Different effects of three interferons L on Toll-like receptor-related gene expression in
HepG2 cells

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Running title: Three IFNLs in HepG2 cells

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ABSTRACT

IFNL1 (IL29), IFNL2 (IL28A) and IFNL3 (IL28B) might play important roles in anti-viral defense. IFNL3 genotypes have been shown to be associated with hepatitis C spontaneous and treatment-induced viral clearance.

The effects of IFNL1, IFNL2 and IFNL3 on innate immunity including Toll-like receptor (TLR)-related pathway in human hepatocytes were examined. After G418 screening, we established the human hepatoma stable cell lines HepG2-IL28A, HepG2-IL28B, and HepG2-IL29, expressing IFNL2, IFNL3, and IFNL1 in conditioned medium, respectively, and a control cell line, HepG2-pcDNA3.1. We performed real-time RT-PCR to investigate 84 Toll-like receptor-related gene expressions in triplicate and, using ddCt methods, compared these gene expressions in each cell line.

IFNL2, IFNL3 and IFNL1 were respectively detected by ELISA in HepG2-IL28A, HepG2-IL28B and HepG2-IL29. Compared to HepG2-pcDNA3.1 cells, 17 (20.2%), 11 (13.0%) and 16 genes (19.0%) were up-regulated 1.5-fold or more ($p < 0.05$); 10 (11.9%), 2 (2.3%) and 10 genes (11.9%) were 1.5-fold or more down-regulated ($p < 0.05$) in HepG2-IL28A, HepG2-IL28B and HepG2-IL29, respectively. EIF2AK2 and SARM1 were up-regulated among all cells. Of interest, TLR3, TLR4 and related molecules CXCL10 (IP10), IL6, EIF2K2, IFNB1, and IRF1,

important genes in the progression of HCV-related pathogenesis and antiviral activities against HCV, in HepG2-IL28B, **presented** different profiles from those of HepG2-IL28A **and** HepG2-IL29.

IFNL3 induces interferon-stimulated genes (ISGs) that are reportedly associated with the progression of HCV-related pathogenesis and antiviral activities against HCV. **IFNL is a powerful modulator of innate immune response and it is supposed that the 3 IFNLs may play different roles in the antiviral activity against HBV and HCV.**

Highlights

- IFNL3 (IL28B) genotypes are associated with HCV clearance.
- Effects of **3 IFNLs on** gene expressions in hepatocytes were examined.
- Three IFNLs induced different actions on TLR-related genes.
- IFNL3 induces ISGs that are associated with HCV clearance.
- Three IFNLs **may** play different roles in liver diseases.

Keywords: Hepatitis C virus; IL28B; innate immune response; ISGs; Toll-like receptor

Abbreviations: See Table S1

1. Introduction

Interferons (IFNs) α/β and related molecules are classified as type I IFNs, and two other types have since been identified: type II (IFN γ) and type III (IFNL) [1]. Interleukin-28A (IL28A), IL28B and IL29, also known as IFNL2, IFNL3 and IFNL1, are recognized as type III IFNs [2]. Type I and II IFN receptors are present on most human cell types while type III IFNL receptors are highly expressed on hepatocytes but not on microvascular endothelium, adipocytes, fibroblasts or central nervous system cells [3]. Binding of IFNL1, IFNL2 or IFNL3 to the membrane-associated IFNL receptor (R) complex leads to the activation of the Janus kinases, Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2), subsequent tyrosine phosphorylation of the IFNLR1 intracellular domain (ICD), and activation of the latent transcription factors, signal transducers and activators of transcription 1 (STAT1) and STAT2 [4], recruiting IFN regulatory factor 9 (IRF9) to form the **trimeric transcription** factor complex IFN-stimulated gene factor 3 (ISGF3), which regulates gene transcription by binding to IFN-stimulated response elements (ISRE) in the promoters of ISGs [5].

Previous comparative cDNA microarray analyses by several groups have shown that the repertoire of genes induced by IFNL is essentially the same as that induced by IFN α/β [5-7]. Exogenous IFNs were used in those studies [6,7]. Disorders of IFN α/β and IFNL production, caused by unc-93 homolog B1 (UNC93B1) and Toll-like receptor

3 (TLR3) mutations, confer a predisposition to herpes simplex encephalitis in otherwise healthy subjects [1,8,9]. IFNL plays a potent role in not only antiviral [10] but also antitumor activities [11]. In host immunity, 5 of the 10 human TLRs (TLR3, TLR4, TLR7, TLR8 and TLR9) can trigger IFN α/β and IFNL, which are critical for antiviral immunity [12]. As the immune system provides the first line of host defenses against microbial pathogens, we chose to study TLR-related genes in the present study.

IFNL3 genotypes have been shown to be associated with hepatitis C spontaneous and treatment-induced viral clearance [13-18]. IFNL1 is capable of antiviral activity against HCV in vivo [19]. There still exist questions as to whether there might be some differences in the actions among the three IFNLs. We also wondered whether TLR signaling is important for viral eradication through induction of IFNLs. We assumed that IFNLs induced by TLR signaling might further strengthen the antiviral response by inducing TLR-related gene expression, and that this might be important for the positive circuit between the TLR-mediated pathways and IFNLs for the control of viral infection.

In the present study, we made HepG2 cells expressing each of the IFNLs, and the effects of IFNL1, IFNL2 and IFNL3 on innate immunity signaling including the TLR-related pathway in human hepatocytes were examined. Altogether, our data

indicate that IFNL is a powerful modulator of innate immune response and suggest that the three IFNLs may play different roles in the antiviral activity against HCV.

2. Materials and methods

2.1. Cells and plasmids

HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) at 37°C, 5% CO₂. Plasmids pcDNA3.1-IL28A, pcDNA3.1-IL28B and pcDNA3.1-IL29 (kindly provided by Prof. T. Betakova, Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic [20]) expressed IFNL2, IFNL3 and IFNL1, respectively. Stable HepG2 cell lines expressing IFNL2, IFNL3 or IFNL1 were made as previously described [21]. Briefly, HepG2 cells were transfected with the expression plasmid pcDNA3.1-IL28A, pcDNA3.1-IL28B, pcDNA3.1-IL29 or pcDNA3.1 in Effectene transfection reagent (Qiagen, Hilden, Germany). After 48 h, G418 (Promega, Madison, WI) was added at 1,000 µg/mL for selection of HepG2-IL28A, HepG2-IL28B, HepG2-IL29, or HepG2-pcDNA3.1. After 3 weeks, to avoid monoclonal selection, all cells were collected for further analysis.

The HBV DNA (4.1kbp) construct containing 1.3 copies of HBV genome (subtype *ayw*), kindly provided by Prof. A. McLachlan, University of Illinois at Chicago, IL, USA [22], was used for transient transfection analysis. Plasmid pHCVrep1bBB7 was kindly provided by Prof. C.M. Rice, Washington University School of Medicine, St.

Louis, MO, USA [23]. The plasmid contained cDNA coding for an HCV subgenomic replicon.

2.2. RNA extraction

Cells were seeded into 35-mm plates, and total cellular RNA was isolated 48 h later by RNeasy Mini kit (Qiagen) according to the manufacturer's instructions [21]. RNA samples were stored at -80°C until use. RNA quality was examined using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. cDNA synthesis, real-time PCR, and PCR array

cDNA synthesis was performed using RT² First Strand Kit (Qiagen). Each 1 µg of RNA was subjected to one reaction. cDNA synthesis reaction was as follows: incubation at 42°C for 15 min and then reaction stoppage by heating at 95°C for 15 min. Quantitative amplification of cDNA was monitored with SYBR Green by real-time PCR in a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions were 95°C for 10 min followed by 40 cycles at 95°C, 15 s for denaturation, and 1 min at 60 °C for annealing and extension. Gene expression profiling of 84 TLR-related genes was performed using RT² profiler PCR arrays

(Qiagen) following the manufacturer's instructions [24]. Genes were annotated by Entrez Gene (NCBI, Bethesda, MD, USA). 84 genes and functional gene grouping are shown in Table S1.

Gene expression was normalized to two internal controls (GAPDH and β -actin) to determine the fold-change in gene expression between the test sample (HepG2-IL28A/28B/29) and the control sample (HepG2-pcDNA3.1) by 2^{-ddCT} (comparative cycle threshold) method, respectively [24]. Three sets of real-time PCR arrays were performed. Data were analyzed with RT2 Profiler PCR Array Data Analysis software (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>).

2.4. HCV subgenomic RNA electroporated into Huh7 cells and inhibition assay with IFNLs from HepG2-derived cell lines

HCV subgenomic RNA (1 μ g) was electroporated into 5×10^6 Huh7 cells using the Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, CA, USA) as described previously [25]. The cells were plated onto 6-well plates. After 48 h, the medium was replaced with conditioned medium from HepG2-pcDNA3.1, HepG2-IL28A, HepG2-IL28B or HepG2-IL29 cells, accompanied by 500 μ g/mL G418. Medium was exchanged every 3 days. Two weeks later, the resultant colonies in each well were

counted.

2.5. *Transfection of HBV DNA construct, HBV DNA extraction and analysis*

Cells were seeded onto 35-mm plates. Transfections of HBV DNA construct (0.4 µg/plate) were performed as previously mentioned [21]. Isolation of DNA was performed by SepaGene (Eidia, Tokyo, Japan). DNA samples were stored at -80°C until use. DNA quality was examined using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific). Quantitative PCR assay for HBV DNA was performed as previously described [26].

2.6. *Enzyme-linked immunosorbent assay (ELISA)*

Cell culture fluids were analyzed for human IFNL2, IFNL3 and IFNL1, respectively, using IFNL2 (Abcam Inc., Cambridge, MA, USA), IFNL3 (USCN Life Science Inc., Wuhan, China) and IFNL1 ELISAs (Abnova, Taipei, Taiwan), according to the manufacturers' protocols. The sensitivities of human IFNL2, IFNL3 and IFNL1 by these ELISA kits were 15 pg/mL, 700 pg/mL and 2 pg/mL, respectively.

2.7. *Statistical analysis*

Student's t-test was used to assess statistical significance. Differences with $p < 0.05$ were considered to be statistically significant. Data are expressed as mean $\pm$ standard deviations of triplicate determination from 3 independent experiments.

3. Results

3.1. *Overexpression of IFNLs in HepG2 cells*

It was well known that hepatocytes and the hepatoma-derived cell line HepG2 expressed similar levels of IL28R α mRNA to the lung epithelial cell line A549, which responds well to IFNLs [4]. We used three protein plasmid vectors under control of the CMV promoter: pcDNA3.1-IL28A, pcDNA3.1-IL28B and pcDNA3.1-IL29 [20,21]. We established three IFN- λ s-overexpressing HepG2 cells, designated as HepG2-IL28A, HepG2-IL28B and HepG2-IL29. We also used pcDNA3.1 for the establishment of a control cell, HepG2-pcDNA3.1. To test the ability of these cells to express IFNL2, IFNL3 and IFNL1, we measured these cytokines by ELISA (Table 1). IFNL2, IFNL3 and IFNL1 could be measured in each cell culture fluid of HepG2-IL28A, HepG2-IL28B and HepG2-IL29, respectively.

3.2. *Different IFN-stimulated gene expressions in HepG2 expressing IFNL2, IFNL3 and IFNL1*

To gain mechanistic insights into IFNL2, IFNL3 and IFNL1 for ISGs, we used a pathway-specific gene array to identify these IFNLs target genes in HepG2-IL28A, HepG2-IL28B and HepG2-IL29 by comparison with control HepG2-pcDNA3.1. We

extracted total RNAs from each of the cells for studying the influence of IFNL2, IFNL3, or IFNL1 overexpression on IFN and cytokine-related genes including TLR signaling pathways using real-time PCR-based array [24]. Compared to HepG2-pcDNA3.1 cells, 17 (20.2%), 11 (13.0%) and 16 genes (19.0%) were up-regulated 1.5-fold or more ($p < 0.05$) (Figure 1A); 10 (11.9%), 2 (2.3%) and 10 genes (11.9%) were down-regulated 1.5-fold or ($p < 0.05$) in HepG2-IL28A, HepG2-IL28B and HepG2-IL29, respectively (Figure 1B). EIF2AK2 and SARM1 were up-regulated in all cells, and PTGS2 and IRAK2 in both HepG2-IL28B and HepG2-IL29, and FOS, IKBKB, IL1A, IRAK1, TLR1, TOLLIP in both IL28A and IL29 ($p < 0.05$) (Figure 1A). EIF2KA2 is also known as PKR, a serine/threonine protein kinase that is activated by autophosphorylation after binding to dsRNA. EIF2KA2 can mediate the antiviral activity of IFNs, and other IFN-inducible genes can mediate the antiviral effects against HBV and HCV [27, 28]. BTK was down-regulated in all cells, CD14 was in both HepG2-IL28B and HepG2-IL29, and CCL2, CD180, CSF2, TLR5 and UBE2N in both HepG2-IL28A and HepG2-IL29 ($p < 0.05$) (Figure 1B).

3.3. *Effects of IFNL2 on IFN and cytokine-related gene expression*

Among TLRs and TLR signaling genes, TLR1, TLR8, SARM1, IRAK1, TOLLIP,

TIRAP, MAP3K7 and NR2C2 were up-regulated 1.5-fold or more in HepG2-IL28A in comparison to HepG2-pcDNA3.1 ($p < 0.05$, Table 2A) but TICAM2, TLR4, TLR5 and CD180 were down-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 3A). TLR8, which senses single-stranded RNA and plays an important role in the immune response to viral infection, can trigger IFN- α/β and IFNL, which are critical for antiviral immunity [12]. Among pathogen-specific response genes, TLR8, IFNG, EIF2AK, IRAK1, IL10 and RIPK2 were up-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 2A) but CCL2, IFNB1 and CD180 were down-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 3A). Recently, we reported that HBV e antigen physically associates with RIPK2 and regulates IL6 gene expression [29]. Among downstream pathways and target genes, IFNG, IKBKB, IRAK1, IL10, IL1A, MAPK8IP3, FOS and MAP3K7 were up-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 2A) but UBE2N, CSF3, CCL2, IFNB1, CSF2 and BTK were down-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 3A). Among regulation genes with adaptive immunity, IFNG, IL10 and MAP3K7 were up-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 2A). Among adapters and TLR interacting protein genes, SARM1, MAPK8IP3, TOLLIP, TIRAP and RIPK2 were up-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 2A) but TICAM2 and

BTK were down-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 3A).

Among effector genes, EIF2AK2, IRAK1, MAP3K7 and RIPK2 were up-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 2A) but UBE2N was down-regulated 1.5-fold or more in HepG2-IL28A ($p < 0.05$, Table 3A).

3.4. *Effects of IFNL3 on IFN and cytokine-related gene expression*

Among TLRs and TLR signaling genes, TLR3, SARM1, IRAK2, MYD88 were up-regulated 1.5-fold or more in HepG2-IL28B, in comparison to HepG2-pcDNA3.1 ($p < 0.05$, Table 2B). Among pathogen-specific response genes, IL6, EIF2AK2, IFNB1, LY96 and PTGS2 were up-regulated 1.5-fold or **more in** HepG2-IL28B ($p < 0.05$, Table 2B) but CD14 was down-regulated 1.5-fold or more in HepG2-IL28B ($p < 0.05$, Table 3B). Among downstream pathways and target genes, IL6, IFNB1, LY96, IRAK2, IRF1, and NFKBIL1 were up-regulated 1.5-fold or more in HepG2-IL28B ($p < 0.05$, Table 2B) but BTK was down-regulated 1.5-fold or more in HepG2-IL28B ($p < 0.05$, Table 3B). Among adapters and TLR interacting protein genes, LY96, SARM1 and MYD88 were up-regulated 1.5-fold or more in HepG2-IL28B ($p < 0.05$, Table 2B) but CD14 and BTK were down-regulated 1.5-fold or more in HepG2-IL28B ($p < 0.05$, Table 3B). Among effector genes, EIF2AK2 and IRAK2 were up-regulated 1.5-fold or more in

HepG2-IL28B ($p < 0.05$, Table 2B). Interestingly, among up-regulated genes (Table 2B), TLR3, TLR4 and related molecules [24, 30], CXCL10 (IP10) [31], IL6 [32], EIF2K2 [28], IFNB1 [30], and IRF1 [33], which play important roles in the progression of HCV-related pathogenesis as well as in antiviral activities against HCV, were observed.

3.5. *Effects of IFNL1 on IFN and cytokine-related gene expression*

Among TLR and TLR signaling genes, TLR1, SARM1, IRAK2, IRAK1, TBK1 and TOLLIP were up-regulated 1.5-fold or more in HepG2-IL29, in comparison to HepG2-pcDNA3.1 ($p < 0.05$, Table 2C), but TLR5 and CD180 were down-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 3C). Among pathogen-specific response genes, PTGS2, FOS, IRAK1, EIF2AK2 and TNFRSF1A were up-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 2C) but CD14, CCL2, HSPA1A and CD180 were down-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 3C). Among downstream pathways and target genes, IL1A, IKBKB, FOS, IRAK2, IRAK1, CASP8, ELK1 and TNFRSF1A were up-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 2C) but MAP3K1, UBE2N, CCL2, CSF2 and BTK were down-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 3C). Among adapters and TLR interacting protein genes, SARM1 and TOLLIP were up-regulated 1.5-fold or more in

HepG2-IL29 ($p < 0.05$, Table 2C) but CD14, HSPA1A and BTK were down-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 3C). Among effector genes, IRAK2, IRAK1, CASP8 and EIF2AK2 were up-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 2C) but UBE2N was down-regulated 1.5-fold or more in HepG2-IL29 ($p < 0.05$, Table 3C).

3.6. Antiviral effects of IFNLs on hepatitis viruses

We previously reported that IFNL1 inhibits HCV JFH1 replication in human hepatoma cell Huh7-derived cell lines [21]. Because it seemed that HCV could not replicate in HepG2 cells, we examined whether IFNLs produced from HepG2-derived cell lines could inhibit HCV subgenomic RNA replication in Huh7 cells using colony formation assay (Figure 2A). Although 8.3 ± 2.0 colonies/field (magnification 40x) were identified in Huh7 cells treated with conditioned medium from HepG2-pcDNA3.1, we did not observe any G418-resistant colonies in Huh7 cells treated with that from HepG2-IL28A, HepG2-IL28B or HepG2-IL29 for 2 weeks, suggesting that the 3 kinds of IFNLs could inhibit HCV subgenomic RNA replication.

Next, we determined whether IFNLs could inhibit HBV replication after HBV transfection. For this, we transfected pCMV-HBV DNA into HepG2-pcDNA3.1, HepG2-IL28A, HepG2-IL28B and HepG2-IL29 cells. Cells were incubated for 10 h

after transfection, washed three times, and then incubated with fresh medium. Medium was exchanged at 24, 48, 72 and 96 h after transfection. Cellular DNA was collected at 96 h after transfection and HBV DNA was tested by real-time PCR. Conditioned medium was also collected for HBV DNA quantitation at 24, 48, 72 and 96 h after transfection. We obtained 14.3% (n=3, $p < 0.05$), 56.2% (n=3, $p < 0.05$) and 80.2% (n=3, $p < 0.05$) of intracellular HBV DNA levels in HepG2-IL28A, HepG2-IL28B and HepG2-IL29, respectively, compared to 100% (n=3) in HepG2-pcDNA3.1 (Figure 2B). In conditioned medium at 96 h after transfection, HBV DNA levels were 8.6, 7.8, 7.9 and 7.8 log copies/mL in HepG2-pcDNA3.1, HepG2-IL28A, HepG2-IL28B and HepG2-IL29, respectively, although we did not observe any differences in HBV DNA levels at 24, 48 and 72 h after transfection.

Discussion

In the present study, we showed that 3 different IFNLs induced expressions of TLR-related genes in human hepatocytes. Our results also showed that IFNL3 induced TLR signaling-related genes that play key roles in the elimination of HCV (Table 2B), supporting the results from recent genome-wide association studies identifying the association between IFNL3 genotypes and natural or treatment-induced HCV elimination [13,14,34,35]. We demonstrated that IFNLs produced by HepG2-derived cell lines could inhibit HBV and HCV replication, supporting the previous study [21].

It has been reported that TLR agonists induce a different expression of type I IFNs and IFNLs [36]. TLR3-, TLR7-, TLR8-, and TLR9-mediated inductions of IFN α/β and IFNL were reported to be important for controlling viral infection [37-41]. Wang et al. [41] reported that TLR3 agonists induce IFNLs and that IFNLs play an important role in anti-HCV activity in hepatic stellate cells (HSCs).

In the present study, we focused on hepatocytes, where HBV and HCV can replicate and which express IFNL receptors. Human HSCs [41] and plasmacytoid dendritic cells [42] can produce IFNLs. Recently, Yoshio et al. [43] reported that human blood dendritic cell antigen 3 (BDCA3)⁺ dendritic cells prodigiously produce IFNL in response to HCV. Are TLR signalings important for viral clearance through induction of

IFNLs? We assumed that IFNLs induced by TLR signaling might further strengthen the antiviral response by inducing the expressions of TLR-related genes. That is, we hypothesized the importance of the positive circuit between the TRL-mediated pathways. To examine whether HepG2 cells over-expressing IFNLs respond more strongly to the corresponding TLR agonists than do control HepG2-pcDNA3.1 cells, we examined IFN- β promoter activities after poly(I-C) transfection into HepG2-IL28B cells [30], which enhanced TLR3 expression (Table 2B). Unexpectedly, however, we did not observe any enhancement of IFN- β activities in HepG2-IL28B, in comparison with HepG2-pcDNA3.1 (data not shown). Further studies will be needed to clarify the roles of IFNLs for viral eradication.

There exist two type I IFNs, IFN- α and IFN- β , which use the same type I IFN receptor. But the clinical effects are a little different between these two type I IFNs. For example, IFN- α and IFN- β are now the first lines of treatment for chronic hepatitis C infection and for relapsing remitting multiple sclerosis, respectively [3]. There are three type III IFNs, IFNL1, IFNL2 and IFNL3, which might use the same IFNL receptor. The present study also indicated that the actions are different among these three IFNLs on ISG expression, although a limitation of our system concerning the dose of each IFN might exist (Table 1). Future studies will be needed to clarify this point and the

underlying mechanisms. Kohli et al. [44] reported that the majority of genes induced by IFN α and IFNL were similar, IFNL exhibited profound, but delayed kinetics of ISGs induction while IFN α induced ISGs more rapidly, and that there were different activities of IFN- α and IFNL between HCV-infected and non-infected cells [44]. Although all three IFNLs induced EIF2K2 and SARM1 (Figure 1A), genes induced by IFNL3 seemed to be different from those by IFNL2 or IFNL1. It was reported that SARM1 was a negative regulator of innate immunity in neuronal morphogenesis [45].

Retinoic acid inducible-gene I (RIG-I)-like receptors (RLRs) are other important innate immune gatekeepers involved in viral clearance. RIG-I is one of the sensors of HCV RNA and plays an important role in anti-HCV defenses [46]. In HCV infection, HCV pathogen-associated molecular pattern (PAMP) triggers production of IFNLs through RIG-I [42]. RLRs, as well as TLRs, might be important in the production of IFNLs [47,48]. Further studies will be needed to determine whether IFNLs are involved in the RLR-mediated signal pathways in antiviral responses against HBV, although we have reported that HBeAg could interact with receptor-interacting serine/threonine protein kinase-2 (RIP2/RIPK2) [29].

Our results supported the previous studies [6,7,19,21,49] showing that IFNL could suppress HCV and HBV replication. These findings and our observation suggest that

the combination of these IFNLs might be useful for the control of chronic hepatitis C and B, as well as other viral infections. Recent studies suggested that a novel IFNL4 also plays an important role in the host response to HCV [50,51]. In conclusion, 3 types of IFNLs induced different expressions of TLR-related genes, suggesting that they play different roles in liver diseases.

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References

- [1] Zhang SY, Boisson-Dupuis S, Chapgier A, Yang K, Bustamante J, Puel A, Picard C, Abel L, Jouanguy E, Casanova JL. Inborn errors of interferon (IFN)-mediated immunity in humans: insights into the respective roles of IFN- α/β , IFN- γ , and IFN- λ in host defense. *Immunol Rev* 2008;226:29-40.
- [2] Zdanov A. Structural analysis of cytokines comprising the IL-10 family. *Cytokine Growth Factor Rev* 2010;21:325-330.
- [3] George PM, Badiger R, Alazawi W, Foster GR, Mitchell JA. Pharmacology and therapeutic potential of interferons. *Pharmacol Ther* 2012;135:44-53.
- [4] Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, Donnelly RP. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 2003;4:69-77.
- [5] Donnelly RP, Kotenko SV. Interferon-lambda: a new addition to an old family. *J Interferon Cytokine Res* 2010;30:555-564.
- [6] Doyle SE, Schreckhise H, Khuu-Duong K, Henderson K, Rosler R, Storey H, Yao L, Liu H, Barahmand-pour F, Sivakumar P, Chan C, Birks C, Foster D, Clegg CH, Wietzke-Braun P, Mihm S, Klucher KM. Interleukin-29 uses a type

- 1 interferon-like program to promote antiviral responses in human hepatocytes. *Hepatology* 2006;44:896-906.
- [7] Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR, Rice CM. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct transduction and gene regulation kinetics. *Gastroenterology* 2006;131:1887-1898.
- [8] Casrouge A, Zhang SY, Eidenschenk C, Jouanguy E, Puel A, Yang K, Alcais A, Picard C, Mahfoufi N, Nicolas N, Lorenzo L, Plancoulaine S, Sénéchal B, Geissmann F, Tabeta K, Hoebe K, Du X, Miller RL, Héron B, Mignot C, de Villemeur TB, Lebon P, Dulac O, Rozenberg F, Beutler B, Tardieu M, Abel L, Casanova JL. Herpes simplex virus encephalitis in human UNC-93B deficiency. *Science* 2006;314:308-312.
- [9] Zhang SY, Jouanguy E, Ugolini S, Smahi A, Elain G, Romero P, Segal D, Sancho-Shimizu V, Lorenzo L, Puel A, Picard C, Chapgier A, Plancoulaine S, Titeux M, Cognet C, von Bernuth H, Ku CL, Casrouge A, Zhang XX, Barreiro L, Leonard J, Hamilton C, Lebon P, Héron B, Vallée L, Quintana-Murci L, Hovnanian A, Rozenberg F, Vivier E, Geissmann F, Tardieu M, Abel L,

- Casanova JL. TLR3 deficiency in patients with herpes simplex encephalitis. *Science* 2007;317:1522-1527.
- [10] Pagliaccetti NE, Robek MD. Interferon-1 in the immune response to hepatitis B virus and hepatitis C virus. *J Interferon Cytokine Res* 2010;30:585-590.
- [11] Tagawa M, Kawamura K, Li Q, Tada Y, Hiroshima K, Shimada H. A possible anticancer agent, type III interferon, activates cell death pathways and produces antitumor effects. *Clin Dev Immunol* 2011;2011:479013.
- [12] Zhang SY, Jouanguy E, Sancho-Shimizu V, von Bernuth H, Yang K, Abel L, Picard C, Puel A, Casanova JL. Human Toll-like receptor-dependent induction of interferons in protective immunity to viruses. *Immunol Rev* 2007;220:225-236.
- [13] Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, Goedert JJ, Kirk GD, Donfield SM, Rosen HR, Tobler LH, Busch MP, McHutchison JG, Goldstein DB, Carrington M. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009;461:798-801.
- [14] Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB:

- Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009, 461:399-401.
- [15] Kanda T, Imazeki F, Yokosuka O. New antiviral therapies for chronic hepatitis C. *Hepatol Int* 2010;4:548-561.
- [16] Nakamoto S, Kanda T, Imazeki F, Wu S, Arai M, Fujiwara K, Yokosuka O. Simple assay based on restriction fragment length polymorphism associated with IL28B in chronic hepatitis C patients. *Scand J Gastroenterol* 2011;46:955-961.
- [17] Miyamura T, Kanda T, Nakamoto S, Wu S, Fujiwara K, Imazeki F, Yokosuka O. Hepatic STAT1-nuclear translocation and interleukin 28B polymorphisms predict treatment outcomes in hepatitis C virus genotype 1-infected patients. *PLoS One* 2011;6:e28617.
- [18] Miyamura T, Kanda T, Nakamoto S, Wu S, Jiang X, Arai M, Fujiwara K, Imazeki F, Yokosuka O. Roles of ITPA and IL28B genotypes in chronic hepatitis C patients treated with peginterferon plus ribavirin. *Viruses* 2012;4:1264-1278.
- [19] Kelly C, Klenerman P, Barnes E. Interferon lambdas: the next cytokine storm. *Gut* 2011;60:1284-1293.

- [20] Svetlikova D, Kabat P, Ohradanova A, Pastorek J, Betakova T. Influenza A virus replication is inhibited in IFN-lambda2 and IFN-lambda3 transfected or stimulated cells. *Antiviral Res* 2010;88:329-333.
- [21] Kanda T, Wu S, Kiyohara T, Nakamoto S, Jiang X, Miyamura T, Imazeki F, Ishii K, Wakita T, Yokosuka O. Interleukin 29 suppresses hepatitis A and C viral internal ribosomal entry site-mediated translation. *Viral Immunol* 2012;25:379-386.
- [22] Oropeza CE, Li L, McLachlan A. Differential inhibition of nuclear hormone receptor-dependent hepatitis B virus replication by the small heterodimer partner. *J Virol* 2008;82:3814-3812.
- [23] Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. *Science* 2000;290:1972-1974.
- [24] Tamura R, Kanda T, Imazeki F, Wu S, Nakamoto S, Tanaka T, Arai M, Fujiwara K, Saito K, Roger T, Wakita T, Shirasawa H, Yokosuka O. Hepatitis C nonstructural 5A protein inhibits lipopolysaccharide-mediated apoptosis of hepatocytes by decreasing expression of toll-like receptor 4. *J Infect Dis* 2011;204:793-801.

- [25] Kanda T, Yokosuka O, Imazeki F, Tanaka M, Shino Y, Shimada H, Tomonaga T, Nomura F, Nagao K, Ochiai T, Saisho H. Inhibition of subgenomic hepatitis C virus (HCV) RNA in Huh-7 cells: ribavirin induces mutagenesis in HCV RNA. *J Viral Hepat* 2004;11:479-487.
- [26] Wu S, Kanda T, Imazeki F, Nakamoto S, Shirasawa H, Yokosuka O. Nuclear receptor mRNA expression by HBV in human hepatoblastoma cell lines. *Cancer Lett* 2011;312:33-42.
- [27] Guidotti LG, Morris A, Mendez H, Koch R, Silverman RH, Williams BR, Chisari FV. Interferon-regulated pathways that control hepatitis B virus replication in transgenic mice. *J Virol* 2002;76:2617-2621.
- [28] Gale M Jr, Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, Korth MJ, Polyak SJ, Gretch DR, Katze MG. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Mol Cell Biol* 1998;18:5208-5218.
- [29] Wu S, Kanda T, Imazeki F, Nakamoto S, Tanaka T, Arai M, Roger T, Shirasawa H, Nomura F, Yokosuka O. Hepatitis B virus e antigen physically associates with receptor-interacting serine/threonine protein kinase 2 and regulates IL-6 gene expression. *J Infect Dis* 2012;206:415-420.

- [30] Kanda T, Steele R, Ray R, Ray RB. Hepatitis C virus infection induces the beta interferon signaling pathway in immortalized human hepatocytes. *J Virol* 2007;81: 12375-12381.
- [31] Romero AI, Lagging M, Westin J, Dhillon AP, Dustin LB, Pawlotsky JM, Neumann AU, Ferrari C, Missale G, Haagmans BL, Schalm SW, Zeuzem S, Negro F, Verheij-Hart E, Hellstrand K; DITTO-HCV Study Group. Interferon (IFN)-gamma-inducible protein-10: association with histological results, viral kinetics, and outcome during treatment with pegylated IFN-alpha 2a and ribavirin for chronic hepatitis C virus infection. *J Infect Dis* 2006;194:895-903.
- [32] Falleti E, Fabris C, Vandelli C, Colletta C, Cussigh A, Smirne C, Fontanini E, Cmet S, Minisini R, Bitetto D, Toniutto P, Pirisi M. Genetic polymorphisms of interleukin-6 modulate fibrosis progression in mild chronic hepatitis C. *Hum Immunol* 2010;71:999-1004.
- [33] Kanazawa N, Kurosaki M, Sakamoto N, Enomoto N, Itsui Y, Yamashiro T, Tanabe Y, Maekawa S, Nakagawa M, Chen CH, Kakinuma S, Oshima S, Nakamura T, Kato T, Wakita T, Watanabe M. Regulation of hepatitis C virus replication by interferon regulatory factor 1. *J Virol* 2004;78:9713-9720.

- [34] Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; 41:1105-1109.
- [35] Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Müller T, Bahlo M, Stewart GJ, Booth DR, George J. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009;41:1100-1104.
- [36] Coccia EM, Severa M, Giacomini E, Monneron D, Remoli ME, Julkunen I, Cella M, Lande R, Uze G. Viral infection and Toll-like receptor agonists induce a differential expression of type I and λ interferons in human plasmacytoid and monocyte-derived dendritic cells. *Eur J Immunol* 2004;34:796-805.

- [37] Ioannidis I, Ye F, McNally B, Willette M, Flano E. Toll-like receptor expression and induction of type I and type III interferons in primary airway epithelial cells. *J Virol* 2013;87:3261-3270.
- [38] Yang K, Puel A, Zhang S, Eidenschenk C, Ku CL, Casrouge A, Picard C, von Bernuth H, Senechal B, Plancoulaine S, Al-Hajjar S, Al-Ghonaïum A, Marodi L, Davidson D, Speert D, Roifman C, Garty BZ, Ozinsky A, Barrat FJ, Coffman RL, Miller RL, Li X, Lebon P, Rodriguez-Gallego C, Chapel H, Geissmann F, Jouanguy E, Casanova JL. Human TLR-7-, -8-, and -9-mediated induction of IFN- α/β and $-\lambda$ is IRAK-4 dependent and redundant for protective immunity to viruses. *Immunity* 2005;23:465-478.
- [39] Stewart CR, Bagnaud-Baule A, Karpala AJ, Lowther S, Mohr PG, Wise TG, Lowenthal JW, Bean AG. Toll-like receptor 7 ligands inhibit influenza A infection in chickens *J Interferon Cytokine Res* 2012;32:46-51.
- [40] Murata K, Sugiyama M, Kimura T, Yoshio S, Kanto T, Kirikae I, Saito H, Aoki Y, Hiramane S, Matsui T, Ito K, Korenaga M, Imamura M, Masaki N, Mizokami M. Ex vivo induction of IFN- λ by a TLR7 agonist determines response to Peg-IFN/Ribavirin therapy in chronic hepatitis C patients. *J Gastroenterol* 2013 Apr 17.

- [41] Wang Y, Li J, Wang X, Ye L, Zhou Y, Ho W. Induction of interferon- λ contributes to Toll-like receptor-3-activated hepatic stellate cell-mediated hepatitis C virus inhibition in hepatocytes. *J Viral Hepat* 2013;20:385-394.
- [42] Stone AE, Giugliano S, Schnell G, Cheng L, Leahy KF, Golden-Mason L, Gale M Jr, Rosen HR. Hepatitis C virus pathogen associated molecular pattern (PAMP) triggers production of lambda-interferons by human plasmacytoid dendritic cells. *PLoS Pathog* 2013;9:e1003316.
- [43] Yoshio S, Kanto T, Kuroda S, Matsubara T, Higashitani K, Kakita N, Ishida H, Hiramatsu N, Nagano H, Sugiyama M, Murata K, Fukuhara T, Matsuura Y, Hayashi N, Mizokami M, Takehara T. Human blood dendritic cell antigen 3 (BDCA3)⁺ dendritic cells are a potent producer of interferon- λ in response to hepatitis C virus. *Hepatology* 2013;57:1705-17015
- [44] Kohli A, Zhang X, Yang J, Russell RS, Donnelly RP, Sheikh F, Sherman A, Young H, Imamichi T, Lempicki RA, Masur H, Kottlilil S. Distinct and overlapping genomic profiles and antiviral effects of interferon- λ and - α on HCV-infected and noninfected hepatoma cells. *J Viral Hepat* 2012;19:843-853.

- [45] Chen CY, Lin CW, Chang CY, Jiang ST, Hsueh YP. Sarm1, a negative regulator of innate immunity, interacts with syndecan-2 and regulates neuronal morphology. *J Cell Biol* 2011;193:769-784.
- [46] Sumpter R Jr, Loo YM, Foy E, Li K, Yoneyama M, Fujita T, Lemon SM, Gale M Jr. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* 2005;79:2689-2699.
- [47] Onoguchi K, Yoneyama M, Takemura A, Akira S, Taniguchi T, Namiki H, Fujita T. Viral infections activate types I and III interferon genes through a common mechanism. *J Biol Chem* 2007;282:7576-7581.
- [48] Ank N, Iversen MB, Bartholdy C, Staeheli P, Hartmann R, Jensen UB, Dagnaes-Hansen F, Thomsen AR, Chen Z, Haugen H, Klucher K, Paludan SR. An important role for type III interferon (IFN- λ /IL-28) in TLR-induced antiviral activity. *J Immunol* 2008;180:2474-2485.
- [49] Robek MD, Boyd BS, Chisari FV. Lambda interferon inhibits hepatitis B and C virus replication. *J Virol* 2005, 79:3851-3854.
- [50] Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H, Hergott D, Porter-Gill P, Mumy A, Kohaar I, Chen S, Brand N,

Tarway M, Liu L, Sheikh F, Astemborski J, Bonkovsky HL, Edlin BR, Howell CD, Morgan TR, Thomas DL, Rehermann B, Donnelly RP, O'Brien TR. A variant upstream of IFN3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat Genet* 2013;45:164-171.

- [51] Bibert S, Roger T, Calandra T, Bochud M, Cerny A, Semmo N, Duong FH, Gerlach T, Malinevemi R, Moradpour D, Negro F, Mullhaupt B, Bochud PY; Swiss Hepatitis C Cohort Study. IL28B expression depends on a novel TT/-G polymorphism which improves HCV clearance prediction. *J Exp Med* 2013;210:1109-1116.

Table 1 Overexpressions of IL28A (IFNL2), IL28B (IFNL3), and IL29 (IFNL1) in human hepatoma cell line HepG2. Enzyme-linked immunosorbent assay (ELISA) was performed for IL28A, IL28B or IL29 at **in** least duplicate.

	HepG2- pcDNA3.1	HepG2-IL28A	HepG2-IL28B	HepG2-IL29
IFNL2 (pg/mL)	< 15	4,100	< 15	< 15
IFNL3 (pg/mL)	< 700	< 700	4,100	< 700
IFNL1 (pg/mL)	< 2	< 2	< 2	1,850

The sensitivities of human IL28A, IL28B and IL29 by these ELISA kits were 15 pg/mL, 700 pg/mL and 2 pg/mL, respectively. 1×10^5 cells were split onto 35-mm plates 48 h before collection of conditioned medium.

Table 2 Up-regulation of Toll-like receptor signaling pathway-related genes in HepG2-IL28A, HepG2-IL28B and HepG2-IL29, compared to HepG2-pcDNA3.1 cells.

(A) Up-regulated genes (1.5-fold or more) in HepG2-IL28A, compared to HepG2-pcDNA3.1 cells.

<i>Genes</i>	Fold Differences	<i>P-values</i>
<i>TLR10</i>	6.35	0.29
<i>CLEC4E</i>	4.78	0.21
<i>CD80</i>	4.69	0.062
<i>TLR1</i>	3.15	0.00016
<i>TLR8</i>	3.03	0.0086
<i>IFNG</i>	2.87	0.011
<i>CD86</i>	2.22	0.38
<i>EIF2AK2</i>	2.20	0.0016
<i>IKBKB</i>	2.11	0.00093
<i>SARM1</i>	2.02	0.0028
<i>TLR2</i>	2.01	0.12
<i>IRAK1</i>	1.92	0.0011
<i>PTGS2</i>	1.87	0.0043
<i>IL10</i>	1.84	0.038
<i>IL1A</i>	1.76	0.0047
<i>MAPK8IP3</i>	1.75	0.00078
<i>TOLLIP</i>	1.70	0.0091
<i>TIRAP</i>	1.62	0.0099
<i>IL12A</i>	1.58	0.066
<i>FOS</i>	1.57	0.0014
<i>MAP3K7</i>	1.56	0.00091
<i>NR2C2</i>	1.56	0.0013
<i>IFNA1</i>	1.54	0.080
<i>RIPK2</i>	1.52	0.0039

(B) Up-regulated genes (1.5-fold or more) in HepG2-IL28B, compared to HepG2-pcDNA3.1 cells.

<i>Genes</i>	Fold Differences	<i>P-values</i>
<i>TLR3</i>	12.10	0.00031
<i>CXCL10 (IP10)</i>	7.44	0.054
<i>IL6</i>	4.11	0.0012
<i>EIF2AK2</i>	4.06	0.021
<i>IFNB1</i>	3.22	0.00069
<i>LY96</i>	2.96	0.027
<i>B2M</i>	2.38	0.00034
<i>CCL2</i>	2.11	0.088
<i>SARM1</i>	2.06	0.00096
<i>IRAK2</i>	1.83	0.0044
<i>TLR10</i>	1.79	0.25
<i>MYD88</i>	1.71	0.0012
<i>IRF1</i>	1.70	0.0014
<i>NFKBIL1</i>	1.63	0.14
<i>PTGS2</i>	1.60	0.025
<i>TLR4</i>	1.58	0.18

(C) Up-regulated genes (1.5-fold or more) in HepG2-IL29, compared to HepG2-pcDNA3.1 cells.

<i>Genes</i>	Fold Differences	<i>P-values</i>
<i>CLEC4E</i>	5.67	0.23
<i>TLR10</i>	4.05	0.052
<i>TLR8</i>	3.98	0.058
<i>IFNG</i>	3.78	0.062
<i>CD86</i>	2.80	0.20
<i>IL1A</i>	2.67	0.0011
<i>TLR2</i>	2.64	1.02
<i>PTGS2</i>	2.55	0.00017
<i>TLR1</i>	2.51	0.022
<i>IFNA1</i>	2.48	0.019
<i>CD80</i>	2.33	0.053
<i>IKBKB</i>	2.30	0.000012
<i>FOS</i>	2.18	0.000015
<i>TLR3</i>	2.14	0.26
<i>SARM1</i>	1.89	0.0060
<i>IRAK2</i>	1.80	0.019
<i>IRAK1</i>	1.76	0.035
<i>CASP8</i>	1.72	0.0071
<i>IL10</i>	1.70	0.15
<i>EIF2AK2</i>	1.61	0.0084
<i>TBK1</i>	1.60	0.0046
<i>TOLLIP</i>	1.57	0.016
<i>RPL13A</i>	1.56	0.00034
<i>ELK1</i>	1.54	0.0031
<i>TNFRSF1A</i>	1.51	0.027

Table 3 Down-regulation of Toll-like receptor signaling pathway-related genes in HepG2-IL28A, HepG2-IL28B and HepG2-IL29, compared to HepG2-pcDNA3.1 cells.

(A) Down-regulated genes (1.5-fold or more) in HepG2-IL28A, compared to HepG2-pcDNA3.1 cells.

<i>Genes</i>	Fold Differences	<i>P-values</i>
<i>UBE2N</i>	0.58	0.012
<i>TLR9</i>	0.48	0.85
<i>CSF3</i>	0.45	0.0071
<i>TICAM2</i>	0.41	0.0049
<i>TLR4</i>	0.38	0.00075
<i>CCL2</i>	0.37	0.0015
<i>IFNB1</i>	0.37	0.013
<i>TLR5</i>	0.25	0.0059
<i>CXCL10 (IP10)</i>	0.23	0.10
<i>CD180</i>	0.12	0.0026
<i>CSF2</i>	0.05	0.00081
<i>BTK</i>	0.05	0.0016

(B) Down-regulated genes (1.5-fold or more) in HepG2-IL28B, compared to HepG2-pcDNA3.1 cells.

<i>Genes</i>	Fold Differences	<i>P-values</i>
<i>CSF3</i>	0.66	0.10
<i>CD14</i>	0.65	0.036
<i>CSF2</i>	0.64	0.084
<i>CD86</i>	0.61	0.32
<i>BTK</i>	0.61	0.034

(C) Down-regulated genes (1.5-fold or more) in HepG2-IL29, compared to HepG2-pcDNA3.1 cells.

<i>Genes</i>	Fold Differences	<i>P-values</i>
<i>MAP3K1</i>	0.58	<i>0.0093</i>
<i>UBE2N</i>	0.52	<i>0.0077</i>
<i>CSF3</i>	0.51	<i>0.064</i>
<i>CD14</i>	0.44	<i>0.0033</i>
<i>CXCL10 (IP10)</i>	0.42	<i>0.16</i>
<i>CCL2</i>	0.34	<i>0.0013</i>
<i>HSPA1A</i>	0.34	<i>0.00046</i>
<i>LTA</i>	0.26	<i>0.017</i>
<i>TLR5</i>	0.23	<i>0.0051</i>
<i>CD180</i>	0.12	<i>0.0027</i>
<i>CSF2</i>	0.06	<i>0.00087</i>
<i>BTK</i>	0.04	<i>0.00079</i>

Fig. 1. Venn diagram representing unique and shared Toll-like receptor signaling pathway-related molecules up-regulated or down-regulated by IL28A (IFNL2), IL28B (IFNL3) and IL29 (IFNL1) in human hepatoma cell lines. (A) Toll-like receptor signaling pathway-related genes up-regulated 1.5-fold or more in HepG2-IL28A/HepG2-IL28B/HepG2-IL29 cells compared to HepG2-pcDNA3.1 cells ($p < 0.05$). (B) Toll-like receptor signaling pathway-related genes down-regulated 1.5-fold or more in HepG2-IL28A/HepG2-IL28B/HepG2-IL29 cells compared to HepG2-pcDNA3.1 cells ($p < 0.05$). Three sets of real-time PCR arrays were performed.

Fig. 2. IFNLs produced from HepG2-derived cell lines could inhibit HCV and HBV replication in human hepatoma cell lines. (A) IFNLs produced from HepG2-derived cell lines could inhibit HCV subgenomic RNA replication in Huh7 cells. HCV subgenomic RNA were electroporated into Huh7 cells [25]. Two weeks after incubation with condition medium from HepG2-pcDNA3.1, HepG2-IL28A, HepG2-IL28B, HepG2-IL29, accompanied with 500 $\mu\text{g/mL}$ G418, the resultant colonies were counted. The number of G418-resistant colonies in the well treated with condition medium from HepG2-pcDNA3.1 was set as 100%. Data are expressed as mean $\pm$ SD. (B) HBV replication was inhibited in HepG2-IL28A, HepG2-IL28B,

HepG2-IL29, compared with HepG2-pcDNA3.1. We transfected pCMV-HBV DNA (0.4 µg) into HepG2-pcDNA3.1, HepG2-IL28A, HepG2-IL28B and HepG2-IL29 cells. Cellular DNA was collected at 96 h after transfection and HBV DNA was examined by real-time PCR [26]. HBV DNA/GAPDH levels from HepG2-pcDNA3.1 were set as 1. Data are expressed as mean $\pm$ SD.

Fig. 1.

(A)

(B)

Fig. 2.

(A)

(B)