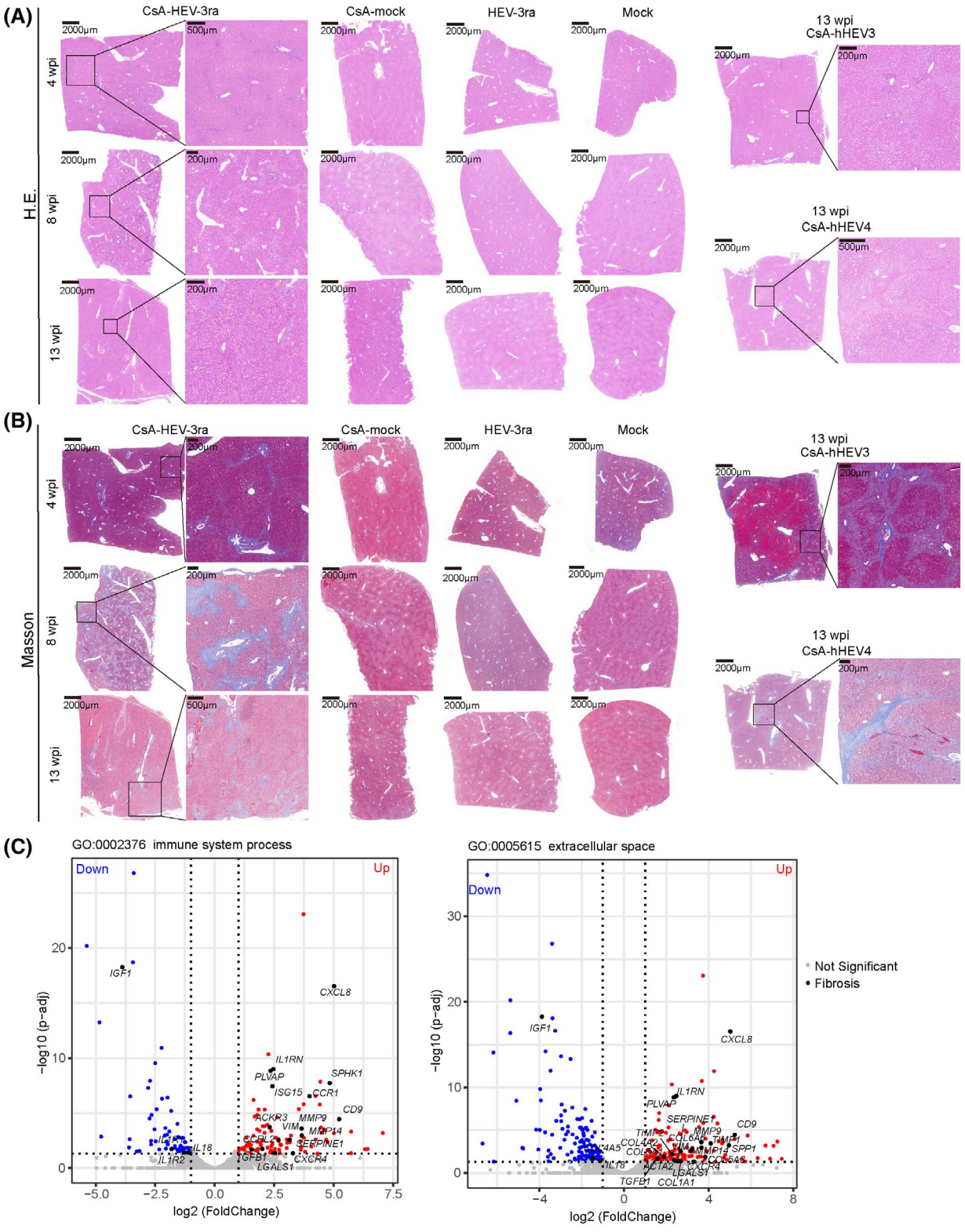


FIGURE 6 HEV-induced liver inflammation and fibrosis. (A,B) Liver histopathology of the rabbits. Representative hematoxylin and eosin (H.E.) and Masson staining of the liver sections from the CsA-HEV-3ra, CsA-mock, HEV-3ra, and mock group at 4, 8 and 13 wpi and the CsA-hHEV3 and CsA-hHEV4 group at 13 wpi, respectively. Scale bars are indicated in the figure. (C) Comparison of gene expressions in obvious fibrosis livers (n = 2) from the CsA-HEV-3ra group versus nonfibrosis livers (n = 3) from the CsA-mock group by volcano plot. Benjamini-Hochberg–corrected p value (p adjust) < 0.05 and |log2 Fold Change| ≥ 1. Selected genes related to fibrosis are marked in black. Data have been prefiltered to enrich for genes implicated in the GO term of immune system process (GO: 0002376, on the left) and extracellular space (GO:0005615, on the right). GO enrichment analysis was performed with up-regulated differentially expressed genes, and these two pathways are both significantly enriched

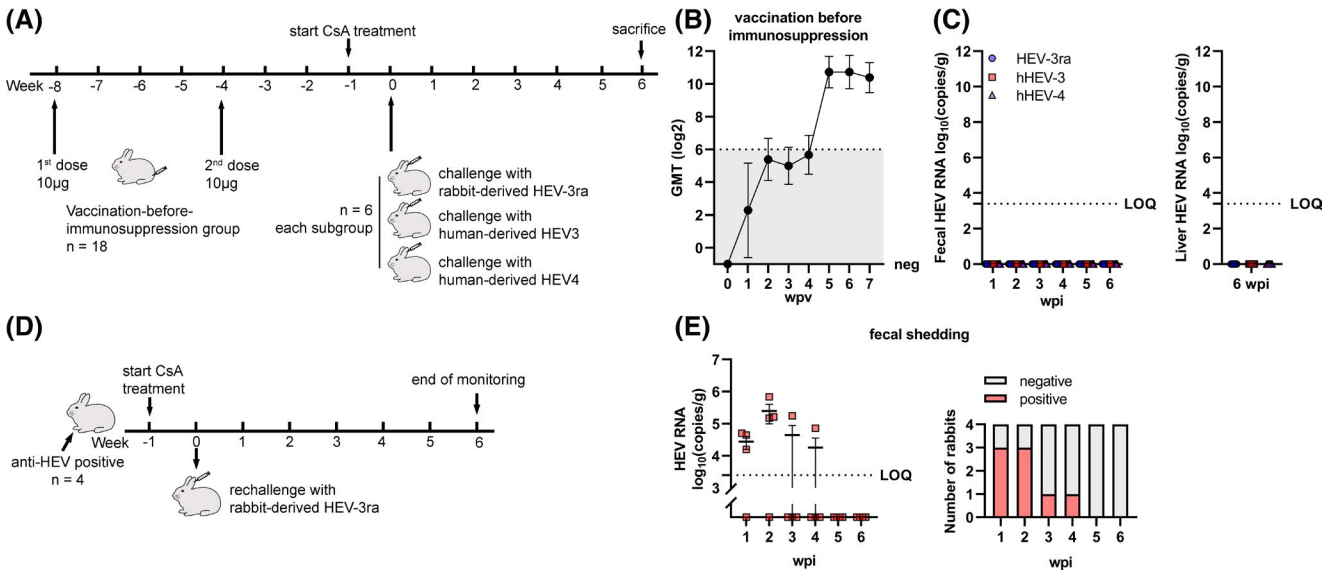


FIGURE 7 Effectiveness of vaccination applied before immunosuppression. (A) Experimental scheme. Rabbits were vaccinated with two 10-μg doses of Hecolin at day 0 and 28 before immunosuppression (vaccination-before-immunosuppression group). (B) Geometric mean titer (GMT) of serum total anti-HEV antibodies during the 7-week vaccination process. (C) Quantification of fecal HEV RNA in the vaccination-before-immunosuppression group over time after inoculation and HEV RNA in liver when sacrificed. (D) Experimental scheme of HEV rechallenge experiment. Rabbits naturally positive for anti-HEV were enrolled (n = 4) and rechallenged with HEV-3ra after immunosuppression. (E) Fecal viral load and number of rabbits with HEV fecal shedding over time after inoculation in the rechallenge experiment. Error bars indicate mean ± SEM. wpv, weeks post vaccination

be impaired by subsequent immunocompromised conditions.

Vaccination during immunosuppression only partially protects against HEV infection

The evaluation of vaccine efficacy under immunosuppression is important. Our vaccination strategy of two 10-μg doses failed to provide full protection to CsA-treated rabbits (Figure 8A,B). Three of four (75%) rabbits showed fecal virus shedding (Figure 8B). A high viral load was observed in the livers of the three rabbits at 9 wpi (Figure 8C). Only one rabbit with the highest anti-HEV antibody level was successfully protected against HEV infection. We next explored whether the efficacy could be improved by increasing the dose and amount of the vaccine. Two additional vaccination strategies (three doses of 10 μg or 30 μg) were tested (Figure 8A). Fifty percent (2 of 4) and 75% (3 of 4) of the rabbits still showed robust HEV infection and chronicity after vaccination 3 times with the 10-μg and 30-μg dose, respectively (Figure 8B,C).

After vaccination was completed and on the day of HEV challenge, the baseline mean anti-HEV antibody levels for the three subgroups of vaccination during immunosuppression were 12.2 ± 15.0, 117.2 ± 167.4, and 8.5 ± 9.4 World Health Organization (WHO) units/ml, respectively. The levels were all significantly lower than the vaccination-before-immunosuppression group (924.4 ± 212.0 WHO units/ml; Figure 8D). Liver fibrosis was also observed in the infected rabbits from the vaccination-during-immunosuppression group sacrificed at 13 wpi, but not in the noninfected rabbits from both vaccination-before-immunosuppression and vaccination-during-immunosuppression groups (Figure 8E and Figure S12). These results demonstrated that vaccination during immunosuppression could not fully protect rabbits from HEV infection and chronicity.

DISCUSSION

In this study, we successfully established chronic infection as defined by viral fecal shedding in 100% of the immunocompromised rabbits using the rabbit-derived HEV-3ra strain. Moreover, chronic infection also developed in immunocompromised rabbits challenged with the human-derived HEV3 or HEV4 strain, with a lower chronicity rate of 66.7% for each strain. Before our study, substantial efforts have been dedicated to the development of animal models of chronic HEV infection. A humanized mouse model based on an immunodeficient genetic background and engraftment of human hepatocytes supported HEV persistence.[21–23] Monkeys[24] and pigs[25] have also been explored as models of chronic HEV infection by treatment with immunosuppressants. These models provide valuable tools for the study of HEV chronicity, and each model has its own advantages. As for

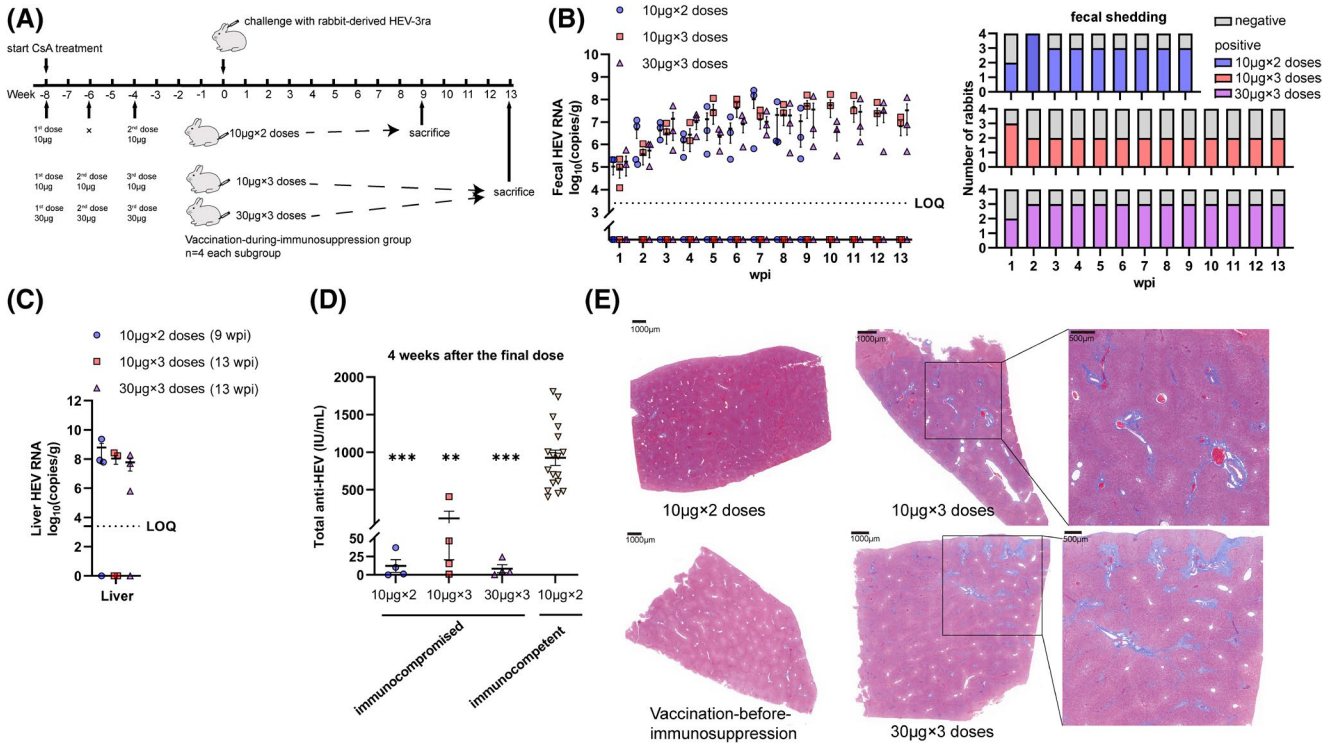


FIGURE 8 Effectiveness of vaccination applied under immunosuppression. (A) Experimental scheme. Rabbits were first induced immunocompromised, then vaccinated during immunosuppression (vaccination-during-immunosuppression group) and challenged with HEV-3ra strain after vaccination completed. The subgroup vaccinated with two 10-μg doses was monitored for 9 weeks, and the other two subgroups for 13 weeks after inoculation (n = 4/subgroup). (B,C) Quantification of fecal HEV RNA and liver HEV RNA in the three subgroups of vaccination-during-immunosuppression group over time after inoculation. Liver HEV RNA was tested at sacrifice (10 μg × 2 subgroups at 9 wpi; the other two subgroups at 13 wpi). (D) Quantification of total anti-HEV antibodies at 4 weeks after the final dose in the three subgroups (n = 4) of vaccination-during-immunosuppression group versus the vaccination-before-immunosuppression group (n = 18). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (E) Masson staining of liver sections from the three subgroups of vaccination-during-immunosuppression group and the vaccination-before-immunosuppression group. Scale bars are indicated in the figure

our immunocompromised rabbit model with HEV-3ra chronic infection presented here, it ideally recapitulates key aspects of patients with chronic hepatitis E, especially organ transplant recipients. Furthermore, previous animal models of HEV chronicity focused primarily on human/swine-derived HEV3,[21–25] whereas emerging evidence has indicated that rabbit HEV3[26,27] and human HEV4[28] can also cause chronic infection in transplant patients. In this respect, our immunocompromised rabbit is capable of modeling both HEV3 chronic infection derived from either rabbits or humans as well as human HEV4 chronic infection. It should be noted that most, but not all, of the animals inoculated with the human-derived strains developed chronicity as defined by viral fecal shedding. This finding agreed with the human clinical setting, in which approximately 60%–70% of transplant patients infected with HEV develop chronic infection.[5,29] In general, the infection level of HEV-3ra was more robust than that of human HEV3 and HEV4, which is intuitive considering the natural adaptation to its host. Moderate levels of persistent viremia were also observed, but were less robust than fecal virus shedding. This is consistent with the clinical observation that fecal HEV shedding is more reliable than viremia in monitoring chronic HEV infection, guiding treatment strategy and predicting relapse.[30–32]

HEV ORF2 capsid protein has at least two forms.[33,34] The secreted form of ORF2 can overwhelm capsid protein in the serum and urine of patients.[33,35] The Wantai ELISA kit primarily detects the secreted form of ORF2,[34] and we depicted the kinetics of ORF2 antigen during chronic HEV infection in rabbits. In immunocompromised rabbits challenged with human-derived strains, incredibly low levels or even no antigenemia or urinary HEV antigen were observed. This might be explained by the lower infectivity of human-derived strains in rabbits. However, high levels of antigenemia and urinary HEV antigen could be detected in immunocompromised rabbits challenged with the rabbit-derived HEV-3ra strain, which agrees well with previous reports on HEV antigens in chronic patients.[8,35,36] It remains debatable whether HEV antigen plays a functional role in pathogenesis and whether it could be a useful marker for diagnosing HEV infection and distinguishing between acute and chronic infections. We believe that our rabbit model provides an essential tool for further investigations of these questions related to ORF2.

Extrahepatic replication and manifestations are reportedly associated with HEV infection.[19] HEV RNA and antigen were detected in the kidney, intestine, CSF, and urine samples from chronically infected rabbits. Recent studies demonstrated that HEV can replicate in human intestinal cells[37] and that protracted fecal HEV shedding is associated with ribavirin treatment failure.[31] The detection of HEV replication in the intestine of HEV-3ra-inoculated rabbits supports the notion that the intestine may serve as another viral reservoir and contribute to virus dissemination. As a natural host, we propose the HEV-3ra-infected rabbit model as a viable option for studying extrahepatic tropism and manifestation of HEV.

Organ transplant patients with chronic HEV infection can rapidly progress to liver fibrosis and cirrhosis,[5,38] which is faster than that observed in HCV-infected patients.[7] In the rabbit model, we observed a similar progression of histological lesions compared to patients with chronic hepatitis E.[5,9,39] RNA sequencing of fibrotic liver tissues also revealed the up-regulation of several known host genes involved in fibrogenesis.[40]

We explored the transcriptional landscape regulated by acute and chronic HEV-3ra infection at different time points. In CsA-untreated rabbits, many DEGs related to both innate and adaptive immune responses were up-regulated in the acute phase (4 wpi) and returned to normal expression during the recovery phase (13 wpi) after virus clearance. Notably, we observed a high overlap of the significantly up-regulated genes in the liver of our rabbits with those previously reported for HEV-infected chimpanzees[20] and humanized mice at 4 wpi.[21] In CsA-treated rabbits, accompanied with lower rates of seroconversion to anti-HEV, less active immune responses were observed after infection, especially at chronic phase (13 wpi). A recent study showed that depletion of CD8+ T cells in rhesus monkeys before HEV challenge only prolonged infection by 1 week and failed to cause chronicity, suggesting that the deficit in CD8+ T cell was compensated by other arms of the adaptive response, most likely neutralizing antibodies and/or CD4+ T cells that have antiviral effector function.[41] In our rabbit model instead, generalized immune suppression induced by immunosuppressants as in transplant patients with HEV chronic infection affects all arms of the adaptive response, which likely explains the successful establishment of HEV chronicity. Of note, host responses primarily related to the regulation of wound healing and extracellular matrix were active during HEV persistent infection. Thus, this rabbit model provides a promising tool for studying the pathogenesis and underlying mechanisms of HEV-induced liver fibrosis and disease progression.

HEV enters and exists in infected host as a mixture of heterogeneous viruses defining quasispecies.[42] In the HEV-3ra-infected rabbit model, we performed an analysis of HEV viral population evolution of different infection time points or under different immune status using the transcriptome data. Although the differences are not statistically significant, SNVs appear more frequently at acute phase of infection or in the absence of immunosuppressants, indicating that the immune activation of the host may somehow contribute to the heterogeneity of the virus. We also found four common nonsynonymous mutations (L190M, P271H, and S634P in ORF1; V581I in ORF2) during HEV-3ra infection, suggesting that these few variants are the dominant ones that survived selective pressure in the rabbits and were critical for HEV-3ra strain to replicate in the liver.

Ribavirin has been used widely to treat patients with chronic HEV infection. The 3-month oral ribavirin treatment, which mimics the clinical treatment strategy,[6] resulted in HEV clearance in all treated rabbits. Although ribavirin is highly effective in treating chronic hepatitis E, treatment failure does occur in some patients.[13] Furthermore, many transplant patients are not eligible or do not tolerate ribavirin treatment, necessitating the development of new anti-HEV therapies.[43] This immunocompromised rabbit model is suitable for testing anti-HEV drug candidates.

The Hecolin HEV vaccine prevented symptomatic HEV1 and HEV4 infections in studies of a large general population.[16,17] Whether this vaccine can prevent chronic infection in immunocompromised patients, including HEV3, is an intriguing issue to assess. In clinical practice, we considered two vaccination strategies applied in patients on the wait list of organ transplantation or patients already receiving the organ. Correspondingly, we tested vaccinations before and during CsA treatment. We found that vaccination completed before CsA treatment conferred full protection against both HEV3 and HEV4 infections. However, only partial protection was achieved when the rabbits were already treated with CsA. Importantly, increased or additional vaccine doses hardly improved the response when the rabbits were immunocompromised. The extremely low anti-HEV antibody levels partially explained the poor performance when Hecolin was administered during immunosuppression. These suggested that transplant patients are best vaccinated before administration of immunosuppressants.

In summary, immunocompromised rabbits challenged with HEV-3ra are an ideal model for recapitulating chronic HEV infection as seen in organ transplant patients, which captures key features of viral kinetics, HEV-host interactions, chronic disease progression, and extrahepatic replication. This model is suitable for antiviral drug testing and, more importantly, for the evaluation of vaccine efficacy and the design of specific vaccination strategies that are effective for protecting immunocompromised patients from infection. Of note, although human-derived HEV3 and HEV4 are less infectious in rabbits, chronic infection as defined by viral fecal shedding was also

established in two-thirds of immunocompromised rabbits, which might facilitate the study of both genotype 3 and 4 chronic infection as well as the study of HEV cross-species transmission.

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CONFLICT OF INTEREST

Nothing to report.

AUTHOR CONTRIBUTIONS

Study design: Qiyu He, Lin Wang, Shuangshuang Li, Fengmin Lu, and Ling Wang. *Experiment design:* Qiyu He. *Assistance with animal experiments:* Fan Zhang, Jingyi Shu, Lin Wang, Zhaochao Liang, Manyu Li, Xing Liu, Xin Yin, and Tianxu Liu. *Assistance with quantification of the inocula of the virus strains:* Xing Liu and Xin Yin. *Assistance with analysis of viral adaptation:* Minghao Du. *Data analysis:* Qiyu He and Fan Zhang. *Manuscript draft:* Qiyu He and Lin Wang. *Manuscript revisions:* Xin Yin, Fengmin Lu, Ling Wang, Qiuwei Pan, and Lin Wang. *Study supervision:* Lin Wang, Ling Wang, and Fengmin Lu.

ETHICS STATEMENT

The animal experiments were approved by the Committee of Laboratory Animal Welfare and Ethics, Peking University Health Science Center and the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

DATA AVAILABILITY STATEMENT

The raw RNA-sequencing data have been deposited in the Genome Sequence Archive in National Genomics Data Center, China National Center for Bioinformation, under accession number CRA004960. All data used in the manuscript are available from the corresponding authors on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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