A small animal model of chronic hepatitis E infection using immunocompromised rats
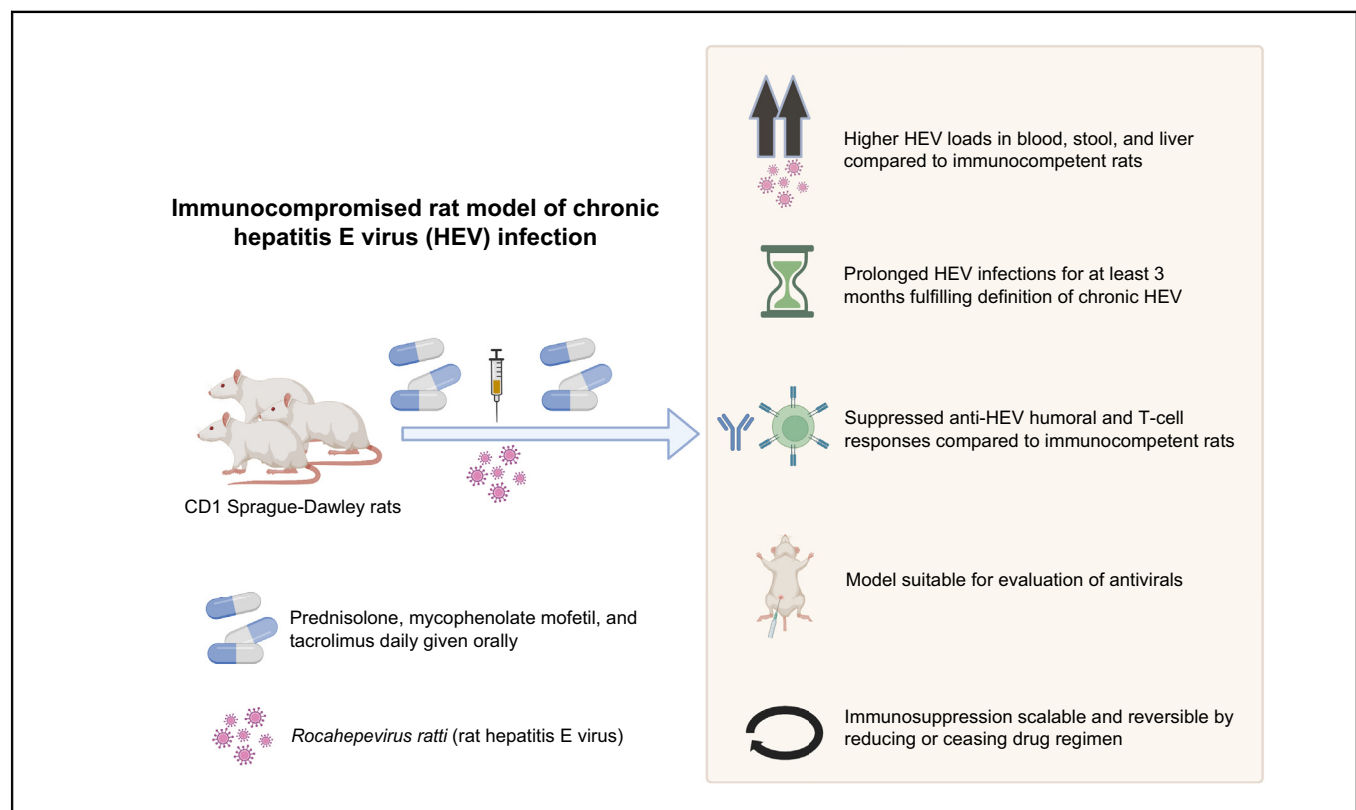
Authors

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Graphical abstract

Highlights

- Chronic HEV infection is challenging to model with small animals.

- Rats can be immunocompromised by transplant rejection drugs taken by patients.

- This model supports chronic rat HEV infection robustly and consistently.

- Immunosuppression in this model is scalable, reversible, and responsive to ribavirin.

Lay summary

Convenient small animal models are required for the study of chronic hepatitis E in humans. We developed an animal model of chronic hepatitis E by suppressing immune responses of rats with drugs commonly taken by humans as organ transplant rejection prophylaxis. This model closely mimicked features of chronic hepatitis E in humans.

A small animal model of chronic hepatitis E infection using immunocompromised rats

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Background & Aims: HEV variants such as swine genotypes within *Paslahepevirus* species *balayani* (HEV-A) and rat HEV (*Rocahepevirus ratti*; HEV-C1) cause chronic hepatitis E in immunocompromised individuals. There are few reliable and accessible small animal models that accurately reflect chronic HEV infection. We aimed to develop an immunocompromised rat model of chronic hepatitis E infection.
Methods: In this animal model infection study, rats were immunosuppressed with a drug combination (prednisolone, tacrolimus, and mycophenolate mofetil) commonly taken by transplant recipients. Rats were challenged with human- and rat-derived HEV-C1 strains or a human-derived HEV-A strain. Viral load, liver function, liver histology, humoural, and cellular immune responses were monitored.
Results: A high-dose (HD) immunosuppressive regimen consistently prolonged human- and rat-derived HEV-C1 infection in rats (up to 12 weeks post infection) compared with transient infections in low-dose (LD) immunosuppressant-treated and immunocompetent (IC) rats. Mean HEV-C1 viral loads in stool, serum, and liver tissue were higher in HD regimen-treated rats than in LD or IC rats (p <0.05). Alanine aminotransferase elevation was observed in chronically infected rats, which was consistent with histological hepatitis and HEV-C1 antigen expression in liver tissue. None (0/6) of the HD regimen-treated, 5/6 LD regimen-treated, and 6/6 IC rats developed antibodies to HEV-C1 in species-specific immunoblots. Reversal of immuno-suppression was associated with clearance of viraemia and restoration of HEV-C1-specific humoural and cellular immune responses in HD regimen-treated rats, mimicking patterns in treated patients with chronic hepatitis E. Viral load suppression was observed with i.p. ribavirin treatment. HD regimen-treated rats remained unsusceptible to HEV-A infection.
Conclusions: We developed a scalable immunosuppressed rat model of chronic hepatitis E that closely mimics this infection phenotype in transplant recipients.
Lay summary: Convenient small animal models are required for the study of chronic hepatitis E in humans. We developed an animal model of chronic hepatitis E by suppressing immune responses of rats with drugs commonly taken by humans as organ transplant rejection prophylaxis. This model closely mimicked features of chronic hepatitis E in humans.

Introduction

HEV is an important cause of hepatitis in humans. The term 'HEV' encompasses a group of virus variants in the family *Hepeviridae*, which is divided into 2 subfamilies: *Orthohepevirinae* and *Parahepevirinae*.[1] *Orthohepevirinae* comprises 4 genera that circulate in terrestrial vertebrates, of which 2, *Paslahepevirus* and *Rocahepevirus*, cause hepatitis in humans. Four genotypes within *Paslahepevirus* species *balayani* (HEV-A; formerly *Orthohepevirus* species A) commonly infect humans: 2 (genotypes 1 and 2) exclusively circulate in humans and 2 (genotypes 3 and 4) circulate in swine and infect humans via the foodborne route. Rodents and ferrets are the natural hosts of *Rocahepevirus* species *ratti* (HEV-C; formerly *Orthohepevirus* species C). We and others have demonstrated that HEV-C genotype 1 (HEV-C1), which circulates in street rats, can infect humans.[2–8]

Immunocompromised individuals contracting HEV frequently develop chronic infection, which can progress to liver fibrosis and cirrhosis if left untreated.[9] Chronic hepatitis E occurs in transplant recipients, people living with HIV, and patients with haematological malignancy. Both HEV-A (genotypes 3 and 4) and HEV-C1 cause chronic hepatitis E in immunocompromised persons with comparable clinical manifestations and ribavirin

responses.[2,10] We have recently shown that HEV-A and HEV-C1 cause similar burdens of chronic hepatitis E among immuno-compromised patients in Hong Kong.[10]

There is a need for accessible small animal models of chronic hepatitis E infection. An immunocompromised pig model of chronic HEV-A infection has been described, but faecal and serum viral loads in this model tended to drop after 8 weeks of infection.[11] Furthermore, pigs are expensive and difficult to maintain. Small animal models of chronic HEV-A infection using human liver chimeric uPA-SCID mice and FRG mice have been developed.[12] These models sustain chronic infection caused by lack of functional adaptive immunity but do not entirely reflect immunocompromised patients who have deficient, but not absent, immune responses to HEV.[13] Furthermore, xeno-transplanting human hepatocytes into mice at a young age is technically challenging. Rabbits and ferrets also shed species-specific cognate HEV variants for long periods, but chronic infection is not consistently observed in all animals.[14–17]

As rats are the natural hosts of HEV-C1, they are ideal candidates for small animal models of hepatitis E. However, HEV-C1 infections in immunocompetent (IC) rats are transient.[18,19]

Persistent HEV-C1 infection in athymic nude rats has been described,[20] but again, such models may not match the more nuanced immunological milieu of immunocompromised patients. In this study, we describe a rat model of chronic HEV-C1 infection using an immunosuppressive drug regimen taken by human solid organ transplant recipients.

Materials and methods

HEV strains

Filtered faecal filtrates containing 2 divergent HEV-C1 strains (CCY and SRN) were used for animal infection. The CCY strain was derived from a transplant recipient with chronic HEV-C1 infection.[2] Across its complete genome (GenBank accession no.: MN450852), CCY shares 95.1% nucleotide identity with the prototype human-derived LCK-3110 strain (MG813927) and, therefore, is a representative of the strain group that has caused most human HEV-C1 infections in Hong Kong.[10] In contrast, the SRN strain was derived from a street rat (*Rattus norvegicus*) captured in Hong Kong. A rectal swab obtained from this rat immersed in virus transport medium (VTM) tested positive for
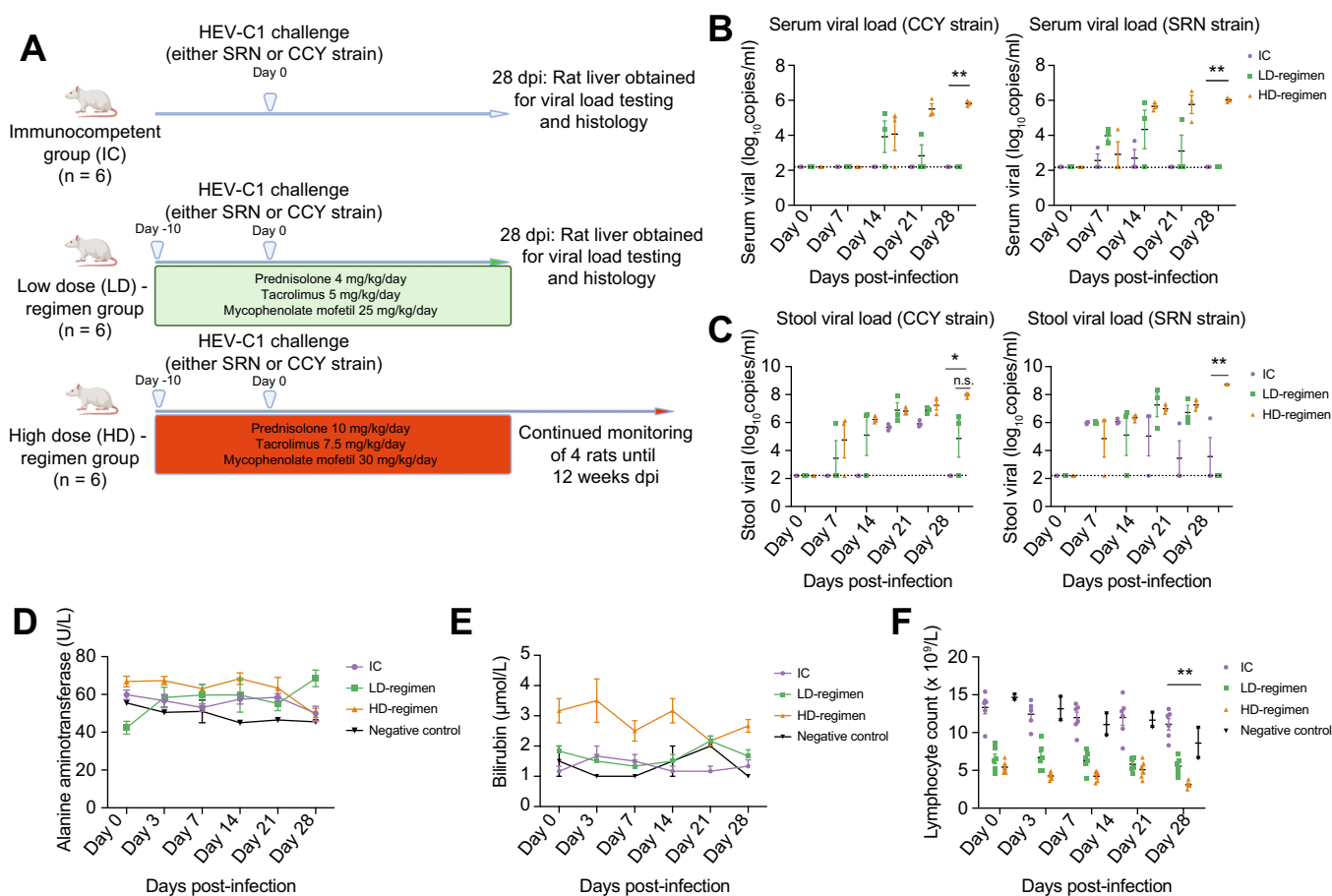

Fig. 1. An immunocompromised rat model of HEV-C1 infection. (A) Scheme of experiment. HEV-C1 viral loads in (B) serum and (C) stool of IC, LD regimen-treated, and HD regimen-treated rats infected with the CCY (n = 3 per group) and SRN virus strains (n = 3 per group). Dotted line represents the limit of detection of the HEV-C1 rRT-PCR assay (2.2 log$_{10}$ copies/ml). Bars represent mean and SEM. Mean viral loads of groups were compared using Student's *t* test at 28 dpi. (D) Alanine aminotransferase, (E) bilirubin, and (F) peripheral blood lymphocyte counts of IC, LD regimen-treated, and HD regimen-treated rats (data of SRN and CCY strain-infected animals in each group are pooled together). Bars represent mean and SEM. Negative controls were 2 uninfected rats. Lymphocyte counts of groups at 28 dpi were compared using Welch's ANOVA. dpi, days post infection; HD, high dose; HEV-C1, HEV-C genotype 1; IC, immunocompetent; LD, low dose; rRT-PCR, real-time reverse-transcription PCR.
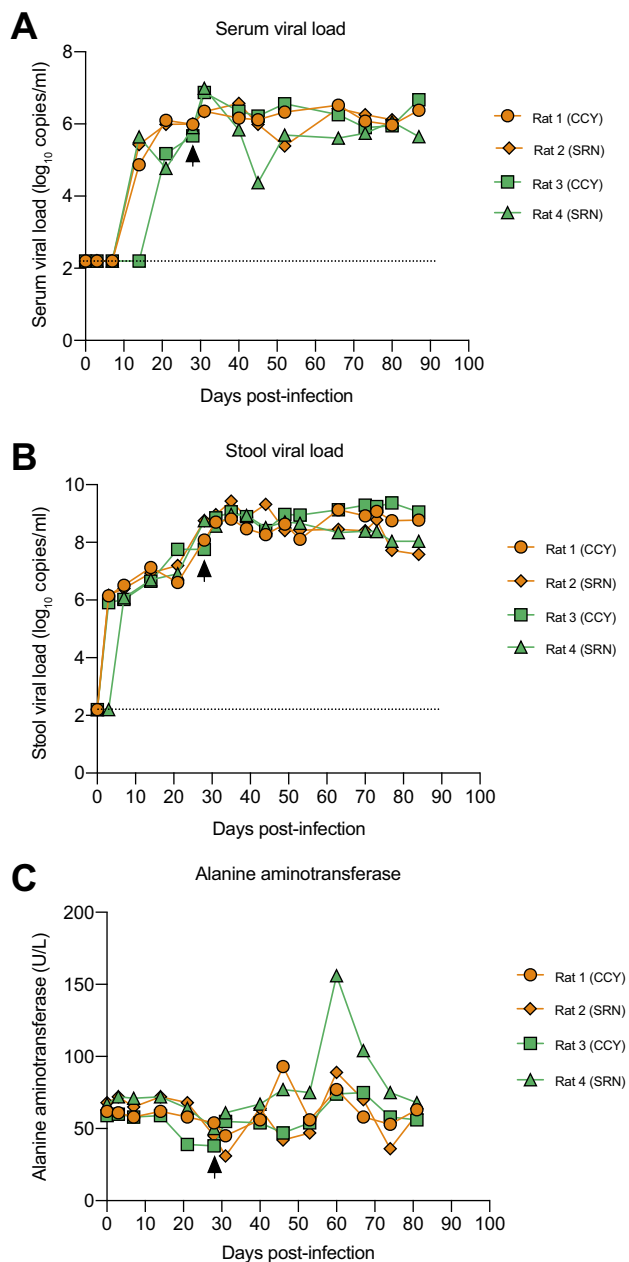
Fig. 2. Immunocompromised rats support chronic HEV-C1 infection. (A) Serum and (B) stool HEV-C1 viral loads of 4 rats followed for 12 weeks post infection. Dotted line represents the limit of detection of the HEV-C1 rRT-PCR assay (2.2 log$_{10}$ copies/ml). (C) Alanine aminotransferase of 4 rats followed for 12 weeks post infection. Rats 1 and 2 (orange lines) were maintained on HD immunosuppressive regimen throughout, whereas rats 3 and 4 (green lines) were transitioned from the HD regimen to the LD regimen at Day 28 post infection (indicated by black arrowhead). Infecting strain of each rat is indicated in parentheses in the legend. HD, high dose; HEV-C1, HEV-C genotype 1; IC, immunocompetent; LD, low dose; rRT-PCR, real-time reverse-transcription PCR.

HEV-C1 by real-time reverse-transcription PCR (rRT-PCR). The VTM was passed through a bacterial filter and i.v. injected into a Sprague–Dawley (*Rattus norvegicus*) rat for viral amplification. Stool from this rat was collected for use in this study. The SRN strain (GenBank accession no.: MG813928) only shares 76.9%

nucleotide identity with CCY. These 2 strains belong to separate genogroups within HEV-C1 (Fig. S1) and enable evaluation of the animal model's ability to support chronic infections by diverse HEV-C1 subtypes. In addition, an HEV-A genotype 4 containing a faecal sample (strain SSW; GenBank accession no.: MK016529) was obtained from a chronically infected heart transplant recipient.[21] A 10% suspension in PBS of each HEV-containing stool was filtered and diluted to a final concentration of 10^6 HEV genome copies/ml.

Animals
Female, 6–8 weeks old, specific-pathogen-free Sprague–Dawley rats (*Rattus norvegicus*) were obtained from the Center for Comparative Medical Research of The University of Hong Kong. Ethics approval for the study was obtained from the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong (protocol: 4817-18). Rats were housed in a biosafety level 2 animal facility and had access to standard pellet feed and water *ad libitum*.

Immunosuppressive drug regimens
The regimen for immunosuppressing rats used 3 drugs commonly taken by solid organ transplant recipients as rejection prophylaxis: prednisolone, tacrolimus, and mycophenolate mofetil (MMF). Prednisolone is a corticosteroid. Tacrolimus is a calcineurin inhibitor; this class of drugs is an established risk factor for chronic hepatitis E and promotes HEV growth *in vitro*.[22,23] Although MMF has been reported to suppress HEV replication, we judged that this would be outweighed by its immunosuppressive effect *in vivo* based on our clinical experience.[2,10,21,23] In addition, the regimen included the H2-receptor blocker ranitidine for gastric ulcer prophylaxis while on prednisolone. Drug suspensions were obtained from the Department of Pharmacy, Queen Mary Hospital, Hong Kong. Drugs were combined in 2 combinations for administration via oral gavage: the low-dose (LD) regimen comprised prednisolone 4 mg/kg/day, tacrolimus 5 mg/kg/day, MMF 25 mg/kg/day, and ranitidine 5 mg/kg/day, whereas the high-dose (HD) regimen comprised prednisolone 10 mg/kg/day, tacrolimus 7.5 mg/kg/day, MMF 30 mg/kg/day, and ranitidine 5 mg/kg/day. Dosages were based on pilot dose escalation experiments exploring tolerability and ability to support chronic HEV infections in individual rats (data not shown).

Model design
For the initial evaluation, rats were divided into 3 groups (n = 6 rats per group). The first group comprised IC rats, the second group was given the LD regimen described above, and the third group was administered the HD regimen. The LD and HD groups began the drug regimen 10 days before infection, and drugs were continued after infection to maintain immunosuppression (Fig. 1A). Within each group, 3 rats were infected with the CCY strain and 3 with the SRN strain. On the day of infection, 0.25 ml of filtered stool suspensions containing either CCY or SRN HEV-C1 strains were administered i.v. such that each rat received an infectious dose of 2.5 × 10^5 genome copies. Two control rats were administered PBS only on the day of infection. After infection, humane endpoints, stool/serum HEV-C1 viral loads, humoral antibody responses, and alanine aminotransferase (ALT) were monitored at regular intervals for a minimum of 28 days post infection (dpi) followed by necropsy. Four rats in the HD group were maintained for longer periods to study viral kinetics up to 3

months dpi (Fig. 1A). Therapeutic effects of reduction or discontinuation of immunosuppression in this model were investigated. HD regimen-treated SRN strain-infected rats were given ribavirin i.p. (30 mg/kg/day) from 7 dpi onwards. Ribavirin was dosed as previously described.[20]

We also attempted HEV-A infection in rats. IC rats and HD immunosuppressed rats were infected with an SSW strain-containing faecal filtrate (infectious dose: 2.5×10^5 HEV-A genome copies).

Histological analysis of liver tissue
H&E staining and immunohistochemical staining using anti-HEV-C1 antisera were performed as previously described.[24] Detailed protocols are included in the Supplementary methods.

HEV rRT-PCR and HEV-C1 genome sequencing
Total nucleic acid was extracted from serum, stool (in VTM), and liver tissue using the EZ1 Virus Mini Kit v2.0 (Qiagen, Hilden, Germany). HEV-A and HEV-C1 rRT-PCR were performed using primers, probes, and protocols described previously.[6] Sequencing

of the near-complete *ORF2* gene of HEV-C1 strains CCY and SRN was performed using the Sanger method as described previously using primers listed in Tables S1 and S2.[2] *ORF2* sequencing was performed because it encodes the viral surface protein and might be expected to be more prone to mutational pressure during chronic infection and interspecies transmission compared with the conserved viral replication machinery encoded by *ORF1*.

Assessment of humoural and cellular immunity
Expression of peptides HEV-A4 p239 and HEV-C1 p241 were performed as previously described.[24] Peptides were expressed in *Escherichia coli* and harvested from inclusion bodies as described in the Supplementary information. These 2 peptides comprise the immunogenic E2s domain of HEV-A and HEV-C1 ORF2, respectively. We have previously shown that immunoblots using these peptides can detect HEV-A- and HEV-C1-specific antibody responses in rat sera.[24] The protocol for immunoblot analysis of rat sera to detect HEV antibodies is described in the Supplementary information. Antibody production was also assessed using a commercial HEV IgG antibody kit (Wantai,
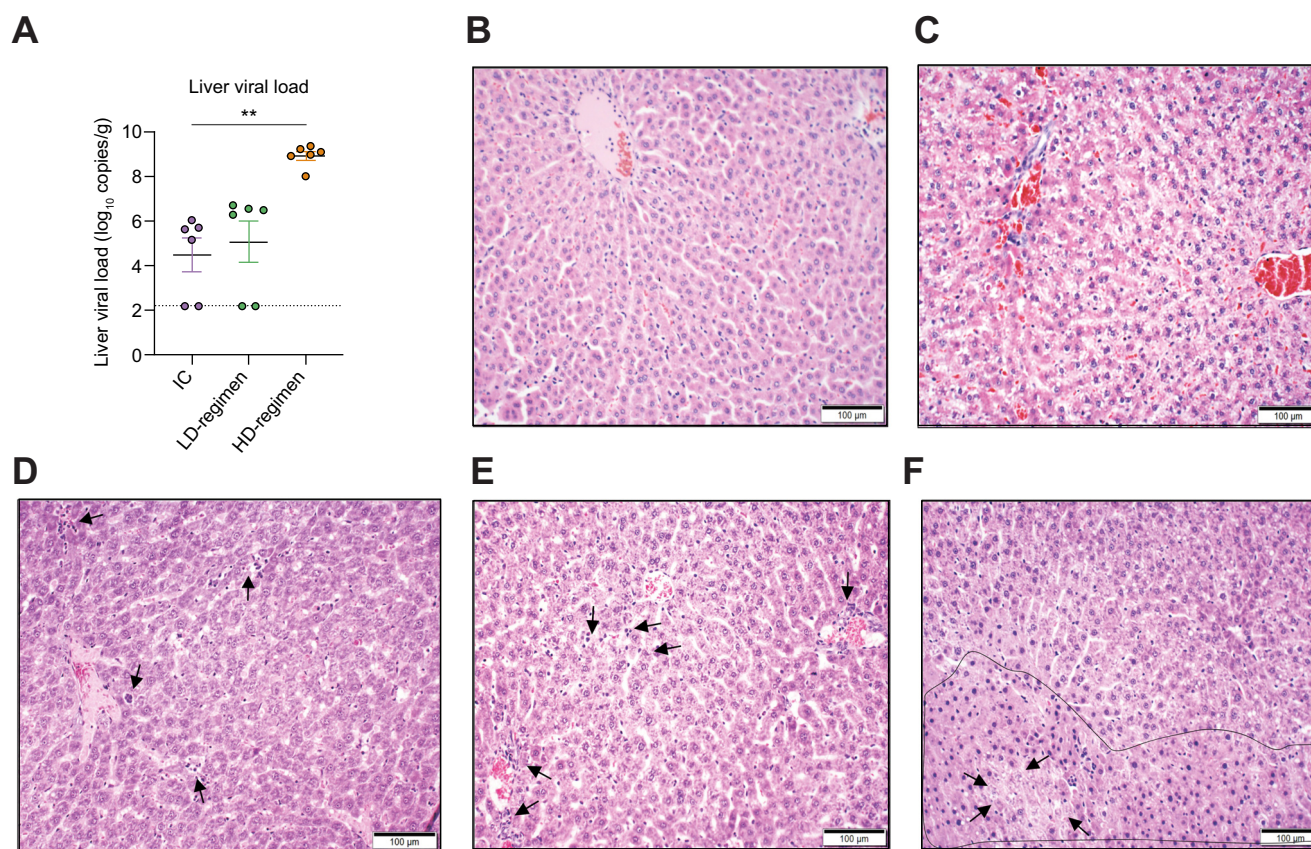

Fig. 3. Analysis of liver tissue viral loads and histology. (A) Liver HEV-C1 viral loads of IC, LD regimen-treated, and HD regimen-treated rats infected with the CCY and SRN virus strains. Dotted line represents the limit of detection of the HEV-C1 rRT-PCR assay (2.2 log$_{10}$ copies/ml). Data of 3 SRN and 3 CCY strain-infected animals in each group are pooled together. Bars represent mean and SEM. IC and LD regimen-treated rat livers were harvested at 28 dpi. Two HD regimen-treated rat livers were harvested at 28 dpi, whereas the other 4 were harvested at 12 weeks post infection. Group means were compared using Welch's ANOVA. H&E-stained liver sections of (B) a normal uninfected rat and (C) an uninfected rat given the HD regimen showing normal architecture of hepatocyte plates. H&E-stained liver section of (D) an infected IC rat and (E) an LD regimen-treated rat showing hepatocyte degeneration with multiple foci of immune cell infiltration (arrows). H&E-stained liver section of (F) an infected HD regimen-treated rat showing hepatocyte degeneration, a large area of nuclear condensation (within dashed line enclosed area), and foci of cell necrosis (arrows). dpi, days post infection; HD, high dose; HEV-C1, HEV-C genotype 1; IC, immunocompetent; LD, low dose; rRT-PCR, real-time reverse-transcription PCR.
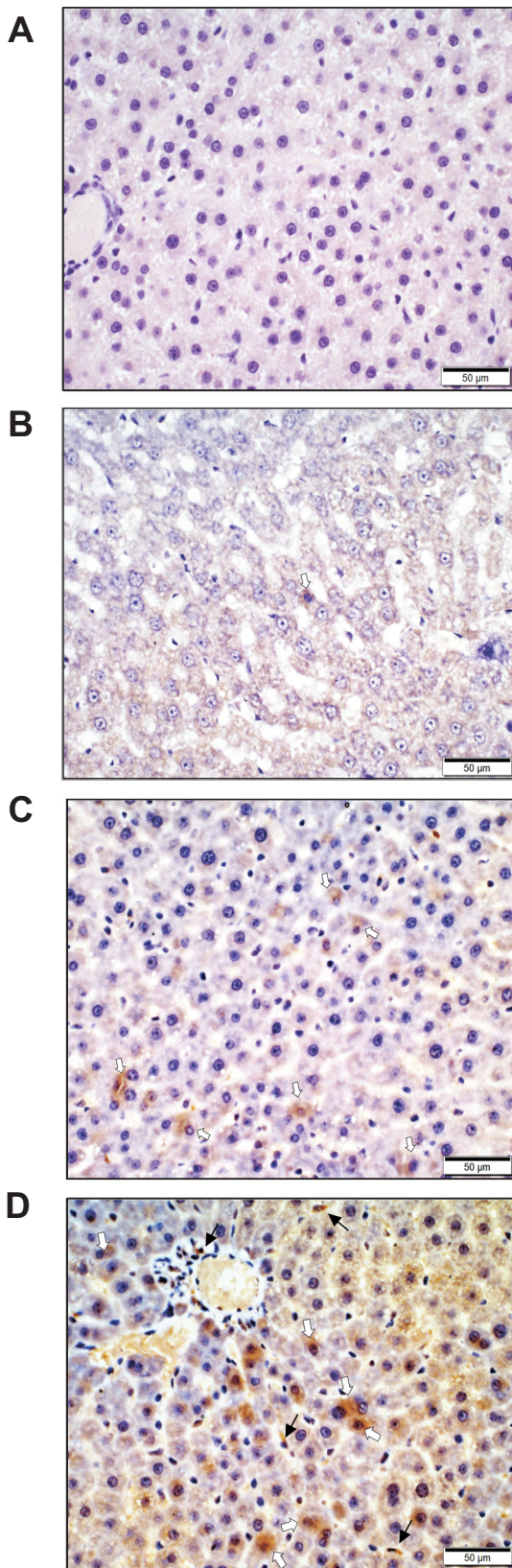
Fig. 4. Immunohistochemical staining of liver sections with murine poly-clonal antisera against HEV-C1 p241. Liver sections of (A) uninfected rats showing no viral antigen staining, (B) IC rats showing occasional cell positivity, (C) LD regimen-treated rats showing cells expressing viral antigen (white arrows) with immune infiltrating cells, and (D) HD regimen-treated rats showing extensive viral antigen expression in hepatocytes (white arrows) and some interstitial cells resembling Kupffer cells (thin black arrows). Some of the antigen-expressing hepatocytes show cytoplasmic vacuolation. HD, high dose; HEV-C1, HEV-C genotype 1; IC, immunocompetent; LD, low dose.

Beijing, China) with modifications (Supplementary methods). We have previously shown that this kit detects antibodies in HEV-C1 infected patient sera with fair sensitivity.[24]

Splenocytes were obtained from selected rats infected with the SRN strain at 35 dpi to measure T-cell responses to HEV-C1. Production of interferon-γ (IFN-γ) by rat splenocytes in response to HEV-C1 p241 and phorbol myristate acetate/ionomycin mitogen stimulation was assessed using an in-house developed ELISpot assay as described in the Supplementary information.

Statistical analysis

Comparisons of means were done using Student's *t* test or Welch's ANOVA. Figures were generated using Prism version 8.1 (GraphPad Software, San Diego, CA, USA). The sample size of animals per group in the initial model (n = 6) was based on an *a priori* estimate that mean faecal viral loads would be at least 2-$\log_{10}$ copies/ml higher in the HD regimen-treated group than in the IC group. This sample size yields a power of 90% at an alpha level of 0.05. For therapeutic trials of reduced immuno-suppression and ribavirin, a sample size of 5 per group was chosen based on an *a priori* estimate that there would be a dif-ference in viral load of 1-$\log_{10}$ copies/ml in the stool between treated and untreated animals.

Results

Characterisation of HEV-C1 infection in rats

Rats in the LD and HD regimen groups tolerated drugs well with no differences from normal IC rats in terms of appearance, behaviour, or feeding patterns. Following infection according to the scheme in Fig. 1A, all rats remained active and did not show signs of infection. Only 2/6 IC rats had detectable viraemia; both had been infected with the SRN strain (Fig. 1B). Most (5/6) LD regimen rats developed viraemia; however, all cleared viraemia by 28 dpi. All HD regimen rats developed viraemia (Fig. 1B) and maintained high viral loads (mean: 5.82 $\log_{10}$ and 6.02 $\log_{10}$ copies/ml for CCY and SRN strain-infected rats, respectively) even at 28 dpi. The same pattern was observed in stool with most IC and LD rats ceasing viral shedding in stool by 28 dpi although viral loads in the latter were higher until 21 dpi (Fig. 1C). In contrast, HEV-C1 loads in stool of HD regimen-treated rats continued to rise during this period with all ani-mals shedding virus loads exceeding 7 $\log_{10}$ copies/ml. At 28 dpi, a near-complete HEV-C1 *ORF2* gene fragment (nt 5,189–6,924) was sequenced from cDNA extracted from stool of HD regimen-treated rats infected with SRN and CCY strains using primers listed in Tables S1 and S2. No mutations were found compared with baseline sequences of either strain.

Infected animals did not develop significantly raised ALT although slight elevations compared with those of uninfected control rats were observed at 14 and 21 dpi (Fig. 1D). LD regimen-treated rats had raised ALT compared with other groups at 28 dpi, coinciding with the time of clearance of virus shedding in stool. Bilirubin of infected animals remained low (Fig. 1E); marginally higher bilirubin in HD regimen-treated animals was probably artefactual as these rats had higher bilirubin at 0 dpi.

Mild liver dysfunction is typical of HEV-C1 infections in humans, which often manifests as anicteric hepatitis.[2] Peripheral blood lymphocyte counts were also monitored (Fig. 1F). For both LD and HD rats, lymphocyte counts were lower than those of IC and negative control rats at 0 dpi (by which time they had received 10 days of the immunosuppressive regimen). By 28 dpi, mean lymphocyte counts in the HD group were significantly lower than those in the IC and LD groups.

Immunocompromised rats support chronic HEV-C1 infection

Four infected rats originally on the HD regimen were kept for 12 weeks post infection (Fig. 1A). At 28 dpi, we maintained 2 rats on the HD regimen (rats 1 and 2) and transitioned the other 2 rats to the LD regimen (rats 3 and 4). Interestingly, all 4 rats maintained high HEV-C1 viral loads in both serum and stool throughout the experiment with no viral load decline in rats 3 and 4 (Fig. 2A and B). This confirms that the model can support chronic hepatitis E infection, defined as 3 months of continuous viraemia.[25] Reduction of immunosuppression after 28 dpi did not rescue rats from chronic infection. This pattern is often observed in chronically immunosuppressed transplant recipients with HEV-C1 infection who seldom clear viraemia with immunosuppression reduction alone.[2,10] All 4 rats had noticeable spikes in ALT above their 28 dpi levels, although these were self-limiting (Fig. 2C).

Liver viral loads and histology

At the end of the above experiments, rat livers were obtained for viral load testing, which showed that HD regimen rats had mean viral loads of 8.92 log$_{10}$ copies/g, which were significantly higher than those of LD and IC rats (Fig. 3A) (p = 0.0008). Histological evaluation by H&E staining of liver at 28 dpi showed hepatocyte degeneration and immune cell infiltration in IC, LD regimen, and HD regimen rats compared with control rats (Fig. 3B–F). HD rat livers additionally showed foci of cell necrosis. On immunohistochemical staining, LD and HD regimen-treated rat livers showed extensive signals indicative of HEV-C1 antigen expression in infected hepatocytes, whereas such staining was scanty in IC rat liver (Fig. 4).

Serological responses

We assessed antibody responses in infected rats using HEV-C1-specific IgG immunoblots. By 14 dpi, 5/6 IC rat sera showed distinct bands in the HEV-C1 p241 peptide immunoblot indicative of a humoral response to HEV-C1 (Fig. 5A). By 28 dpi, all 6 IC rats had seroconverted. All LD regimen rats remained seronegative at 14 dpi, but 5 seroconverted by 28 dpi. In contrast, none of the HD regimen rats seroconverted at 28 dpi. The Wantai HEV IgG assay corroborated the immunoblot results with weak or absent IgG responses in LD and HD regimen-treated rats compared with IC rats (Fig. 5B). The immunosuppressive regimen both abrogated and delayed the development of a humoral response to HEV-C1.

Effect of reducing immunosuppression and ribavirin

Management of chronic hepatitis E involves reduction of immunosuppression and, failing this, ribavirin.[26] As shown above, HD regimen-treated rats were unable to clear infection even when immunosuppression was reduced after 28 dpi (Fig. 2A and B). To assess the impact of earlier immunosuppression reduction, we infected 15 HD regimen-treated rats with the SRN strain (Fig. 6A). Five animals were maintained on the HD regimen throughout the study period (group 1). To model treatment of hepatitis E in immunocompromised patients, we trialled early reduction of the HD regimen to the LD regimen (group 2) or complete cessation of HD regimen at 7 dpi (group 3). Mean serum and stool viral loads of group 1 rats remained high at 28 dpi as expected. Immunosuppression reduction or cessation was associated with declines in stool viral shedding and plateauing of serum viral loads (Fig. 6B and C). Most animals in groups 2 and 3 cleared viraemia by 28 dpi.

We then examined HEV-C1 antibody responses of group 1, 2, and 3 rats (1 randomly selected animal per group). Within 2 weeks of immunosuppression reduction or reversal (i.e. 21 dpi), strong antibody responses were apparent in rats with reduced or ceased immunosuppression (Fig. 6D). Blot intensity was comparable with the IC control infected animal. At 35 dpi, splenocytes were harvested from the same randomly selected animal in groups 1, 2, and 3 for measurement of IFN-γ production
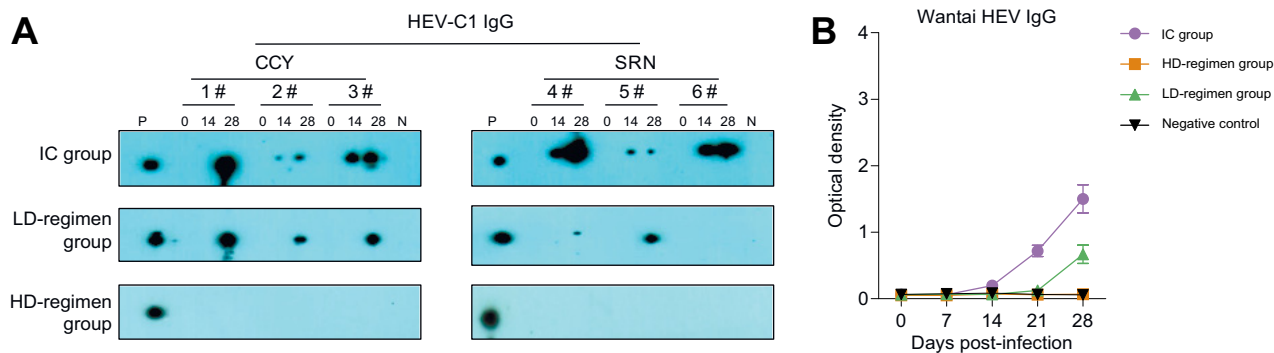

Fig. 5. Humoral responses to HEV-C1 infection. (A) Anti-HEV antibody responses of individual IC, LD regimen-treated, and HD regimen-treated rats infected with CCY and SRN strains at 0, 14, and 28 days post infection assessed using HEV-C1 p241 species-specific immunoblot. P represents positive control using an anti-His antibody, whereas N represents negative control using uninfected rat sera. (B) Anti-HEV antibody responses of IC, LD regimen-treated, and HD regimen-treated rats at 0, 7, 14, and 28 days post infection assessed using the Wantai HEV IgG assay. Data of SRN and CCY strain-infected animals in each group are pooled together. Bars represent mean and SEM. HD, high dose; HEV-C1, HEV-C genotype 1; IC, immunocompetent; LD, low dose.
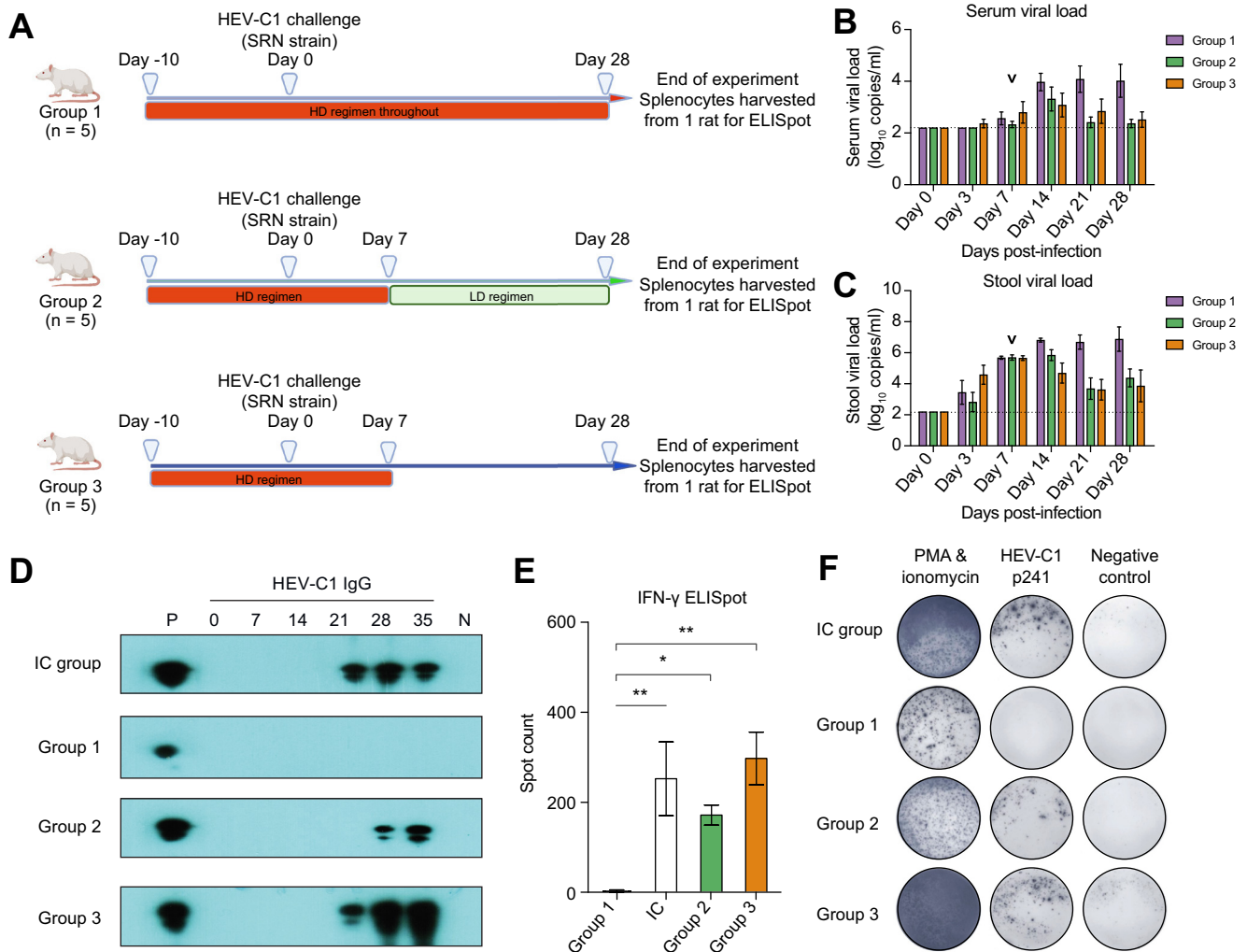
Fig. 6. Immunosuppression in HD regimen-treated rats is reversible. (A) Scheme of experiment (n = 5 rats per group). (B) Serum and (C) stool viral loads of rats infected with the SRN virus strain. All rats were initially given the HD regimen, but immunosuppression was either reduced or stopped at 7 dpi (down arrow). Bars represent mean and SEM. Dotted line represents the limit of detection of the HEV-C1 rRT-PCR assay (2.2 $\log_{10}$ copies/ml). (D) HEV-C1 p241 species-specific immunoblot showing recovery of serological responses after reduction or cessation of immunosuppression at 7 dpi in groups 2 and 3. P represents positive control using an anti-His antibody, whereas N represents negative control using uninfected rat sera. (E) Mean spot count of IFN-γ ELISpot using splenocytes stimulated by HEV-C1 p241. Mean of 3 replicates of 1 animal per group presented. Groups means were compared using Student's t test. (F) Representative pictures of IFN-γ ELISpot membranes of IC, group 1, 2, and 3 rat splenocytes stimulated with PMA/ionomycin mitogen, HEV-C1 p241, and no mitogen (negative control) are shown. dpi, days post infection; HD, high dose; HEV-C1, HEV-C genotype 1; IC, immunocompetent; IFN-γ, interferon-γ; LD, low dose; rRT-PCR, real-time reverse-transcription PCR.

following HEV-C1 p241 stimulation using ELISpot. Immune restitution was clearly observed with group 2 and 3 animals having higher spot counts than the group 1 animal (Fig. 6E and F) when stimulated with both HEV-C1 p241 and PMA/ionomycin; their ELISpot responses were attenuated (group 2) or similar (group 3) to an IC control infected animal. The group 1 rat had nearly undetectable responses on HEV-C1 p241 stimulation. These results show that immunosuppression in this model is reversible, provided that drugs are stopped by 7 dpi.

We then assessed the effect of i.p. ribavirin (given 30 mg/kg/day) in infected immunocompromised rats (Fig. 7A). Viral loads of individual ribavirin-treated and control animals were compared with their 7-dpi baseline for a period of 3 weeks. As shown in Fig. 7B and C, a suppressive effect on viral load was apparent in ribavirin-treated animals at the second week of

treatment. This effect was maintained in 3 animals into the third week of treatment, but rebound was observed in 2 other treated animals. None of the ribavirin-treated animals cleared the infection.

HEV-A infection

Lastly, we assessed whether IC and HD regimen-treated rats could be infected with an HEV-A genotype 4 strain derived from a chronically infected transplant recipient (Fig. S2A). Only 1 of the 3 IC rats showed a low viral load in the stool of 4.19 $\log_{10}$ copies/ml at a single time point (3 dpi). None of the 3 HD regimen-treated HEV-A-injected rats had detectable HEV-A in stool, serum, or liver. Furthermore, none of the rats developed a humoral response to HEV-A in either immunoblot or Wantai IgG assays (Fig. S2B and C).
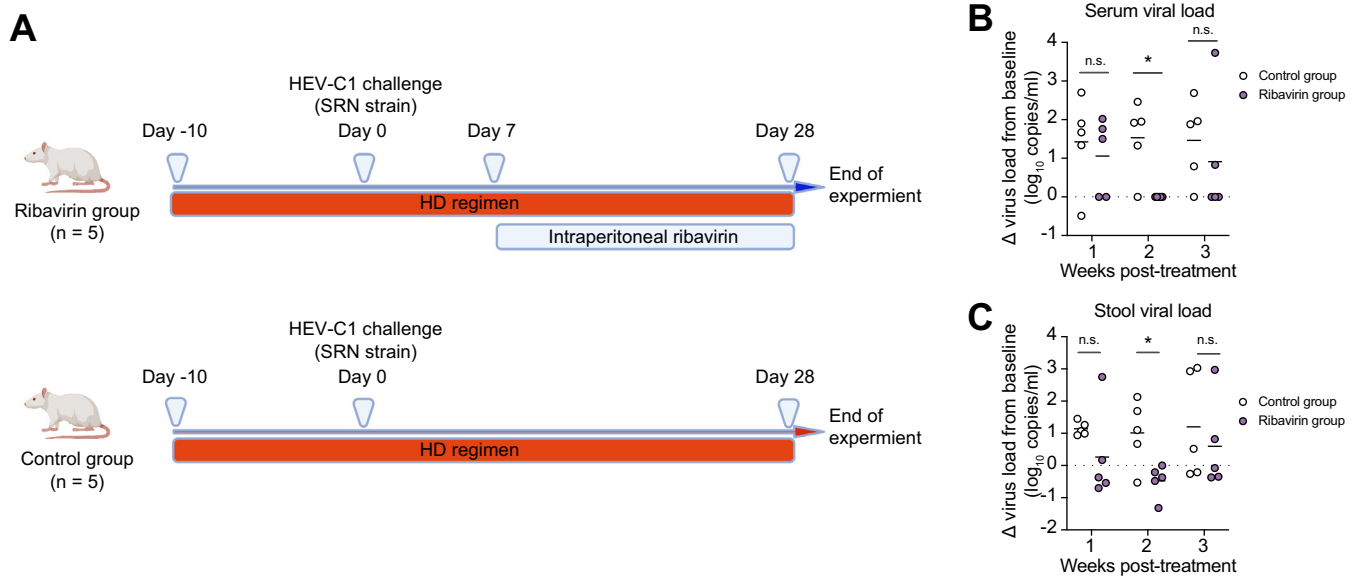
Fig. 7. Therapeutic effect of ribavirin in HD regimen-treated rats. (A) Scheme of experiment (n = 5 rats per group). HD regimen-treated rats in group 1 in a previous experiment served as the control group. Changes in (B) serum and (C) stool viral loads of individual animals at each week of treatment compared with their baseline viral loads at 7 days post infection using Student's *t* test. Mean differences are shown for each group. HD, high dose; HEV-C1, HEV-C genotype 1.

Discussion

Animal models are crucial tools for studying chronic hepatitis E. Immunocompromised cynomolgus macaque and pig models of chronic HEV-A infection have been described.[11,27] An HEV-C1 model in non-human primates has also been described, which could potentially be adapted to model chronic infection.[28] However, large animal models are cumbersome and limited to a few facilities. Accessible small animal models of chronic hepatitis E infection are difficult to develop. Successful models have used chimeric human-liver mice such as uPA^{+/+}-SCID mice, uPA^{+/+}-NOG mice, and humanised FRG mice.[29–32] All 3 models lack humoral and cellular immunity, allowing them to be transplanted with human hepatocytes. Lack of a functional immune response ensures that they support chronic HEV infection. However, these models are cumbersome. uPA^{+/+}-SCID mice require human hepatocyte infusion at a very young age, which is technically challenging. Furthermore, cryopreserved human hepatocytes for xenotransplantation are expensive. The lack of immunity in these mice is also unlike the more nuanced immunodeficiency state of transplant recipients.[13] Ferrets and rabbits can harbour chronic HEV-C and HEV-A infections, respectively, under natural and experimental conditions.[15,16,33,34] However, chronicity is not consistently observed.[15,16,35] Rabbits immunosuppressed with cyclosporin A support chronic HEV-A genotype 3 (rabbit variant) infection.[17] This is a useful surrogate of chronic HEV-A infection, although viraemia in this model tends to be transient with human-derived HEV-A genotype 3 and genotype 4 infection.[17]

The recent recognition of HEV-C1 as a cause of chronic hepatitis E necessitates a representative animal model for this infection. Such a model would also mimic chronic HEV-A infection because HEV-C1 is similar to HEV-A in terms of rates of progression to chronicity, clinical manifestations, and treatment outcomes in immunocompromised patients.[10] However, IC rats only sustain transient HEV-C1 infections.[18,19] Here, we demonstrate consistent prolongation of HEV-C1 infection duration using a common strain of laboratory rat fed immunosuppressants. The model sustained high levels of HEV-C1 replication owing to near-absent humoral and T-cell immunity against HEV, but this was also reversible, provided immunosuppression was reduced early, which is not the case with athymic rats.[20] This relative scalability enables analysis of adaptive immune responses during HEV infection.

We successfully infected rats with human- and rat-derived HEV-C1 strains. CCY, the human strain used in this study, is a representative of the LCK-3110 strain group that has caused nearly all human HEV-C1 infections in Hong Kong.[10] The route of transmission of HEV-C1 infections to humans is unknown but is presumed to be directly from rodents without involving an intermediate host based on the relatedness of human and rat HEV-C1 strains during a community outbreak in Hong Kong.[2] This study further supports this by demonstrating that the CCY human-derived HEV-C1 strain is highly adapted to rats. Rat infections by CCY did not require any adaptive mutations in *ORF2*.

As HEV-C1 is a newly recognised cause of chronic hepatitis E, susceptibility to ribavirin is a key question that needs to be addressed. We used a dose of 30 mg/kg/day as per previous publications.[17,20] Ribavirin suppressed viral loads at the second week of treatment although rebound was observed at the third week in 2/5 treated animals. Ribavirin administered soon after virus inoculation also did not always rescue athymic rats from HEV-C1 infection.[20] Even in human patients with HEV-C1, delayed or absent responses to ribavirin are not uncommon.[10] Future dose escalation studies of ribavirin with longer treatment duration are required in rats.[32]

Even heavily immunosuppressed rats were not susceptible to HEV-A infection. Susceptibility of rats to HEV-A infection is controversial,[36] but our findings corroborate other researchers who have documented that rats are not susceptible to HEV-A, even with immunosuppression.[18,37] Even studies reporting

success in infecting rats with HEV-A find very low viral loads and absence of seroconversion.[38] The factors leading to resistance to HEV-A infections in rats is unknown and worthy of investigation. Identification of such factors could enable development of knockout models of HEV-A infection. Mongolian gerbils have recently been reported to support both HEV-A and HEV-C1

infections.[39] Immunosuppressed gerbils may serve as a useful rodent model that can support both HEV-A and HEV-C1 infections.

In summary, we describe a convenient accessible rat model of chronic hepatitis E, which can be used for studies of HEV immunopathogenesis, extrahepatic manifestations, vaccines, and antiviral therapeutics.

Conflicts of interest
SS has received speaker's honoraria from Abbott Medical Laboratories. The other authors report that there are no competing interests to declare.

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

Authors' contributions
Conceptualised and drafted the study: SS. Performed the experiments and provided critical input into the study: SW, JW, EHKS, ZL. Analysed the liver histology and immunohistochemical staining: AJXZ. Analysed the raw data: KH, NFSC. Aided design and execution of the ELISpot experiments: CHYF, VKMP. Performed sequencing of the SRN strain: WMC. Designed the phylogenetic tree: CCYY. Designed the species-specific immunoblots: JPC. Critically reviewed the manuscript: KYY.

Data availability statement
Sequences of SRN, CCY, and SSW strains have been uploaded to GenBank with accession numbers included in the methods. Raw data of viral loads, liver function tests, lymphocyte counts, and ELISpot responses of treated animals are available upon request.

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