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Treatment induced clearance of hepatitis E viruses by interferon-lambda in liver-humanized mice

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Abstract

Background: Hepatitis E viruses (HEV) are an underestimated global cause of enterically transmitted viral hepatitis, which may persist in immunocompromised hosts, posing a risk for progressive liver fibrosis with limited treatment options. We previously established liver-humanized mice as a model for chronic HEV infections, which can be cleared by a 2-week pegylated (peg)-Interferon(IFN)α treatment course. However, severe side effects may hamper the use of IFNα in immunocompromised transplant recipient patients. IFNλ may be a valuable alternative, as its receptor is less ubiquitously expressed.

Aims: In this study, we assess the in vitro and in vivo potency of pegIFNλ to induce innate immune signalling in liver cells and to clear a persistent HEV infection in liver-humanized mice.

Methods & Results: We found that human liver cells expressed the IFNλ receptor (IFNLR1) and are responsive to pegIFNλ. Treatment with pegIFNλ of liver-humanized mice persistently infected with HEV genotype 3 showed that pegIFNλ was well tolerated. Dose escalation studies showed that although HEV was not cleared at pegIFNλ doses up to 0.12 mg/kg for a maximum of 8 weeks, a dose of 0.3 mg/kg pegIFNλ treatment resulted in complete clearance of HEV antigen and HEV RNA from the liver in 8 out of 9 liver-humanized mice.

Conclusions: PegIFNλ is well tolerated in mice and leads to clearance of a persistent HEV infection in liver-humanized mice.

KEYWORDS

cytokines, hepatitis E virus, interferon alpha, interferon lambda, viral hepatitis

Abbreviations: HBV, Hepatitis B Virus; HCV, Hepatitis C Virus; HDV, Hepatitis D Virus; HEV, Hepatitis E Virus; IFN, Interferon; IFNLR, Interferon Lambda Receptor; ISG, Interferon Stimulated Gene; PEG, Pegylated; PHH, Primary Human Hepatocyte; RBV, Ribavirin.

1 | INTRODUCTION

Human hepatitis E virus (HEV) infections are one of the leading causes of acute viral hepatitis and result in up to 70,000 deaths worldwide each year.[1] HEV is a non-enveloped single-stranded RNA virus with a broad host range. Of eight identified genotypes (gt), gt1 to 4 and gt7 can infect humans,[2] resulting in waterborne HEV gt1 and two epidemics in developing countries, or in zoonotic HEV gt3, gt4 and gt7 infections, characterized by a mostly self-limiting acute hepatitis. In addition, a chronic HEV gt3 infection in patients with an immunocompromised status, such as patients who received a solid organ transplant, with autoimmune disease or human immunodeficiency virus infection has recently emerged as a significant health problem.[2-10] No controlled clinical studies have determined the optimal treatment for chronic HEV infections. Expert guidance lists dose reduction of immunosuppressive drugs, ribavirin (RBV) monotherapy or pegylated-Interferon α (pegIFNα) as therapeutic options.[11] The latter is hampered by severe side effects and increases the risk of acute rejection in HEV-infected solid organ transplant recipients.

As an alternative to pegIFNα, pegIFNλ has been proposed as an antiviral for other chronic viral hepatitis infections, including hepatitis B, C and delta viruses.[12-14] Both IFNα, also called type I IFN, and IFNλ, also called type III IFN, belong to a family of cytokines that are key effectors of the innate immune response by inciting antiviral, anti-proliferative and immunomodulatory effects in target cells, via a similar downstream signalling cascade, the JAK/STAT pathway. Even though both type I and type III IFNs are produced by all cell types, the distribution of the IFNλ receptor (IFNLR1) is less abundant than the IFNα receptor and mostly present on epithelial cells, hepatocytes and a limited number of immune cell types.[15,16] Therefore, IFNλ treatment has the potential to spare patients from the systemic side effects of pegIFNα (reviewed in Ref. [17]) as shown in a randomized controlled trial in patients with chronic hepatitis C virus (HCV) infection.[14]

The biological activity of both type I and III IFNs are assessed via their relative induction of intracellular interferon-stimulated genes (ISGs). Previous data in HCV-infected cell lines and patients showed a lower relative sensitivity to exogenous IFNa when endogenous IFNa activity was already relatively high, as evidenced by, for instance, high ISG expression.[18-20] Whether similar differential sensitivity also occurs during HEV infection, and whether the type and magnitude of the innate immune response impact the sensitivity to exogenous IFNλ are unknown. Importantly, it was recently reported that the expression of IFNλ and ISG were induced by HEV infection in vitro.[21-23]

We and others developed a liver-humanized mouse model that is susceptible to patient derived clinical HEV strains and allows the study of HEV's biology and evaluation of antiviral treatment candidates.[21,24-27] In this model, HEV infections do not elicit an ISG response, persist for the life span of the mouse host and can be cleared by stimulating the innate immune response by injections with pegIFNα.[28]

Lay Summary & Key Points

Our pre-clinical findings show that pegIFNλ clears a persistent HEV infection in liver chimeric mice. Treatment with pegIFNλ induces innate immune signalling in liver cells and liver-humanized mice. PegIFNλ was well tolerated, and no liver-specific side effects were recorded.

Since no data is available on the therapeutic potential of pegIFNλ for HEV, we examined the potential of pegIFNλ as a new treatment candidate against chronic gt3 HEV infections. We documented baseline IFNLR mRNA expression by primary human hepatocytes (PHH) and showed that PHH are responsive to exogenous pegIFNλ. Treatment with pegIFNλ of liver-humanized mice persistently infected with HEV showed that pegIFNλ was well tolerated. Dose escalation studies showed that although HEV was not cleared at pegIFNλ doses up to 0.12 mg/kg for a maximum of 8 weeks, a dose of 0.3 mg/kg pegIFNλ treatment resulted in complete clearance of HEV antigen and HEV RNA from the liver in eight out of nine liver-humanized mice. These pre-clinical data suggest that pegIFNλ might be a valuable alternative to treat HEV infection in patients.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Thawed cryopreserved primary human hepatocytes (PHH, Lonza, Lot:9F3003(donor 1), Basel, Switzerland and BD Gentest, Lot:342 (donor 2) and Lot:345 (donor 3), Corning, Corning, NY, USA) (Table S1) or HepG2 cells (ATCC) were seeded in a 6-well plate in growth medium containing Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Greiner Bio-one, Kremsmünster, Austria), 2 mM l-glutamine (Lonza, Basel, Switzerland), 1% penicillin-streptomycin (Lonza, Basel, Switzerland), and 20 mM HEPES (Lonza, Basel, Switzerland). HepG2 cells were incubated overnight and washed once with phosphate-buffered saline (PBS, Oxoid, Hampshire, UK). Human hepatocyte cultures were used immediately after seeding. HepG2 and PHH cell cultures were supplemented with 0.01 mg/L pegIFNα (Pegasys, Roche, Basal, Switzerland) or 0.1 mg/L pegIFNλ (kindly provided by Eiger BioPharmaceuticals, Palo Alto, CA, USA) or PBS (Oxoid, Hampshire, UK) up to 5 hours.[15] After incubation for the indicated duration, cells were collected in Qiazol (Qiagen, Hilden, Germany).

2.2 | Mouse model, infections and treatment

NOD. Cg-Prkdcscid Il2rgtm1Sug Tg(Alb-Plau)11-4/ShiJic mice (uPA-NOG) and NOD. Cg-Prkdcscid Il2rgtm1Sug Tg(Alb-UL23)7-2/ShiJic (TK-NOG) mouse embryos were provided by Dr Suemizu, Central

Institute for Experimental Animals, Kawasaki, Japan.[29,30] Mice were bred at the Central Animal Facility of the Erasmus Medical Centre (Animal Ethical Committee approval nr 141-12-11). Homozygous uPA$^{+/+}$ or TK+mice were anesthetized and transplanted with 0.5 to 2 × 10^6 viable cryopreserved PHH (Lonza, Lot:9F3003(donor 1), Basel, Switzerland, and BD Gentest, Lot:342 (donor 2) and Lot:345 (donor 3), Corning, Corning, NY, USA) via intrasplenic injection, as described.[24,25,29,30] At day −7 and −5 before transplantation, TK+mice received an intraperitoneal (ip) ganciclovir injection to initiate liver damage.[29]

Hepatocyte engraftment was determined by quantifying human albumin levels (hALB) in mouse serum with ELISA as previously described (Bethyl laboratories, Montgomery, TX, USA).[24,25] Successfully engrafted mice (as defined by hALB >0.1 mg/L[31]) were intravenously inoculated with 10^6 IU of a patient-derived faecal HEV gt3c strain (GenBank accession number ORF1:JQ015423, ORF2:KP895854),[24,28] or left non-infected as negative control. After viral inoculation, mice were housed individually.

Hepatitis E virus-infected mice were treated with pegIFNα (0.03 mg/kg unless stated otherwise) every 3-4 days via subcutaneous (sc) injection. Non-treated HEV-infected and -non-infected animals were used as controls.[28] PegIFNλ was administered at dosages up to 0.3 mg/kg every 3-4 days for up to 8 weeks, via sc or ip injection. HEV-infected mice received 60 μg/kg pegIFNλ for 4 weeks and during the last 12 days of this 4-week pegIFNλ treatment period, animals also received daily RBV injections at a dose of 25 mg/kg. Body weight and the assessment of clinical symptoms were determined 2-3 times a week (Table S2 and Figure S3). Mouse liver and bile samples were collected at euthanasia. A liver fragment was stored into RNA later at −80°C (Qiagen, Hilden, Germany). For some animals, weekly mouse faecal samples were obtained. HEV RNA levels were determined using an ISO15189:2012-validated, internally controlled qRT-PCR, as described previously.[24,32]

2.3 | RNA isolation, cDNA synthesis and qPCR analysis

RNA was phenol-chloroform extracted from less than 30 mg liver tissue or cell lysates. cDNA was prepared with PrimeScript reverse transcriptase master mix (Takara Bio Inc, Kusatsu, Japan) according to manufacturer's protocol. Primer sets (using SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA)) and TaqMan primer-probes for mRNA expression analysis are listed in Table 1. GAPDH was used as housekeeping gene control. Relative expression levels were calculated using the 2^{-dCt} conversion. Cross-reactivity of primers was checked using C57BL/6 and non-transplanted NOG mouse liver samples.

2.4 | Confocal microscopy imaging

Mouse livers were fixed in 4% formaldehyde (Merck Millipore, Burlington, MA, USA). For microscopy imaging, 4-5 μm cuts were prepared from paraffin-embedded blocks. Immunofluorescent staining of the livers was performed using rabbit anti-ORF2[33] and goat anti-human albumin (A80-229A, Bethyl Laboratories, Montgomery, TX, USA) antibodies followed by Alexa Fluor-488 or 594-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA). Nuclei were counterstained with DAPI (Thermo Fisher Scientific, Waltham, MA, USA). Slides were viewed with an EVOS fluorescence microscope (Thermo Fisher Scientific, Waltham, MA, USA).

TABLE 1 Target genes and Taqman probe IDs used for qPCR analysis (Thermo Fisher Scientific, Waltham, MA, USA)

Target Gene	Taqman Probe	Target Gene	Taqman Probe
CXCL10	Hs01124251_g1	MX1	Hs00895608_m1
DDX58	Hs01061436_m1	OAS1	Hs00973637_m1
IFIT1	Hs01911452_s1	RSAD2	Hs00369813_m1
IFNLR1 (IL28RA)	Hs00417120_m1	STAT1	Hs01013996_m1
ISG15	Hs01921425_s1	TLR3	Hs01551078_m1
GAPDH	Hs00266705_g1		

2.5 | Statistical analysis

GraphPad Prism version 9.00 for Windows (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and illustrations. Unpaired Student t tests were used to obtain P values between groups and $P < .05$ was accepted as statistically significant. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$.

3 | RESULTS

3.1 | Primary human hepatocytes are responsive to pegIFNλ treatment in vitro

We previously showed that HEV clearance from liver-humanized mice by pegIFNα treatment is dependent on a robust induction of ISG.[28] To examine whether pegIFNλ can result in comparable ISG induction as pegIFNα, we first studied the IFNLR1 expression in PHH at baseline and upon stimulation with both interferons. As seen in Figure 1A, IFNLR1 mRNA was expressed by PHH and its expression levels further increased following pegIFNλ and pegIFNα treatment. We next examined the in vitro ISG response to exogenous pegIFNλ. As HepG2 cells are known to respond to IFNα, we used HepG2 cells as a positive control and we incubated PHH or HepG2 with pegIFNλ and pegIFNα and determined the change in mRNA expression levels of STAT1, ISG15, IFIT1 and OAS1 (Figure 1 and Figure S1). Albeit that some difference in the expression levels of individual ISG were

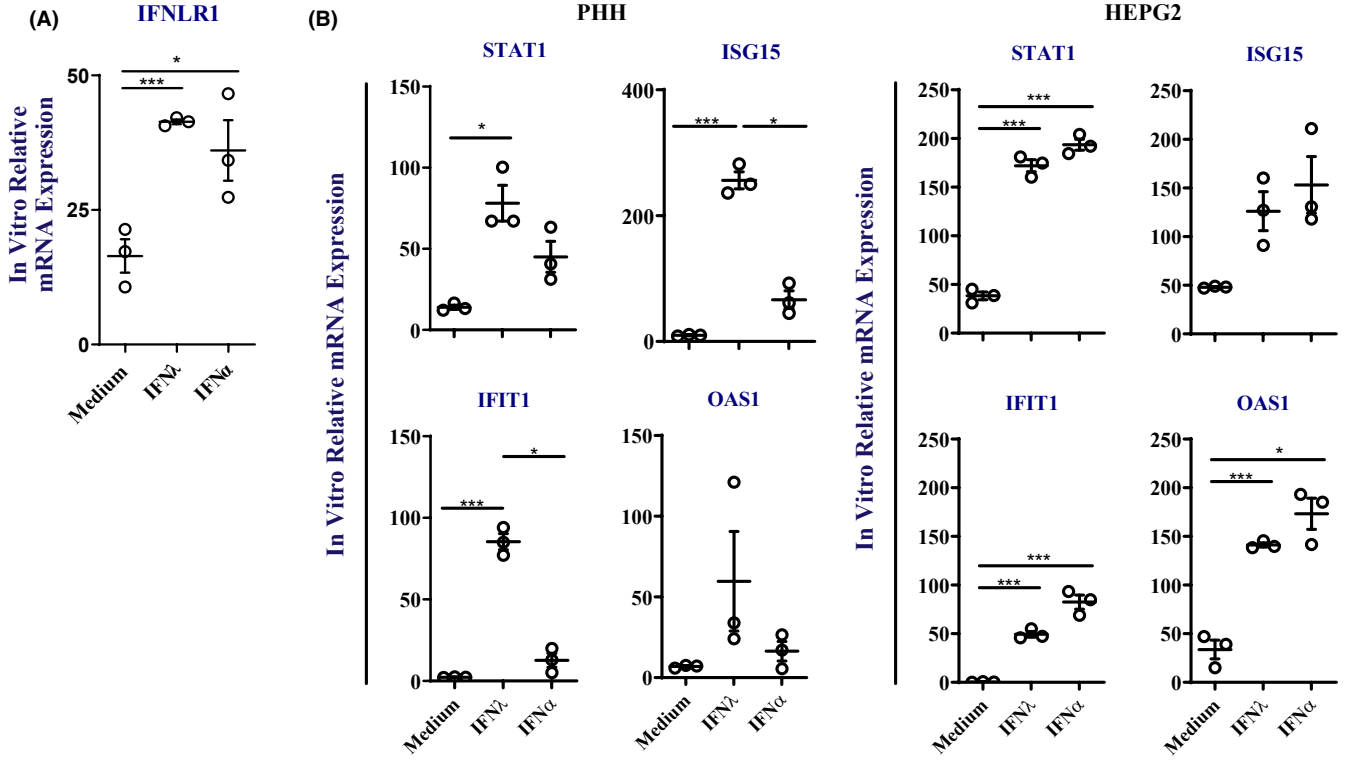


FIGURE 1 Primary human hepatocytes and hepG2 cells are responsive to exogenous IFNλ treatment in vitro. (A) IFNλ receptor, IFNLR1, mRNA expression levels of primary hepatocytes (Donor 2) and HepG2 cells after incubation with 0.01 mg/L pegIFNα or 0.1 mg/L pegIFNλ for 4 h. Each dot represents the average of three independent replicates. (B) Interferon stimulated gene (ISG) mRNA induction of primary human hepatocytes (PHH, donor342) and HepG2 cells following incubation with 0.01 mg/L pegIFNα, 0.1 mg/L pegIFNλ or PBS for 5 h. Each dot shows a biological replicate and lines show Mean±SEM, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

observed upon stimulation, both exogenous pegIFNα and pegIFNλ were potent inducers of ISG in PHH and HepG2 cells. In summary, exogenous IFNλ leads to an increase of both the IFNLR1 expression levels and ISG in vitro in PHH.

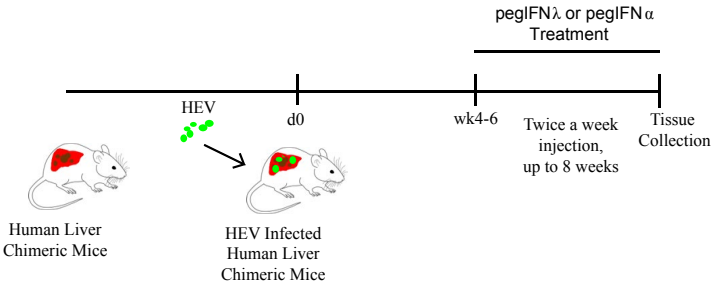
3.2 | PegIFNλ treatment leads to clearance of a persistent HEV infection in liver-humanized mice

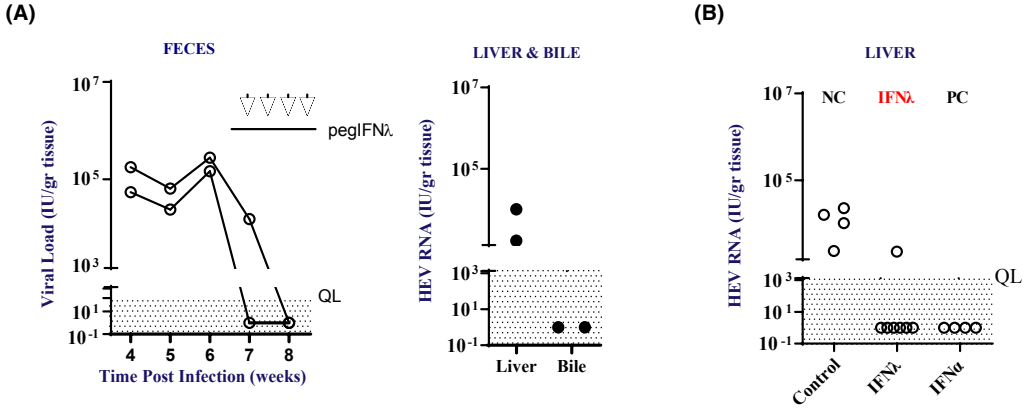
Given the in vitro induction of ISGs in both HepG2 and PHH by IFNλ, we next examined whether pegIFNλ was able to clear a HEV gt3 infection in vivo in liver-humanized mice. We first applied 0.03 mg/kg pegIFNλ twice weekly for 2 weeks, similar to the pegIFNα dose applied in our previous antiviral efficacy studies.[28] As shown in Figure 2A, HEV faecal shedding dropped after two doses of pegIFNλ. Bile and liver samples, collected at sacrifice after four injections, showed a sterilization in bile, but HEV RNA persisted in liver fragments. HEV faecal shedding therefore is sensitive to pegIFNλ treatment, but faecal or bile sterilization data is not reflective of liver clearance.

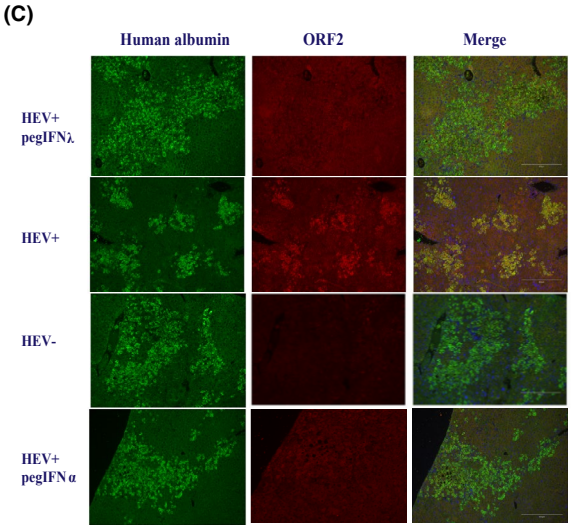
Given the reported delayed kinetics of ISG induction by IFNλ compared to IFNα,[34,35] we next examined different pegIFNλ doses and treatment durations and specifically analysed liver samples to determine the optimal HEV clearance strategy. All applied doses of pegIFNλ (up to 0.3 mg/kg) and durations (up to 8 weeks) were well tolerated by both infected and non-infected mice, without weight loss or behavioural changes (Figure 2 and Figure S3). A complete sterilization of HEV liver titres required 4 twice-weekly injections of pegIFNλ at a dose of 0.3 mg/kg (Figure S2). In independent confirmatory experiments, HEV clearance was observed in eight out of nine mice treated with 0.3 mg/kg for 2 weeks (Figure 2B). Mice treated with pegIFNα (n = 4) served as positive controls and untreated controls (n = 4) remained HEV positive (Figure 2B). Confocal images of liver sections showed no HEV ORF2 positive cells in pegIFNλ-treated animals, while human albumin expressing islands in liver fragments of non-treated controls remained HEV ORF2 positive (Figure 2C).

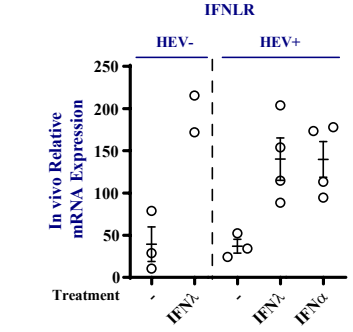
As expected, IFNLR1 mRNA was expressed in livers of humanized mice infected with HEV. Treatment with both pegIFNλ and pegIFNα further strongly increased IFNLR1 expression (Figure 2D). We next examined whether the differential sensitivity for exogenous pegIFNλ vs pegIFNα to clear HEV in vivo, could be ascribed a high baseline type III IFN responses. However, HEV gt3c infection did not induce an intrahepatic ISG, nor a type III IFN response (Figures 2E, Figure S4 and S5 and Table S4). Nevertheless, the applied pegIFNλ treatment strongly induced intrahepatic ISG mRNA expression, with a comparable profile to that of pegIFNα treatment (Figure 2F).

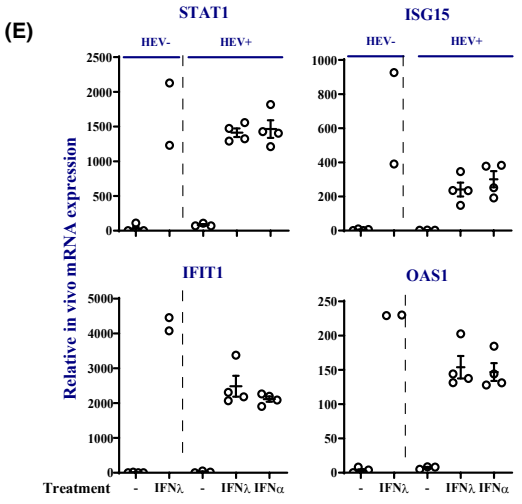
In conclusion, treatment with pegIFNλ leads to intrahepatic ISG induction,loss of HEV ORF2 protein expression in human albumin


pegIFNλ or pegIFNα
Treatment
HEV
d0
wk4-6
Twice a week
injection,
up to 8 weeks
Tissue
Collection
Human Liver
Chimeric Mice
HEV Infected
Human Liver
Chimeric Mice


(A)
FECES
LIVER & BILE
Viral Load (IU/gr tissue)
pegIFNλ
QL
Time Post Infection (weeks)
HEV RNA (IU/gr tissue)
Liver
Bile
(B)
LIVER
NC
IFNλ
PC
HEV RNA (IU/gr tissue)
QL
Control
IFNλ
IFNα


(C)
Human albumin
ORF2
Merge
HEV+
pegIFNλ
HEV+
HEV-
HEV+
pegIFNα


(D)
IFNLR
HEV-
HEV+
In vivo Relative
mRNA Expression
Treatment
IFNλ
IFNλ
IFNα


(E)
STAT1
ISG15
IFIT1
OAS1
HEV-
HEV+
Relative in vivo mRNA expression
Treatment
IFNλ
IFNλ IFNα

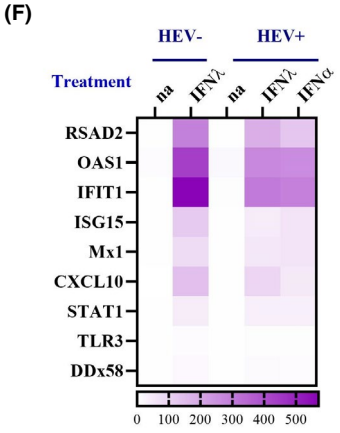

(F)
HEV-
HEV+
Treatment
na
IFNλ
na
IFNλ
IFNα
RSAD2
OAS1
IFIT1
ISG15
Mx1
CXCL10
STAT1
TLR3
DDx58
0 100 200 300 400 500

FIGURE 2 Pegylated interferon (pegIFN)λ treatment clears hepatitis E viruses (HEV) infection in liver-humanized mice. (A) Liver-humanized mice were infected with HEV gt3c for 6 weeks, after which they received an sc injection of 0.03 mg/kg pegIFNλ, every 3-4 days, for 2 weeks. HEV RNA titres in faeces (left), bile and liver (right) are shown. (B) Treatment with pegIFNλ and pegIFNα at optimal doses. HEV gt3c-infected liver-humanized mice were treated with sc 0.3 mg/kg pegIFNλ (n = 9) or sc 0.03 mg/kg pegIFNα (n = 4) injections, every 3-4 days, for 2 weeks. HEV gt3-infected control animals were left untreated (n = 4). Each dot represents an individual mouse. (C) Confocal images of liver sections stained for human albumin (anti-human albumin, A80-129A) and HEV viral antigens (anti-ORF2). Scale bar: 10 μm. IFNLR1 (D) and ISG (E) expression, and ISG expression heatmap in comparison to non-infected (F) following 2 weeks of pegIFNλ or pegIFNα treatment in vivo. The colour scale bar indicates the relative mRNA expression level in comparison to non-treated and non-infected animals (first column). Dark purple represents a significant increase, and white-light purple represent a very low expression. Non-infected (n = 2) and HEV gt3c-infected mice received 0.3 mg/kg pegIFNλ (n = 4), or 0.03 mg/kg pegIFNα sc injections (n = 4), every 3-4 days for 2 weeks. Treatment naïve, non-infected (n = 4) and HEV gt3c-infected (n = 3) mice were included as controls. Each dot represents an individual mouse. Lines show Mean ± SEM. Taqman probe details of ISGs are provided with Table S1

positive clusters and HEV RNA liver clearance in liver-humanized mice.

4 | DISCUSSION

In this study, we identified for the first time, in vivo antiviral efficacy of pegIFNλ as a new treatment candidate against persistent gt3 HEV infections in liver-humanized mice. Exogenous IFNλ stimulated ISG mRNA expression in PHH and hepatoma cells in vitro and resulted in complete clearance of HEV antigen and HEV RNA from the liver of liver-humanized mice persistently infected with HEV. Additionally, pegIFNλ was well tolerated and no systemic side effects were recorded.

Interferon-lamda has been considered to be an alternative for IFNα because both cytokines show antiviral, anti-proliferative and immunomodulatory effects and are potent inducers of the JAK/STAT pathway, which is initiated following their engagement with their respective receptors (reviewed in Ref. [17]). Differently, ISG induction and antiviral activity by IFNλ treatment were reported to be delayed and milder compared to IFNα.[34,35] Similarly, we here observe that the required pegIFNλ dose for ISG induction and HEV clearance is higher compared to pegIFNα. A differential sensitivity for exogenous IFNα has previously been ascribed to high baseline levels of intrahepatic ISG and IFNLR1 in HCV infections.[36] We here could not observe a high baseline ISG nor type III IFN expression in the livers of animals infected with a HEV gt3c strain, as possible explanation for a higher required pegIFNλ dose. Interestingly, animals infected with the Kernow strain (HEV subtype gt3a), did show a type III IFN induction (Figure S5), similar to the response observed in vitro in HepG2 cells[23] and seemed more resistant to pegIFNλ-treatment with prolonged faecal shedding despite 8 weeks of treatment (Figure S6). The reason for this strain difference is currently unclear but might be a peculiarity of the Kernow strain.[37] Our in vivo data might therefore be more representative for clinical HEV infections. The difference in dose response between pegIFNλ and pegIFNα observed here, require additional studies into the signalling cascades and feedback cycles initiated by IFNα and IFNλ receptor engagement. Nevertheless, pegIFNα and pegIFNλ resulted in a comparable dose-dependent viral RNA decrease in HCV trials,[35,38,39] thereby pointing to additional effects of pegIFNλ, for example on B cells and macrophages,[15,16] in the clinical setting that could not be modelled in our immunocompromised mouse model.

Treatment options for chronic HEV-infected immunocompromised patients are based on dose reduction of immunosuppressive drugs, RBV monotherapy or pegIFNα, which can cause severe side effects such as severe depression and neutropenia.[40] Because the expression of IFNLR is less abundant, IFNλ treatment has a great potential to spare patients from the systemic side effects of pegIFNα. Previous clinical trials have already documented that tolerability of pegIFNλ was better than pegIFNα while comparable antiviral activity against HCV and HDV was recorded (reviewed in Ref. [14,41,42]). Our data also indicates that pegIFNλ could be an alternative to pegIFNα against chronic HEV infections since less side effects are expected, and no side effects were recorded in liver-humanized animals following pegIFNλ treatment up to 8 weeks and up to 0.3 mg/kg. Our pre-clinical findings showing that pegIFNλ clears HEV infection in liver chimeric mice may have important consequences for future treatment strategies in HEV-infected patients. The here tested optimal mouse dose would be equivalent to a human pegIFNλ dose of ±24.4 μg/kg.[43] However, controlled trials are necessary to determine whether these findings can be extrapolated to patients.

5 | TRANSPARENCY DECLARATIONS

This research has not been submitted for publication nor has it been published in whole or in part elsewhere. We attest to the fact that all authors listed on the title page have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and its interpretation and agree to its submission to Liver International.

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CONFLICT OF INTEREST

AB has received research grants from GlaxoSmithKline, Janssen Pharmaceuticals, Gilead Sciences, Inc and Fujirebio. TV has participated in Advisory Committees or Review Panels for: Gilead

Sciences, Abbvie, BMS. He has also received grant/research support from: Gilead Sciences, BMS and speaking and teaching support from: Gilead Sciences, BMS.

AUTHOR CONTRIBUTIONS

Designed research: GS, AB, TV. Performed experiments: GS, CEM, JZ, GWO, AB, TV. Analysis and interpretation of the data: all. Provided essential research tools: JJCKV, ZF, AB. Wrote manuscript: GS, AB, TV. Critical revision of the manuscript for important intellectual content: all. Approved final version of manuscript: all.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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