HHS Public Access

Author manuscript

Antiviral Res. Author manuscript; available in PMC 2025 January 01.

Published in final edited form as:

Antiviral Res. 2024 January ; 221: 105779. doi:10.1016/j.antiviral.2023.105779.

Interferon lambda receptor-1 isoforms differentially influence gene expression and HBV replication in stem cell-derived hepatocytes

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Abstract

Background: In the tolerogenic liver, inadequate or ineffective interferon signaling fails to clear chronic HBV infection. Lambda IFNs (IFNL) bind the interferon lambda receptor-1 (IFNLR1) which dimerizes with IL10RB to induce transcription of antiviral interferon-stimulated genes (ISG). IFNLR1 is expressed on hepatocytes, but low expression may limit the strength and antiviral efficacy of IFNL signaling. Three *IFNLR1* transcriptional variants are detected in hepatocytes whose role in regulation of IFNL signaling is unclear: a full-length and signaling-capable form (isoform 1), a form that lacks a portion of the intracellular JAK1 binding domain (isoform 2), and a secreted form (isoform 3), the latter two predicted to be signaling defective. We hypothesized that altering expression of IFNLR1 isoforms would differentially impact the hepatocellular response to IFNLs and HBV replication.

Methods: Induced pluripotent stem-cell derived hepatocytes (iHeps) engineered to contain FLAG-tagged, doxycycline-inducible IFNLR1 isoform constructs were HBV-infected then treated with IFNL3 followed by assessment of gene expression, HBV replication, and cellular viability.

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Declarations of Competing Interests: No conflicts of interest to report.

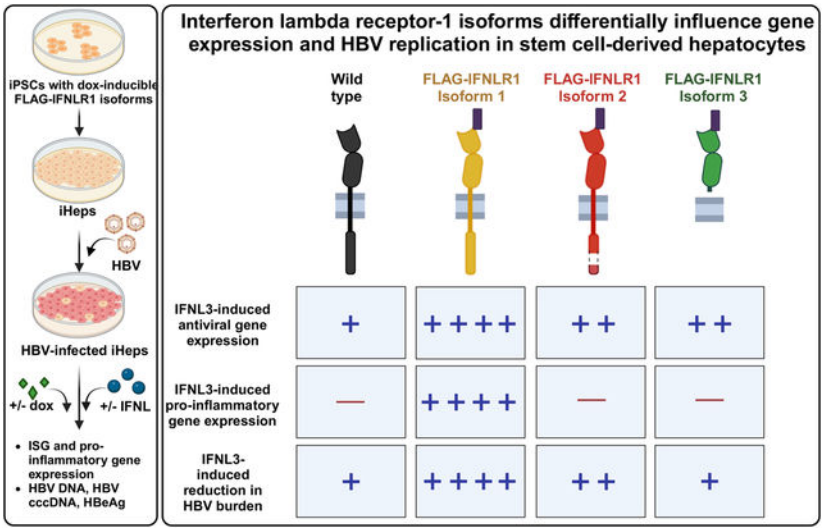
Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Results: Minimal overexpression of IFNLR1 isoform 1 markedly augmented ISG expression, induced *de novo* proinflammatory gene expression, and enhanced inhibition of HBV replication after IFNL treatment without adversely affecting cell viability. In contrast, overexpression of IFNLR1 isoform 2 or 3 partially augmented IFNL-induced ISG expression but did not support proinflammatory gene expression and minimally impacted HBV replication.

Conclusions: IFNLR1 isoforms differentially influence IFNL-induced gene expression and HBV replication in hepatocytes. Regulated IFNLR1 expression *in vivo* could limit the capacity of this pathway to counteract HBV replication.

Graphical Abstract



Keywords

IFNLR1; interferon lambda; induced hepatocytes; HBV DNA; cccDNA

1.0 INTRODUCTION

Hepatitis B virus (HBV) chronically infects 296 million persons worldwide and causes liver cirrhosis and hepatocellular carcinoma[1]. Interferons (IFN) are secreted when cells detect pathogens and promote cellular antiviral defense. While HBV activates an endogenous IFN response in some experimental systems[2–6], *in vivo* HBV is a stealth virus that avoids detection and resultant IFN production[7–10]. Exogenous administration of type-I IFN is the only approved therapy that can silence or eradicate the episomal covalently closed circular DNA (cccDNA), but this outcome occurs infrequently[11]. As IFNs remain in clinical testing as components of functional HBV cure strategies that combine antiviral and immunomodulatory therapies[12–15], further exploration of how IFN signaling can be modulated to influence HBV replication is warranted.

Lambda interferons (IFNL) are a distinct family of proteins composed of four ligands (IFNL1–4) that bind the interferon lambda receptor-1 (IFNLR1) which then dimerizes with interleukin 10 receptor beta subunit (IL10RB)[16–18]. The resultant JAK-STAT signaling

cascade results in expression of hundreds of interferon-stimulated genes (ISGs), similar to type-I IFN signaling[16,19–22]. However, while type-I IFN signaling leads to rapid induction of both antiviral and inflammatory genes and is quickly inhibited by negative regulators, IFNL signaling induces a less inflammatory transcriptional profile that is slower in onset and less susceptible to negative regulation[21,23–25]. While the type-I IFN receptor is broadly expressed by all cells, IFNLR1 has low and restricted expression in hepatocytes, epithelial cells at mucosal surfaces, and select immune cells, suggesting a critical role for IFNLs in promoting immunity at barrier sites without causing excessive, deleterious inflammation[21,26–32].

While IFNLs are not currently approved for treatment of any infection, they demonstrated antiviral activity against SARS-CoV-2, hepatitis C virus, hepatitis D virus, and HBV in clinical trials[33–37]. Pegylated (PEG)-IFNL1 promoted a significant decline in HBV DNA and HBV surface antigen (HBsAg) and had favorable tolerability compared to PEG-IFN alpha-2, a type-I IFN[38]. However, this antiviral effect plateaued after repeated dosing and viral rebound occurred post-treatment, for reasons that are poorly understood. It is unknown if variable IFNLR1 expression or induction of regulatory pathways during prolonged treatment influenced these outcomes.

IFNLR1 detection has largely been based on mRNA levels as IFNLR1 protein has low expression and highly specific commercial reagents are limited[39,40], a constraint that may be overcome with the recent characterization of monoclonal antibodies that target protein[41]. Multiple *IFNLR1* transcriptional splice variants are expressed in cells and are predicted to encode distinct protein isoforms.[18,27,28]. *IFNLR1* isoform 1 encodes a full-length protein that is signaling-capable, whereas isoform 2 and 3 are predicted to be signaling defection due to lack of part of the intracellular JAK1 binding domain and the transmembrane domain, leading to secretion, respectively[16,22,28,42]. *IFNLR1* isoform 1 has the highest mRNA expression in epithelial cells, including hepatocytes, and work by ourselves and others showed that overexpression of this protein augments IFNL-induced antiviral gene expression and permits de novo expression of inflammatory genes similar to type-I IFN signaling[28,40,43,44]. Contrary to expectations, using HEK293T cells with doxycycline-inducible expression of FLAG-IFNLR1 isoforms, we recently showed that IFNLR1 isoform 2 or isoform 3 overexpression in the presence of endogenous IFNLR1 supported a partial increase in IFNL3-induced ISG induction but did not support pro-inflammatory gene expression[44]. These findings identified unique functional roles for IFNLR1 isoforms and suggested varied isoform expression could be a mechanism cells use to influence antiviral responses without causing excessive inflammation. We thus hypothesized that IFNLR1 isoforms could differentially influence the response of IFNL-exposed hepatocytes and HBV replication.

To test this hypothesis, we generated induced pluripotent stem cells (iPSCs) that expressed doxycycline-inducible IFNLR1 isoform expression constructs and differentiated them into hepatocyte-like cells (iHeps) which are physiologically like primary hepatocytes, have intact IFN signaling pathways, and are permissive for HBV replication[45–50]. We evaluated the impact of IFNLR1 isoform overexpression on gene expression and HBV replication in iHeps after IFNL treatment.

2.0 MATERIALS AND METHODS

2.1 Cell lines

The K3 iPSC line (a gift from Stephen Duncan) was used to generate the cell lines described in this work[45–47]. The design and cloning strategies of FLAG-tagged IFNLR1 isoform constructs are described in[44]. K3 iPSCs were electroporated with 30μg PvuI-linearized plasmid DNA followed by plating on Matrigel-coated plates in mTeSR plus zFGF for 48h. Clones with integrated expression constructs were selected with puromycin (1μg/ml) and surviving clones picked, screened for doxycycline-induced expression of FLAG by immunofluorescent stain, and positive clones expanded. iPSCs were then maintained in mTeSR plus zFGF and puromycin under low oxygen conditions (4% O_2/5% CO_2). Resultant colonies (FLAG-Iso1, FLAG-Iso2 and FLAG-Iso3) with FLAG-positive stain and a vector-only control (EV) that had growth rates comparable to wild-type (WT) line were selected for further experimentation.

To generate an *IFNLR1* knock-out (*IFNLR1*-KO) iPSC line, a CRISPR guide RNA targeting exon 4 of *IFNLR1* was designed (CCTGGTGCTCACCCAGACGG), cloned into the PX459 pSPCas9(BB)-2A-Puro vector (#48139; Addgene)[51], and transfected into K3 iPSCs using Viafect (Promega). Cells were selected with puromycin (1 μg/ml) and propagated. Genomic DNA was extracted from surviving clones using QuickExtract™ DNA extraction solution (Epicenter) and targeted regions were amplified using Herculase Fusion Polymerase (Agilent) and target area-specific primers (Fwd: CCTCGGGATTACTGACAGGTCC; Rev: CAGGTTCTTCTCACCCTCCCAG). Amplicons were restriction digested and sequenced to confirm the identity of insertion-deletion events. A clone with a −2 bp deletion and + 1 bp insertion, which introduced a premature stop codon, that had high Tracking of Indels by Decomposition and Inference of CRISPR Edits scores was selected for future work.

iPSC clones were differentiated to iHeps according to an established 20-day, four-step process as previously described[45–47]. To prevent expression construct silencing during differentiation, culture medium was supplemented with 10ng/ml doxycycline (dox; Sigma Aldrich)[52,53].

2.2 Characterization of iHeps by protein and gene expression

To detect expression of the hepatocyte lineage markers α-fetoprotein (AFP) and human nuclear factor 4α (HNF4α), WT iPSC or iHeps were fixed with 4% paraformaldehyde, permeabilized with 0.5% TritonX-100, then blocked with 3% BSA-DPBS at 25°C. Cells were stained with antibodies against AFP and HNF4α in 1% BSA-DPBS overnight at 4°C (Suppl. Table 1) followed by species-specific secondary antibodies and counterstain with VectaShield with DAPI (Vector). Images were captured with an EVOS Cell Imaging System (ThermoFisher).

To quantitate gene expression, RNA was isolated from iPSC and iHep lines using a Qiagen RNeasy kit and quantitated by Nanodrop. cDNA was transcribed with a high-capacity cDNA reverse transcription kit and qRT-PCR was performed using commercially available TaqMan primer-probe sets listed in Suppl. Table 2 (ThermoFisher).. qRT-PCR to detect construct expression, HBV DNA, and HBV cccDNA used custom primer-probe sets (Suppl. Table

3). Experiments were performed with biological and technical duplicates with expression relative to *GAPDH*. Technical duplicates were averaged and data presented in graphs represent the average of biological replicates.

Flow cytometry was used to evaluate dox-induced FLAG expression. Differentiated iHeps were incubated 24h in Hepatocyte Culture Medium (HCM; Lonza) without dox supplementation then cultured in +/− dox (100 ng/ml) for 24h. Cells were released using undiluted TrypLE Select Enzyme (10X; Gibco) then stained with Live-or-Dye 615/740 fixable viability stain (Biotium). Cells were fixed with 2% paraformaldehyde and then surface stained for FLAG or permeabilized with Triton X-100 to permit intracellular FLAG staining (Suppl. Table 1). Twenty thousand live events per sample were collected on a Millipore Guava easyCyte 8HT flow cytometer and data were analyzed with FlowJo X v10.0 (BD Life Sciences).

An immunoblot was performed to detect FLAG-tagged protein secreted into culture supernatants. Supernatants collected from iHeps exposed to 100ng/ml dox for 24h were clarified by centrifugation and applied to nitrocellulose membrane by vacuum aspiration using a BioRad Bio-Dot apparatus. The membrane was blocked with 5% BSA in TBS, probed with murine monoclonal anti-FLAG M2 (Sigma Aldrich), revealed with goat anti-mouse IgG-HRP (Novus Biologicals) and developed with ECL (ThermoFisher). Imaging was performed with a FluorChem R instrument (Protein Simple).

2.3 HBV infection and IFNL3 treatment of iHeps.

HBV viral stock was precipitated from HepG2.2.15 cell supernatant using a PEG Virus Precipitation kit (Abcam) and quantitated by qPCR using an HBV DNA primer-probe set relative to M-HBsAg plasmid DNA (#103012, Addgene)[54]. HBV was suspended in HCM supplemented with oncostatin M (OSM), 10ng/ml dox, and 4% PEG 8000 and iHeps were inoculated with 1,000 HBV genome equivalents (GEq)/cell. After 24h iHeps were extensively washed and cultured in media without PEG 8000.

After 14-days of incubation to allow HBV propagation, one of four treatments diluted in HCM+OSM was applied: 1) medium only, 2) 100ng/ml dox, 3) 100ng/ml IFNL3 (R&D Systems), or 4) 100ng/ml dox plus 100ng/ml IFNL3. Treatments were freshly applied for eight consecutive days followed by collection of cells and supernatant for HBV quantitation and gene expression analysis. IFNL3 was selected as a representative IFNL due to its strong affinity and antiviral activity[55].

To quantitate HBV, culture supernatants were clarified by centrifugation, viral DNA purified using the Qiagen QIAamp Blood DNA kit, and qPCR performed. Data are presented as HBV gEq per volume of supernatant and each biological sample was assayed in technical duplicate.

To evaluate gene expression and quantitate cellular HBV viral load, cells were harvested for a) extraction of RNA using a Qiagen RNeasy kit or b) extraction of DNA using a Qiagen QIAamp Blood DNA kit. To quantitate HBV cccDNA, extracted DNA was heated to 85°C for 5min, then incubated with plasmid-safe ATP-dependent DNase for 16h at 37°C prior to

inactivation of enzyme by heating to 70°C for 30min[35]. Quantitation of total HBV DNA was determined by qPCR using the primers described in[54] or HBV cccDNA using the primers described in[35] and in Suppl. Table 3.

HBeAg was quantitated in clarified culture supernatants by ELISA (Creative Diagnostics) to evaluate active HBV replication. Supernatant from HepG2 and HepG2.2.15 cultures served as negative and positive controls. Experiments met quality control standards and samples with values ≥1.0 were considered positive for HBeAg.

2.4 Statistical analyses.

Data are presented as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism v9.1.0 software with statistical significance set at $p \leq 0.05$.

3.0 RESULTS

3.1 Expression of FLAG-tagged IFNLR1 isoform constructs in iHeps

To examine how altering expression of IFNLR1 isoforms impacts hepatocytes treated with IFNLs during HBV infection, we first differentiated primary human fibroblast-derived K3 iPSCs into iHeps[45–47]. iHeps expressed HNF4α and AFP, characteristic markers of hepatocytes, whereas iPSCs did not (Fig. 1A). We next generated stable iPSC lines that expressed dox-inducible, FLAG-tagged IFNLR1 isoform constructs, henceforth referred to as FLAG-Iso1, FLAG-Iso2 and FLAG-Iso3. Each iPSC clone could be differentiated to iHeps, evidenced by higher relative expression of *ALB*, an indicator of mature hepatocytes, and reduced relative expression of the pluripotent stem cell marker *OCT4* (Fig. 1B). WT iHeps which stably expressed empty vector (EV) and iHeps in which endogenous *IFNLR1* was abrogated by CRISPR-Cas9 gene editing (*IFNLR1*-KO) similarly expressed hepatocyte markers. All iHep lines expressed *NTCP*, the functional receptor for HBV (Fig. 1B).

To evaluate FLAG-IFNLR1 expression in iHeps, dox was eliminated from culture medium for 24h followed by dox (100ng/ml) induction for 24h. FLAG-Iso1, -2 and -3 gene expression were significantly greater in dox-induced iHeps relative to cells maintained in medium or EV-iHeps (Fig. 1C). A low level of FLAG-isoform construct gene expression was detected in dox-free conditions compared to respective EV-iHeps, suggesting a degree of non-specific transcription from the tet-promoter, as has been previously observed[44,56], or residual transcript from the dox (10 ng/ml) included during differentiation. Neither dox-treatment nor construct overexpression influenced endogenous *IFNLR1* or *IL10RB* gene expression (Suppl. Fig. 1). Endogenous *IFNLR1* isoform 1 was more highly expressed than isoforms 2 or 3, consistent with prior observations in epithelial cells, including hepatocytes[28].

We next examined FLAG-Iso1, -2, and -3 protein expression by flow cytometry using anti-FLAG antibody (gating strategy shown in Suppl. Fig. 2A). FLAG-Iso1 and FLAG-Iso2 were detected on the cell surface and intracellularly in dox-induced iHeps, whereas FLAG-Iso3 was only observed intracellularly, as predicted (Fig. 1D). No FLAG staining was observed in iHeps maintained in medium or in EV-iHeps. Overexpression of FLAG-isoform constructs did not negatively impact iHep health with >92% viability of all lines (Suppl. Fig. 2B). By

dot blot, we confirmed that only FLAG-Iso3 iHeps secreted protein into culture supernatants after dox induction, as anticipated for this IFNLR1 isoform (Suppl. Fig. 3)[44,57].

3.2 Differential expression of antiviral and pro-inflammatory genes by iHeps

To evaluate how IFNLR1 isoform overexpression influences the cellular response to IFNLs, we quantitated expression of the antiviral ISG, *MX1*. WT iHeps, which express endogenous IFNLR1, induced *MX1* after IFNL3 treatment, while no *MX1* induction was observed in *IFNLR1*-KO iHeps (Suppl. Fig. 4). IFNL3 treatment of FLAG-Iso1 iHeps led to a marked increase in *MX1* expression relative to IFNL3-treated EV-iHeps (Fig. 2A). *MX1* induction in IFNL3-treated FLAG-Iso1 iHeps was similar irrespective of dox-treatment, indicating that the amount of FLAG-Iso1 produced in dox-minus conditions was sufficient to support a maximal response that was not further augmented by higher receptor expression, comparable to our prior observations in HEK293T cells[42]. An increase in *MX1* expression was also detected in IFNL3-treated FLAG-Iso2 iHeps irrespective of dox-inclusion, and to a lesser extent in dox-treated FLAG-Iso3 cells. These findings were unexpected, as lacking a portion of the cytoplasmic signaling domain (FLAG-Iso2) or the transmembrane domain (FLAG-Iso3) are predicted to prohibit the capacity for IFNL signaling; of note, these findings could be influenced by retained endogenous IFNLR1 expression. Notably, *MX1* induction in FLAG-Iso2 and FLAG-Iso3 iHeps was significantly less than FLAG-Iso1 iHeps.

IFNLR1 isoform 1 overexpression was previously shown to not only support higher IFNL-induced expression of antiviral genes in immortalized hepatocytes, but also de novo transcription of pro-inflammatory genes like *CXCL10*[40]; in this prior work, the capacity of IFNLR1 isoform 2 or 3 to support proinflammatory gene expression was not examined. Our work with HEK293T cells showed that FLAG-Iso1 overexpression supported *de novo* expression *CXCL10* after IFNL3 treatment; however, and unexpectedly, FLAG-Iso2 and -Iso3 did not[44]. To evaluate these findings in iHep clones, we quantitated expression of the proinflammatory gene *CXCL10*. IFNL3-stimulated FLAG-Iso1 cells exhibited *CXCL10* induction, while IFNL3-treated EV, FLAG-Iso2, FLAG-Iso3 iHeps did not (Fig. 2B). Collectively, these data demonstrate that IFNLR1 isoforms differentially influence both the magnitude and breadth of gene expression imparted by IFNL treatment in iHeps.

3.3 Endogenous IFNLR1 supports IFNL-mediated inhibition of HBV replication

Because IFNLR1 isoforms differentially influence antiviral and pro-inflammatory gene expression, this suggests their relative expression *in vivo* could have influenced the plateau in efficacy observed when PEG-IFNL1 was tested for treatment of chronic HBV infection[38]. As no clinical samples exist to test this hypothesis, we evaluated the possibility of this mechanism using an *in vitro* iHep-HBV infection model. WT and *IFNLR1*-KO iHeps were HBV infected and incubated for fourteen days to allow for propagation of infection (Fig. 3A). HBV DNA in culture supernatants increased comparably in both lines indicating productive HBV infection and suggesting no induction of a meaningful IFNL response in WT iHeps (Fig. 3B), consistent with HBV replicating in hepatocytes as a stealth virus[7–10]. HBV-infected iHeps were then mock or IFNL3-treated daily for eight days. IFNL3 treatment of WT iHeps reduced HBV DNA in supernatants and cell lysates compared to mock-treated WT iHeps, consistent with prior reports[58,59], whereas IFNL3 treatment had

no effect on HBV replication in *IFNLR1*-KO iHeps (Fig. 3C). Quantitation of HBeAg in culture supernatant as an indicator of active HBV replication revealed a significant reduction in IFNL3-treated WT iHeps, whereas no change was observed in *IFNLR1*-KO iHeps (Fig. 3D). These data demonstrate that endogenous levels of IFNLR1 support a reduction in HBV burden in response to IFNL3 treatment.

3.4 IFNLR1 isoforms differentially impact IFNL3-mediated inhibition of HBV replication

We next evaluated how IFNLR1 isoform overexpression influences the impact of IFNL3 treatment during HBV infection in FLAG-Iso1, -2 and -3 iHeps with comparison to WT, *IFNLR1*-KO, and EV iHep controls (Fig. 4A). Quantitation of HBV DNA in supernatants during infection identified no differences between lines (Fig. 4B). After eight days of IFNL3 treatment, HBV DNA declined in WT and EV iHeps compared to mock-treated iHeps, while no HBV reduction was observed in *IFNLR1*-KO iHeps (Fig. 4C). An accelerated and greater magnitude of HBV DNA decline was observed in IFNL3-treated FLAG-Iso1 iHeps (Fig. 4C). FLAG-Iso2 and FLAG-Iso3 iHeps had partial or no augmentation of HBV decline, respectively, relative to EV and WT iHeps (Fig. 4C). On days 19 and 21 post-infection in FLAG-Iso1 iHeps and on day 21 in FLAG-Iso2 iHeps, significantly less HBV DNA was detected in supernatants relative to similarly treated EV iHeps. Microscopic observation of iHep lines at this time revealed intact cultures and cellular integrity; thus, despite HBV infection, FLAG-isoform overexpression, and repeated IFNL3 treatment, iHep cultures remained viable (Suppl. Fig. 5).

We next examined the relative burden of cellular HBV DNA and cccDNA after treatment. Consistent with culture supernatant results, WT and EV iHeps had reduced HBV DNA and cccDNA after IFNL3 treatment compared to mock-treated iHeps or IFNL3-treated *IFNLR1*-KO iHeps (Fig. 4D). HBV DNA and cccDNA were more markedly reduced in IFNL3-treated FLAG-Iso1 iHeps, and this response was further enhanced upon IFNLR1 overexpression with dox. IFNL3 treatment of FLAG-Iso2 cells also had reduced HBV DNA compared to EV iHeps, but overexpression did not augment this response and the decline was less marked than FLAG-Iso1 iHeps. There was no difference between FLAG-Iso3 and EV iHeps for these parameters.

Quantitation of HBeAg revealed that only IFNL3-treated FLAG-Iso1 iHeps showed a significant reduction relative to IFNL3-treated EV iHeps, a reduction that was further augmented by dox-induced IFNLR1 overexpression. These data collectively demonstrate that IFNL3 engagement of FLAG-Iso1 promoted an antiviral response beyond that imparted by endogenous IFNLR1 expression, and higher FLAG-Iso1 further enhanced this phenotype.

3.5 Expression of antiviral and pro-inflammatory genes in HBV-infected, IFNL3-treated iHeps

To evaluate the hepatocellular response of HBV-infected, IFNL3-treated iHeps upon completion of treatment, we quantitated *MX1, APOBEC3G, ISG15, USP18, CXCL10*, and *IRF1* expression as representative ISGs and mediators of anti-HBV response. WT, EV, and all FLAG-Isoform expressing iHeps treated with IFNL3 had augmented expression of *MX1, APOBEC3G* (Fig. 5A&B), *ISG15* and *USP18* (Suppl. Fig. 6A&B) compared to mock-

treated cells, and there was no response in *IFNLR1*-KO iHeps. IFNL3-treated, dox-induced FLAG-Iso1 cells had significantly greater expression of *MX1* and *APOBEC3G* whereas IFNL3-treated FLAG-Iso2 and -Iso3 iHeps had higher *MX1* and *ISG15* expression in both non- and dox-induced conditions, compared to EV iHeps. Expression of *MX1, ISG15*, and *USP18* in IFNL3-treated FLAG-Iso2 and -3 iHeps was comparable in magnitude to FLAG-Iso1, in contrast to what had been observed in non-HBV infected iHeps treated with a single dose of IFNL3 (Fig.2A); this difference could relate to HBV infection, repeated IFNL3 stimulation, and/or the prolonged culture conditions. Notably, only IFNL3-treated FLAG-Iso1 iHeps induced both *CXCL10* and *IRF1* expression (Fig. 5C&D). These data suggest that proinflammatory gene expression supported by FLAG-Iso1, but not FLAG-Iso2 or -Iso3, may be critical for maximal suppression of HBV replication by IFNLs.

4.0 DISCUSSION

In this study, we demonstrate that overexpression of IFNLR1 isoforms uniquely and differentially influences IFNL3-induced gene expression and HBV replication in iHeps. This study builds upon our prior work using HEK293T cells with stable expression of FLAG-Isoform constructs, wherein minimal overexpression of FLAG-isoform 1 induced a maximal IFNL3-induced antiviral activity and a concomitant dampening of type-I IFN response[44]. Overexpression of FLAG-isoform 2 permitted moderate IFNL3-induced antiviral gene activity and minor IFNL3 specific antiviral gene expression was observed by cells that overexpressed FLAG-Isoform 3. Intriguingly, whereas overexpression of FLAG-Isoform 1 resulted in expression of pro-inflammatory genes, overexpression of FLAG-isoform 2 or -3 did not. In our current work, we employed an iPSC-derived hepatocyte cell culture model that was more physiologically similar to primary hepatocytes and also susceptible to infection with HBV. Overexpression of canonical IFNLR1 (FLAG-Iso1) led to a marked increase in IFNL3-induced expression of the antiviral ISGs *MX1* and *APOBEC3G*, and the pro-inflammatory genes *CXCL10* and *IRF1*. FLAG-Iso1 cells had greater inhibition of HBV infection relative to control iHeps. This demonstrates a direct influence of receptor expression levels on replication of a hepatotropic virus and suggests that tight hepatocellular control of IFNLR1 expression may limit pathway activity and excessive inflammation in a tolerogenic organ, but with a cost of restricting the antiviral benefit of IFNL exposure. In contrast, iHeps expressing non-canonical IFNLR1 isoforms (FLAG-Iso2 and -Iso3) with endogenous IFNLR1 exhibited increased expression of antiviral ISGs, but no induction of proinflammatory genes and reduced or no impact on HBV replication relative to IFNLR1 isoform 1 after IFNL3 treatment. This suggests non-canonical IFNLR1 isoforms, either autonomously or in concert with endogenous IFNLR1, may influence antiviral ISG expression without supporting induction of proinflammatory genes. Taken together, these data suggest relative expression of IFNLR1 isoforms may control the nature of the transcriptional response of hepatocytes after IFNL exposure through mechanisms that directly influence cellular capacity to inhibit HBV replication.

We posit that the mechanism for varied responses relates to the signaling capabilities supported by each IFNLR1 isoform. IFNLR1 isoform 1 is a full-length protein comprised of all extra- and intracellular components, and thus should support maximal engagement of the JAK-STAT signaling cascade[16,22,42]. While IFNL signaling through endogenous IFNLR1

results in STAT1:STAT2 heterodimers, overexpression of IFNLR1 was shown to support formation of STAT1:STAT1 homodimers leading to interferon regulatory factor-1 (*IRF1*) and *CXCL10* expression[40]. FLAG-Iso1 iHeps demonstrated increased expression of *IRF1* and *CXCL10*, correlating with our prior observation in HEK293T cells, therefore whether this specific signaling mechanism accounts for the FLAG-Iso1-mediated IFNL induction of antiviral and proinflammatory genes that was highly effective in reducing HBV burden in this study merits further investigation.

IFNLR1 isoform 2 lacks a portion of the intracellular Box 1 domain and the entire Box 2 domain that together promote JAK1 binding and stabilization[40,60]. Although predicted to be incapable of signaling due to these truncations, IFNL3-treated FLAG-Iso2 iHeps expressed *MX1* and had reduced HBV burden, albeit to a lesser extent than FLAG-Iso1 cells. We speculate this could relate to the capacity of IFNLR1 isoform 2 to form less stable IFNLR1-IL10RB receptor complexes upon IFNL3 binding. We posit that this weaker IFNL signaling, manifested as a reduced *MX1* response compared to FLAG-Iso1 cells, may not support a shift toward STAT1 homodimer formation and pro-inflammatory gene expression that is required for enhanced HBV inhibition. IFNLR1 isoform 3 is missing both transmembrane and intracellular domains, and while predicted to be incapable of signaling[42], overexpression of FLAG-Iso3 on iHeps expressing endogenous IFNLR1 did promote low level induction of *MX1*. IFNLR1 isoform 3 has been shown to bind and sequester IFNL at the cell surface[28], potentially making the cytokine available to interact with endogenous receptor by proximity. However, the minimal antiviral response and lack of proinflammatory gene expression by FLAG-Iso3 iHeps did not result in HBV clearance herein. The molecular mechanisms by which IFNLR1 isoforms 2 and 3 influence signaling in the presence of endogenous IFNLR1 and whether they can autonomously support signaling without endogenous IFNLR1, using cells with abrogated endogenous expression, is an important area of future work.

Collectively, this study identifies differential IFNL signaling capabilities for each IFNLR1 isoform that directly influence HBV replication in iHeps. These data suggest a possible mechanism for the outcome of prior clinical trials in which PEG-IFNL therapy for chronic HBV showed promise to activate immune cells and reduce HBV burden[38,61], yet this response plateaued over time. It is possible that variable IFNLR1 expression levels and/or differential expression of IFNLR1 isoforms over the course of repeated treatment could have contributed to an attenuated response to exogenous PEG-IFNL. While this work utilized a cell culture model comprised of a single cell type without ancillary cells found within the liver itself, the iHep model will be useful for continued efforts to dissect the cellular response to HBV infection and IFNL3-IFNLR1 activation, particularly when considering how to augment the antiviral response of non- or minimally reactive hepatocytes in an effort to resolve HBV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

We thank Paige Lamprecht-McGinnis for assistance with iHep reagents and Troy Dearth for technical assistance (Medical University of South Carolina).

Funding:

EGM received support from NIGMS (P20GM130457), NIDDK (P30DK123704), and the South Carolina Clinical & Translational Research (SCTR) Institute (NCATS Grant Number UL1TR001450). JGE received support from NCATS (TL1TR001451). HG received support from NIAID (R01AI110762, R01AI150255, and R01AI134818).

Abbreviations

cccDNA	covalently closed circular DNA
dox	doxycycline
EV	empty vector
IFN	interferon
IFNL	interferon lambda
IFNLR1	interferon lambda receptor-1
iHep	induced hepatocyte
iPSC	induced pluripotent stem cell
ISG	interferon-stimulated gene
WT	wild type

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Highlights

1. IFNLR1 isoforms differentially support expression of lambda interferon-induced genes in hepatocytes.
2. IFNLR1 isoform 1 supports antiviral and inflammatory gene expression and maximal inhibition of HBV replication.
3. IFNLR1 isoforms 2 and 3 support antiviral, but not inflammatory, gene expression and have less impact on HBV replication.

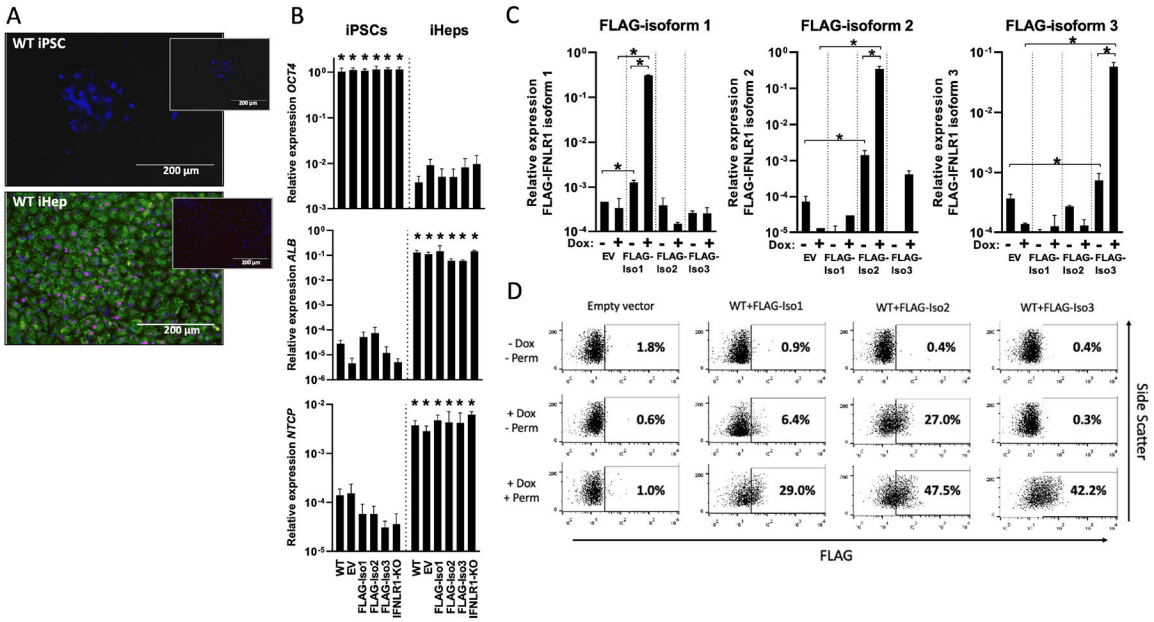

Fig 1. Characterization of iHep lines.

(A) Immunofluorescent staining of WT iPSCs and iHeps for expression of HNF4α (red) and AFP (green). HNF4α colocalization with DAPI nuclear stain (blue) yielded pink color. Insets, iPSC or iHeps stained with isotype antibody controls. Scale bar, 200 μm. **(B)** Expression of *OCT4*, *ALB*, and *NTCP* in iPSC and iHep lines determined by qRT-PCR relative to *GAPDH*. * = p≤0.05 by Student's t-test comparing iPSCs to corresponding iHeps. **(C)** Expression of each FLAG-IFNLR1 construct by iHep lines after induction with doxycycline relative to *GAPDH* determined by qRT-PCR. * = $p \leq 0.05$ by Student's t-test as indicated. **(D)** Flow cytometry scatter plots of viable iHeps showing the proportion of each population with dox-induced expression of FLAG-IFNLR1 construct. Quantitative data are shown as mean ± standard deviation of biological duplicates assayed as technical replicates. Abbreviations: iPSC, induced pluripotent stem cell; iHep, induced hepatocyte; WT, wild-type; HNF4α, human nuclear factor 4α; AFP, a-fetoprotein; *OCT4*, octamer binding transcription factor; *ALB*, albumin; *NTCP*, sodium taurocholate co-transporting polypeptide; dox, doxycycline; perm, permeabilized.

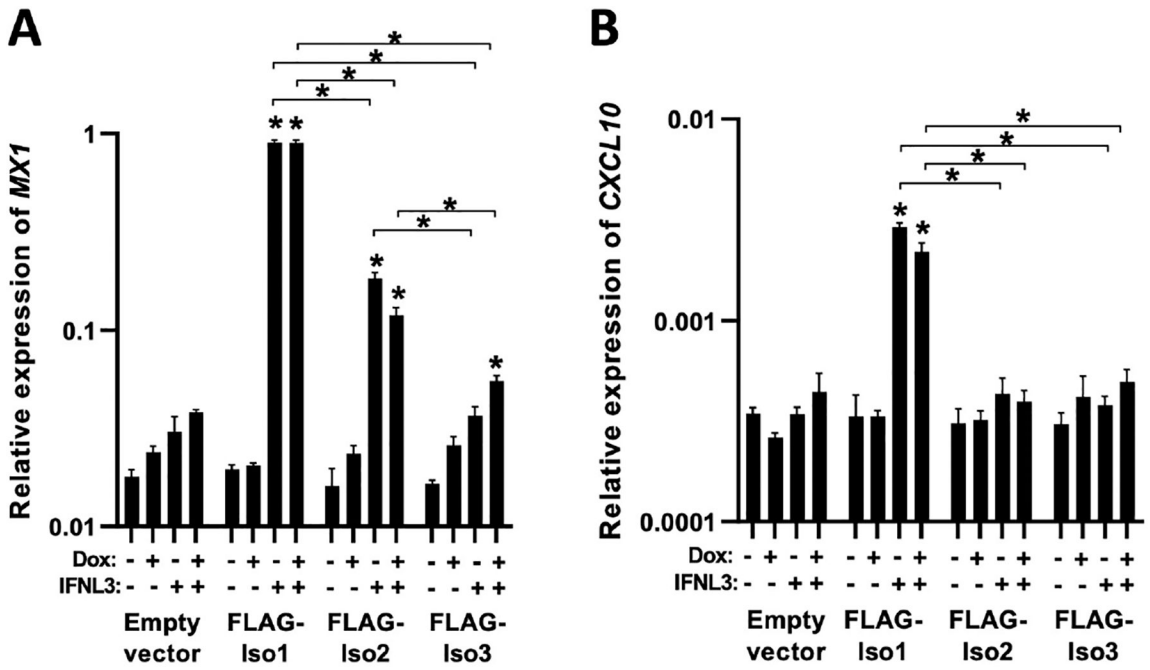

Fig 2. Differential gene expression in IFNL3-stimulated, FLAG-IFNLR1 isoform expressing iHeps.

Expression of **(A)** *MX1* and **(B)** *CXCL10* relative to *GAPDH* determined by qRT-PCR. * = p≤ 0.05 by Student's t-test as indicated. Individual * indicate significance compared to respective EV control. Quantitative data are shown as mean ± standard deviation of biological duplicates assayed as technical replicates. Representative data are shown from two independent experiments. Abbreviations: iHep, induced hepatocyte; dox, doxycycline; IFNL3, interferon lambda, *MX1*, MX dynamin-like GTPase; *CXCL10*, C-X-C motif chemokine ligand 10.

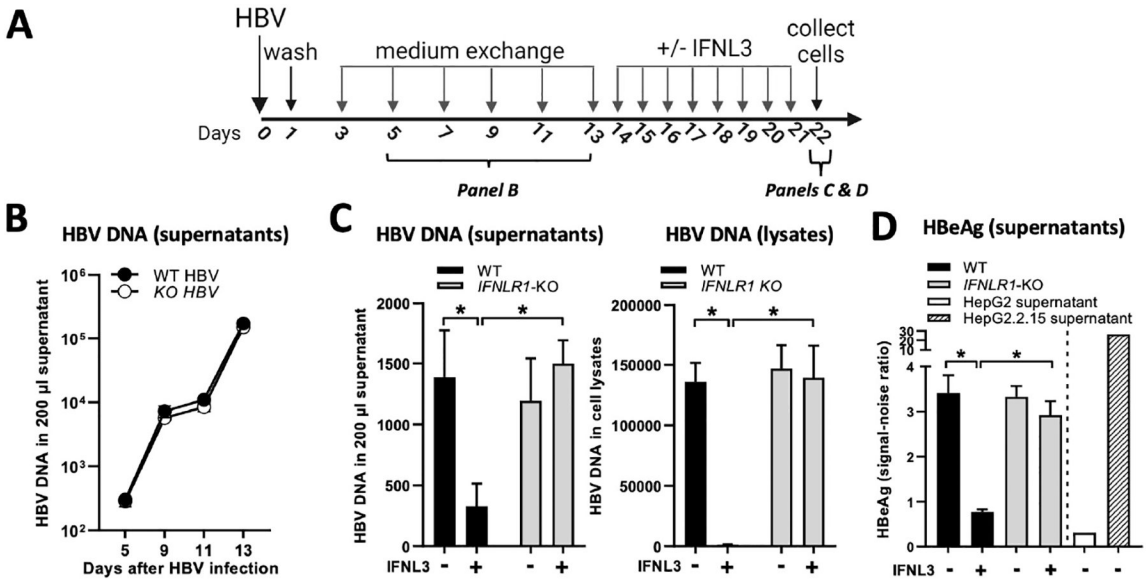

Fig 3. IFNL3 treatment of HBV-infected WT, but not *IFNLR1*-KO, iHeps reduced viral burden.

(A) Schematic depicting HBV infection and IFNL3 treatment of WT and *IFNLR1*-KO iHeps. Quantitation of HBV DNA in supernatants collected **(B)** during progression of HBV infection and **(C)** in supernatants and cell lysates upon completion of IFNL3 treatment was determined by qPCR. **(D)** Detection of HBeAg in clarified culture supernatants collected on day 22 was determined by ELISA. Supernatants from HepG2 and HepG2.2.15 cell lines cultured for 7 days without medium exchange are shown as negative and positive controls, respectively. HBeAg values ≥1.0 indicate an HBeAg positive sample. Quantitative data are shown as mean ± standard deviation of biological duplicates assayed as technical replicates. * = p≤ 0.05 by Student's t-test. Abbreviations: IFNL3, interferon lambda 3; WT, wild type; *IFNLR1*-KO, interferon lambda receptor-1 knock out.

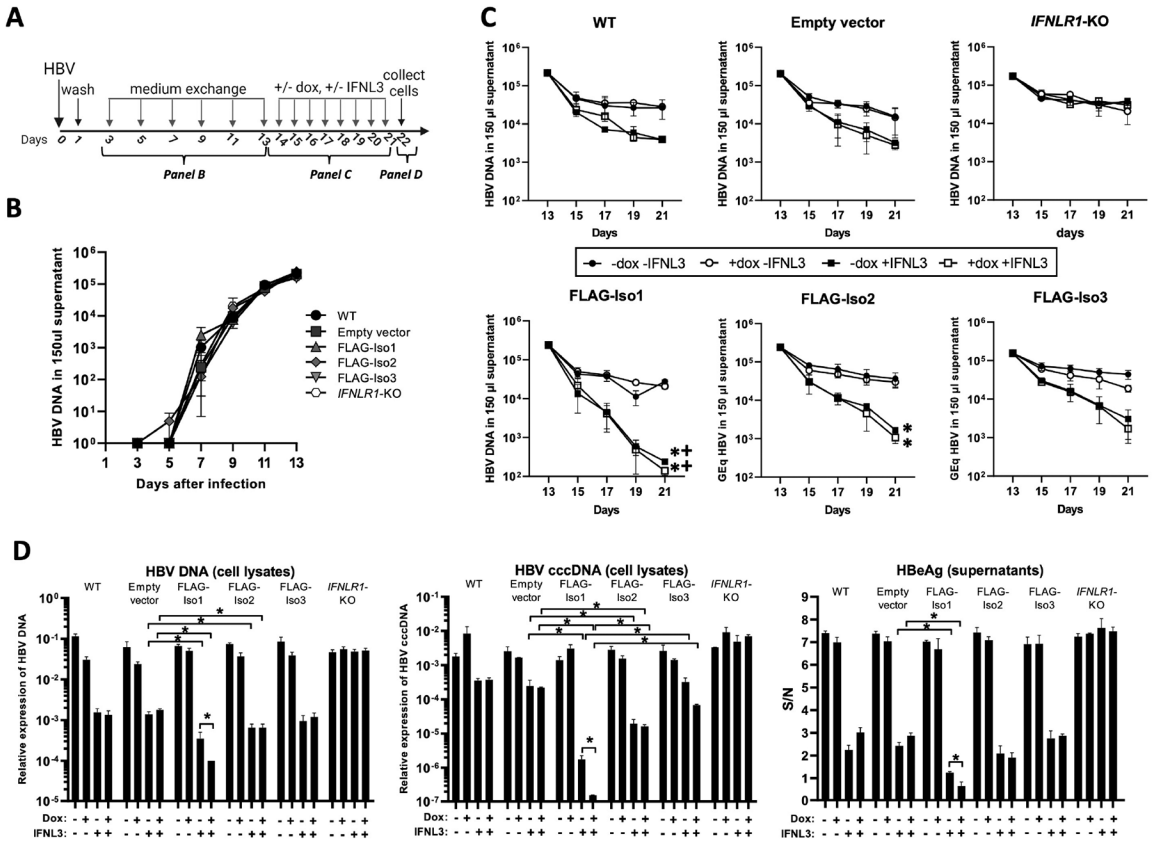

Fig. 4. Differential expression of FLAG-IFNLR1 isoforms influenced IFNL3-mediated inhibition of HBV replication.

Schematic to depict HBV infection of iHep lines and IFNL3 treatment **(A)**. Quantitation of HBV DNA in clarified culture supernatants collected **(B)** during the 13-day progression of infection and **(C)** during 8-days of IFNL3 treatment as determined by qRT-PCR. * = p≤ 0.05 compared to similarly treated EV line and + = p≤ 0.05 relative to mock-treatment within the same iHep line on the indicated day. **(D)** Quantitation of HBV DNA and HBV cccDNA in cell lysates collected on day 22 relative to *GAPDH as* determined by qPCR. HBeAg was quantitated in clarified culture supernatants collected on day 22 by ELISA with values ≥1.0 indicating an HBeAg-positive sample. All lines other than the *IFNLR1*-KO line had a significant drop in total HBV DNA and HBeAg after IFNL3 treatment relative to mock-treatment; for figure clarity, statistics representing these results are omitted from the figure. * =p≤ 0.05 as indicated for significant differences between similarly treated lines. Quantitative data are shown as mean ± standard deviation of biological duplicates assayed as technical replicates. Data are representative of two independent experiments. Student's t-test was used for statistical analysis. Abbreviations: dox, doxycycline; IFNL3, interferon lambda 3; WT, wild type; *IFNLR1*-KO, interferon lambda receptor-1 knock out; cccDNA, covalently closed circular DNA.

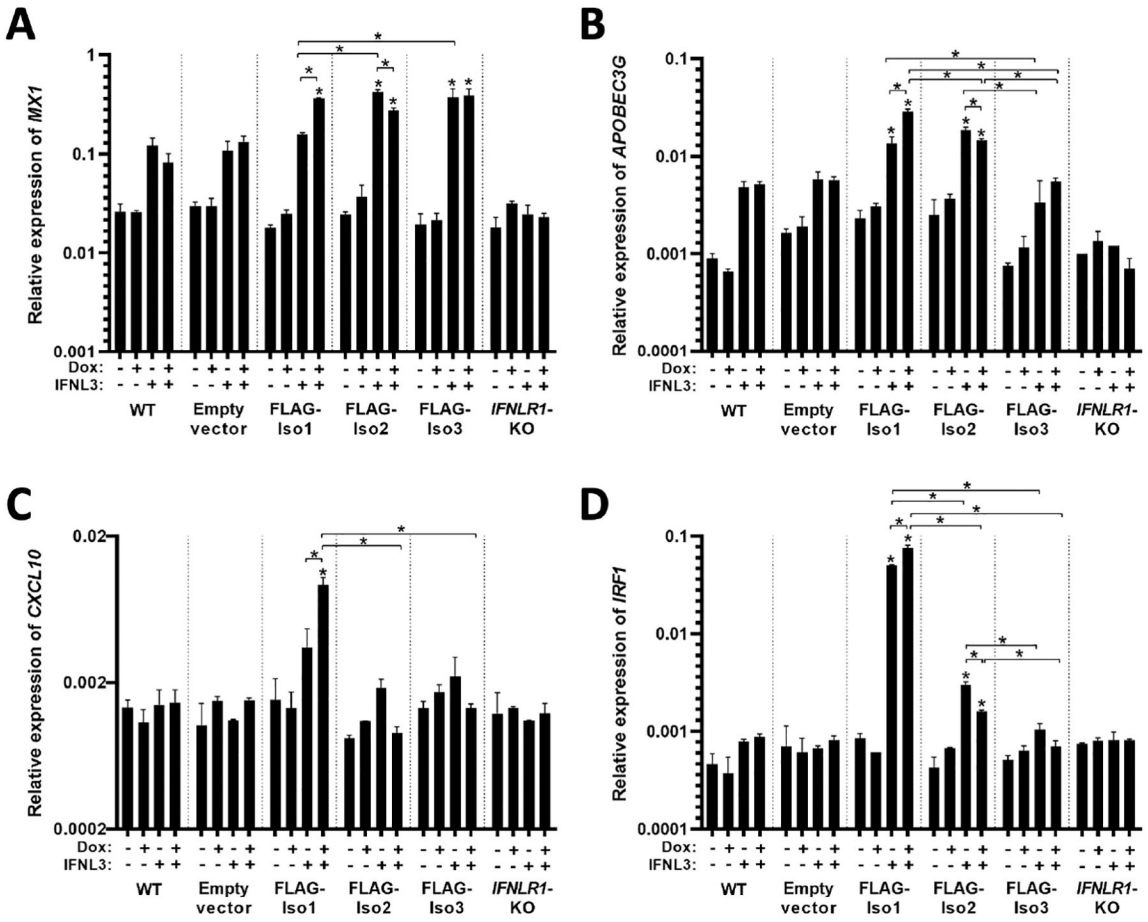

Figure 5: Expression of antiviral and proinflammatory genes by HBV-infected iHeps upon completion of IFNL3 treatment.

Expression of **(A)** *MX1*, **(B)** *APOBEC3G*, **(C)** *CXCL10*, and **(D)** *IRF1* relative to *GAPDH* determined by qRT-PCR at day 22, as shown in Figure 4A. All lines other than the *IFNLR1*-KO line had a significant increase in *MX1* and *APOBEC3G* after IFNL3 treatment relative to mock-treated conditions in the same line; for figure clarity, statistics representing these results are omitted from the figure. * = $p \leq 0.05$ by Student's t-test as indicated. Individual * indicate significance compared to respective EV control. Quantitative data are shown as mean ± standard deviation of biological duplicates. Representative data is shown from two independent experiments. Abbreviations: iHep, induced hepatocyte; dox, doxycycline; IFNL3, interferon lambda, *MX1*, MX dynamin-like GTPase; *CXCL10*, C-X-C motif chemokine ligand 10; *APOBEC3G*, apolipoprotein B mRNA editing enzyme catalytic subunit 3G; *IRF1*, interferon regulatory factor 1.