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IL-28B (IFN-λ3) and IFN-α synergistically inhibit HCV replication

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SUMMARY. Genetic variation in the IL-28B (interleukin-28B; interferon lambda 3) region has been associated with sustained virological response (SVR) rates in patients with chronic hepatitis C treated with peginterferon-α and ribavirin. However, the mechanisms by which polymorphisms in the IL-28B gene region affect host antiviral responses are not well understood. Using the HCV 1b and 2a replicon system, we compared the effects of IFN-λs and IFN-α on HCV RNA replication. The anti-HCV effect of IFN-λ3 and IFN-α in combination was also assessed. Changes in gene expression induced by IFN-λ3 and IFN-α were compared using cDNA microarray analysis. IFN-λs at concentrations of 1 ng/mL or more exhibited concentration- and time-dependent HCV inhibition. In combination, IFN-λ3 and IFN-α had a synergistic anti-HCV effect; however, no synergistic enhancement was observed for interferon-stimulated response element (ISRE) activity or upregulation of interferon-stimulated genes (ISGs). With respect to the time course of ISG upregulation, the peak of IFN-λ3-induced gene expression occurred later and lasted longer than that induced by IFN-α. In addition, although the genes upregulated by IFN-α and IFN-λ3 were similar to microarray analysis, interferon-stimulated gene expression appeared early and was prolonged by combined administration of these two IFNs. In conclusion, IFN-α and IFN-λ3 in combination showed synergistic anti-HCV activity *in vitro*. Differences in time-dependent upregulation of these genes might contribute to the synergistic antiviral activity.

Keywords: HCV, IFN-λ, IL-28B, ISG, synergistic inhibition, microarray.

INTRODUCTION

In 2009, reports from three genome-wide association studies revealed that several single-nucleotide polymorphisms (SNPs) (rs12979860, rs12980275 and rs8099917) around the IL-28B (interleukin-28B; interferon lambda 3) gene are strongly associated with sustained viral response (SVR) to PEG-IFN and RBV treatment for chronic hepatitis C [1–3]. Specifically, patients with the TG or GG genotype at rs8099917 infected with genotype 1b are more resistant to PEG-IFN and RBV treatment than patients with the TT genotype. IL-28B haplotypes were also reported to be strongly associated with spontaneous HCV clearance [1, 4, 5].

IL-28B is a member of the type III IFN family [6], consisting of IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). IFN-λs bind to their cognate receptor, composed of IL28R1 and IL10R2, and then activate the receptor-associated Janus-activated kinases (Jak) 1 and tyrosine kinase (Tyk) 2, leading to the activation of downstream signal transducer and activator of transcription (STAT) proteins, STAT1 and STAT2. Similar to type I IFN signalling, the Jak-STAT signalling pathway activates the IFN-stimulated response element (ISRE) within the promoter region of interferon-stimulated genes (ISGs) [7].

Concerning the functional role of IL-28B in HCV infection, two of *in vivo* studies assessed the correlation of IL-28A/B mRNA levels in whole blood and peripheral blood mononuclear cells (PBMC) with IL-28B haplotypes at position rs8099917. IL-28A mRNA and IL-28B mRNA levels in subjects with the TT genotype were higher than in subjects with other genotypes (TG or GG), suggesting an association between higher amounts of endogenous IFN-λs and HCV clearance [2, 3]. On the other hand, subjects

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; ISG, interferon-stimulated genes; MTS, dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium; PBMC, peripheral blood mononuclear cells; SNP, single-nucleotide polymorphisms; STAT, signal transducer and activator of transcription; SVR, sustained viral response.

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with the TT genotype at SNP rs8099917 were reported to have lower expression levels of ISGs in the liver during the pretreatment period as compared with subjects with the TG or GG genotypes [8]. Several *in vitro* studies support a direct role of IFN-λs in the control of HCV replication through the innate immune pathway. In a cell culture system, Marcello *et al.* [9] showed that IFN-λ1 inhibited HCV replication with similar kinetics to that of IFN-α, although IFN-λ1 induced stronger upregulation of ISGs and this effect lasted longer. Moreover, combinations of IFN-λ1 and IFN-α had a greater inhibitory effect on HCV replication compared with the individual agents [10].

As suggested by the studies performed to date, a change in IFN-λ3 expression might be a key mechanism by which IL-28B SNPs determine the response to PEG-IFN and RBV. Considering that IFN-λ1 plays a direct role in the control of HCV replication and that IFN-λ1 enhances the antiviral activity of IFN-α, it seems reasonable to speculate that IFN-λ3 plays a similar antiviral role. Therefore, in this study, we investigated the direct antiviral role of IFN-λ3 alone and in combination with IFN-α using an HCV replicon system. In addition, we used microarray analysis to investigate the influence of IFN-λ3, alone or in combination with IFN-α, on the regulation of ISG-mediated antiviral pathways.

MATERIALS AND METHODS

Cell culture and HCV replicon

The human hepatoma cell lines OR6 and Huh7.5.1 were maintained in Dulbecco's modified Eagle's medium (DMEM: Sigma, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS) at 37°C in 5% CO_2. JFH-1-infected Huh7.5.1 cells were grown in DMEM supplemented with 10% FBS. The OR6 cell line, harbouring full-length genotype 1b HCV RNA and co-expressing *Renilla* luciferase (ORN/C-5B/KE) [11], was established in the presence of 500 μg/mL G418 (Promega, Madison, WI, USA).

Reagents

IL-28A (IFN-λ2), IL-28B (IFN-λ3) and IL-29 (IFN-λ1) were obtained from R&D Systems (Minneapolis, MN, USA). IL-28A and IL-29 are recombinant proteins generated from an NSO-derived murine myeloma cell line, and IL-28B is a recombinant protein generated from the CHO cell line. Interferon alpha-2b (INTRON®A 300 IU) was obtained from Schering-Plough Corporation (Kenilworth, NJ, USA).

Reporter plasmids and luciferase assay

HCV replication in OR6 replicon cells was determined by monitoring *Renilla* luciferase activity (Promega). To monitor IFN signalling directed by the interferon-stimulated response element (ISRE), the plasmids pISRE-luc expressing firefly luciferase were cotransfected using FuGENE®6 Transfection Reagent (Roche, Indianapolis, IN, USA) following the manufacturer's protocol. Luciferase activity was quantified using the dual-luciferase assay system (Promega) and a GloMax 96 Microplate Luminometer (Promega). Assays were performed in triplicate, and the results were expressed as mean ± SD percentage of the control values.

Quantification of HCV core protein and RNA

We quantified HCV core protein in culture supernatant using Lumipulse Ortho HCV Ag (Ortho Clinical Diagnostics, Tokyo, Japan) as specified by the manufacturer. The principle of the measurement method is based on the chemiluminescent enzyme immunoassay (CLEIA) [12].

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Intracellular genomic JFH-1 HCV RNA as well as cellular mRNA of IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18 was quantified by TaqMan RT-PCR. The cells were lysed and subjected to reverse transcription without purification of RNA using a Cells-to-Ct kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Quantitative PCR was performed in triplicate using a 7500 Real-Time PCR System (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. The sequences of the sense and antisense primers and the TaqMan probe for 5'UTR region of HCV were 5'-TGCGG AACCGGTGAGTACA-3', 5'-CTTAAGGTTTAGGATTCGTGCT CAT-3' and 5'-(FAM)CACCCTATCAGGCAGTACCACAAGG CC(TAMRA)-3', respectively. TaqMan probes for IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18 were purchased from Applied Biosystems. Primers for 18s rRNA (Applied Biosystems) were used as internal control.

Microarray analysis

OR6 replicon cells were harvested by centrifugation after exposure to 0.01 ng/mL IFN-α, 10 ng/mL IFN-λ3 or a combination of both for 6, 12, 24 and 48 h. Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). On-column DNase digestion was performed using the RNase-Free DNase Set (Qiagen). Quality control of extracted RNA was performed with the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

The RNA was then amplified and labelled using the Ambion® WT Expression Kit and GeneChip® WT Terminal Labelling and Control Kit (Affymetrix, Santa Clara, CA, USA). cDNA was synthesized, labelled and hybridized to the GeneChip® array according to the manufacturer's protocol, starting with 200-ng total RNA. The GeneChips were finally washed

and stained using the GeneChip® Fluidics Station 450 (Affymetrix) and then scanned with the GeneChip® Scanner 3000 7G (Affymetrix).

Affymetrix CEL files were imported into GeneSpring GX v.11.5 (Agilent Technologies, Santa Clara, CA, USA) analysis software. Data were normalized using robust multichip average analysis (RMA).

Dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium (MTS) assays

To evaluate the cell viability, dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. *P* values of < 0.05 were considered to be statistically significant.

RESULTS

IFN-λ1, IFN-λ2 and IFN-λ3 demonstrate antiviral activity against HCV

To determine the antiviral effect of IL-29 (IFN-λ1), IL-28A (IFN-λ2) and IL-28B (IFN-λ3) against HCV, OR6/ORN/C-5B/KE cells were seeded in 96-well plates for 24 h and then treated with IFN-λ1, IFN-λ2, IFN-λ3 or IFN-α at various concentrations for another 24, 48 and 72 h. In this system, the *Renilla* luciferase activity reflects the amount of HCV RNA synthesized. As shown in Fig. 1, at concentrations of 1 ng/mL or more, all IFN-λs led to a concentration- and time-dependent decrease in luciferase activity of the OR6/C-5B replicon. IFN-λ3 at 10 ng/mL inhibited HCV replication (32% reduction, $P < 0.05$) to a similar extent as 0.01 ng/mL IFN-α (49% reduction, $P < 0.05$) by 48 h.

We also assessed the effects of IFN-λ1, IFN-λ2 and IFN-λ3 on Huh7.5.1/JFH-1 cells. JFH-1 cells were seeded in 96-well plates for 24 h and then treated with IFN-λ1, IFN-λ2, IFN-λ3 or IFN-α at various doses for another 48 h. To determine their antiviral effect, HCV core protein in the medium and intracellular HCV RNA were measured by CLEIA and quantitative real-time RT-PCR, respectively. HCV RNA quantitative PCR assays were multiplexed for 18s ribosomal RNA to control for the amount of input RNA. As shown in Fig. 2, all IFN-λs inhibited HCV replication in JFH-1 cells in a concentration-dependent manner. Similarly, all of the IFN-λs caused suppression of HCV core protein secretion into the cell culture medium (Figure S1).

In C-5B system, there was no evident cytotoxicity below 100 ng/mL in any interferons except for IFN-λ1 (Figure S2). On the other hand, cytotoxicity was observed in lesser concentrations by those IFNs in JFH-1 system. However, as demonstrated in Fig. 2 and Figure S3, antiviral effect exceeded the cytotoxicity in the JFH-1 system.

Synergistic inhibition of HCV replication by IFN-λ3 and IFN-α in combination

We examined whether the combination of IFN-λ3 and IFN-α induces greater antiviral activity as compared with the individual cytokines alone. OR6/ORN/C-5B/KE cells were treated with the combinations of IFN-λ3 and IFN-α at various concentrations for 48 h. As shown in Fig. 3a, the relative concentration–inhibition curves of IFN-α were plotted for each fixed concentration of IFN-λ3, and the curves shifted to the left with increasing concentrations of IFN-λ3. The results indicate a synergistic effect of IFN-λ3 and IFN-α against HCV replication. We confirmed the synergistic effect of IFN-λ3 and IFN-α by isobologram (Fig. 3b). The inhibitory effects of the combination were quantified according to the method of Chou *et al.* using the CalcuSyn software program (Biosoft, Cambridge, UK). At the ED_{50} of each drug, the combination index was 0.40-0.61, indicating significant synergism. We also assessed the effect of the combination on Huh7.5/JFH-1 cells by HCV RNA quantitative PCR assays and HCV core protein secretion. At the ED_{50} of each drug, the combination index was 0.55 and 0.48, respectively (Table S1). The cytotoxicity was not observed at the range of concentration tested (Fig. S2E, S3E).

IFN-λ3 induces ISRE promoter activity

We used the ISRE luciferase reporter assay to assess activity downstream of the JAK-STAT signalling pathway. The ISRE-firefly luciferase plasmid was transfected into OR6/ORN/C-5B/KE cells for 24 h, and these cells were cultured with various concentrations of IFN-λ3 and IFN-α for another 12, 24 or 48 h. Firefly and *Renilla* luciferase activity was then measured.

IFN-λ3 induced ISRE luciferase activity in a time-dependent manner: activity was elevated threefold after treatment with 100 ng/mL IFN-λ3 for 48 h (Fig. 4). In contrast, IFN-α induced ISRE luciferase more rapidly, producing maximal activation of the response to IFN-α at 12 h. The combination of IFN-λ3 and IFN-α induced ISRE luciferase activity similarly to IFN-λ3 alone.

IFN-α and IFN-λ3 induce expression of similar genes in HCV 1b replicon cells

OR6/ORN/C-5B/KE cells were stimulated for 6, 12, 24 and 48 h with 0.01 ng/mL IFN-α, 10 ng/mL IFN-λ3 or a combination of both, while controls were left unstimulated for the same time interval. Induction of gene expression by IFNs was analysed in microarray experiments.

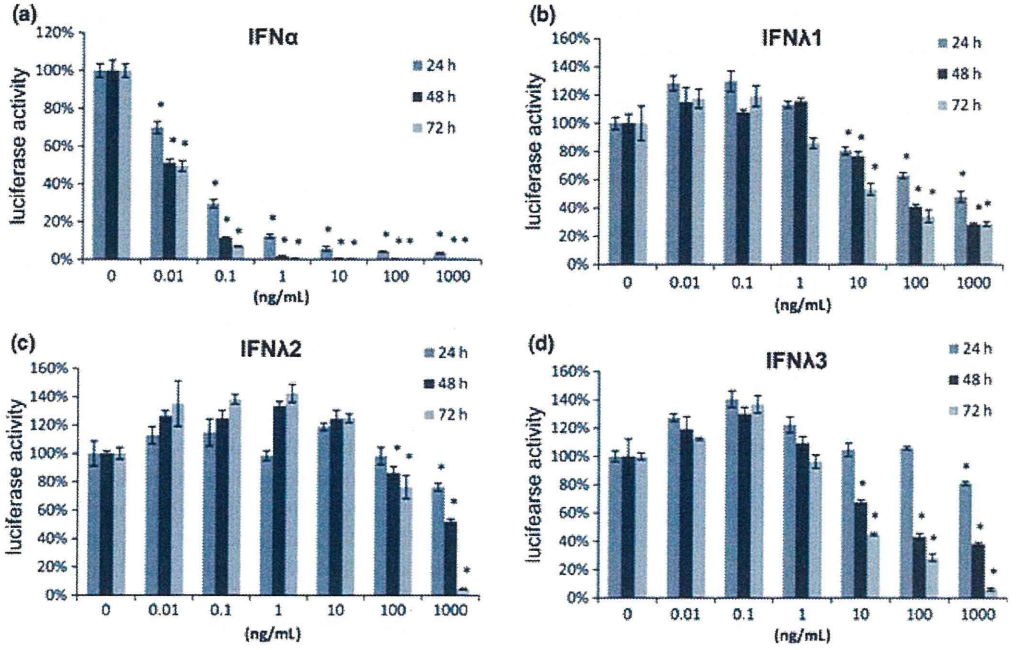

Fig. 1 IFN-α and IFN-λs inhibit HCV replicon in OR6 cells. Specific inhibition of the replication of a full-length HCV genotype 1b replicon by (a) IFN-α and (b) IFN-λ1. (c) IFN-λ2. (d) IFN-λ3 were quantified on the basis of luciferase activity. Symbols show the mean value of triplicate wells; error bars show the SD. *: $P < 0.05$ *vs* control (IFN 0 ng/mL) of each time point.

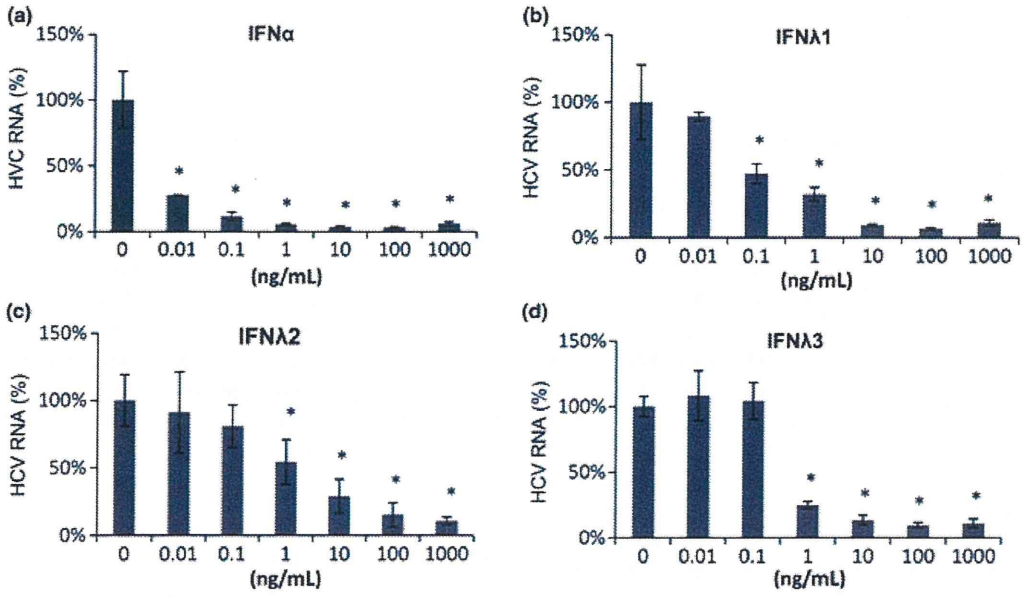

Fig. 2 IFN-α and IFN-λs inhibit HCV replicon in Huh7.5.1 cells. JFH1-infected Huh7.5.1 cells were incubated with various concentrations of (a) IFN-α and (b) IFN-λ1. (c) IFN-λ2. (d) IFN-λ3. After 48 h of treatment, total RNA was isolated and reverse transcribed, after which quantitative PCR was performed. Symbols show the mean value of triplicate wells; error bars show the SD. *: $P < 0.05$ *vs* control (IFN 0 ng/mL).

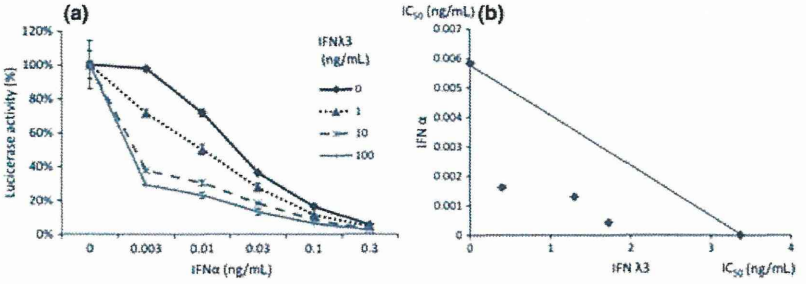

Fig. 3 Synergistic inhibitory effect of IFN-λ3 with IFN-α on hepatitis C virus replication. OR6/ORN/C-5B/KE cells were treated with combinations of IFN-λ3 with IFN-α at various concentrations. (a) The relative concentration–inhibition curves of IFN-α plotted for each fixed concentration of IFN-λ3 (0, 1, 10 and 100 ng/mL). (b) Classic isobologram for IC_{50} of IFN-λ3 with IFN-α in combination.

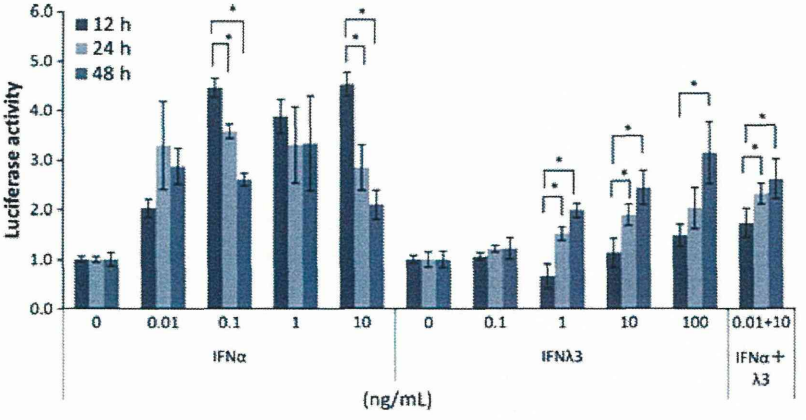

Fig. 4 IFN-stimulated response element (ISRE) promoter activity induced by IFN-α, IFN-λ3 or combination of IFN-α and IFN-λ3. OR6/ORN/C-5B/KE cells transfected with ISRE-firefly luciferase were cultured with various concentrations of IFN-α alone, IFN-λ3 alone or 0.01 ng/mL IFN-α plus 10 ng/mL IFN-λ3. ISRE-firefly luciferase activity at 24 h after transfection. Symbols show the mean value of triplicate wells; error bars show the SD. *: $P < 0.05$.

At all time points, the IFN-λ3-treated samples showed a tendency for the induction of a larger number of genes than samples treated with IFN-α. However, as shown in Table 1 listing the top 25 genes that were upregulated by both IFN-α and IFN-λ3 at 12 h, most of the upregulated genes are previously identified ISGs and the genes with high ranks were similar irrespective of the type of IFN or time point.

The time course of ISGs regulation differs between IFN-α and IFN-λ3

By microarray analysis, ISGs were more rapidly induced after the addition of IFN-α *vs* IFN-λ3 (data not shown). To confirm the rapid induction of ISGs by IFN-α, six ISGs, that is, IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18, were quantified for time-dependent expressional change by real-time RT-PCR. Expression of most of the genes upregulated by IFN-α peaked at 12 h and fell thereafter. In contrast, expression of IFN-λ3-induced genes peaked at 24 h and lasted up to 48 h. Combination of IFN-α and IFN-λ3 induced ISG with peak effects occurring at 12–24 h and lasting up to 48 h (Fig. 5).

DISCUSSION

In this study, we demonstrated that IFN-λ family members have distinctive time-dependent antiviral activities in an HCV replicon system and that IFN-λ3 and IFN-α have a synergistic effect in combination. Moreover, we attempted to identify the antiviral mechanism of IFN-λ3 by conducting a cDNA microarray analysis.

In previous studies, anti-HCV activity of IFN-λ1, IFN-λ2 and IFN-λ3 was reported in JFH-1 and OR6/C-5B systems [13]. Time-dependent anti-HCV activity has also been observed with IFN-λ1 [9]. In this study, we confirmed the previous results and added the further finding that time-dependent antiviral activity is not limited to IFN-λ1, but rather is common among all IFN-λs.

Table 1 Top 25 genes that were upregulated by both IFN-α and IFN-λ3 at 12 h

Gene bank ID	Gene symbol	Gene description	IFN-α 0.01 ng/mL fold increase	IFN-λ3 10 ng/mL fold increase	IFN-α+IFN-λ3 fold increase
BC007091	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	4.01	4.49	4.87
BC049215	OAS2	2′-5′-oligoadenylate synthetase 2, 69/71kDa	3.06	3.88	4.48
M33882	MX1	Myxovirus (influenza virus) resistance 1	3.24	3.29	3.69
AF095844	IFIH1	Interferon induced with helicase C domain 1	2.73	3.02	3.54
BC038115	DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	2.70	2.92	3.51
BC011601	IFI6	Interferon, alpha-inducible protein 6	3.07	3.24	3.42
BC042047	HERC6	Hect domain and RLD 6	2.56	2.75	3.34
AF442151	RSAD2	Radical S-adenosyl methionine domain containing 2	1.32	2.59	3.28
U34605	IFIT5	Interferon-induced protein with tetratricopeptide repeats 5	2.47	2.91	3.25
AY730627	OAS1	2′,5′-oligoadenylate synthetase 1, 40/46kDa	2.32	2.57	3.05
AB006746	PLSCR1	Phospholipid scramblase 1	2.37	2.51	3.03
AF307338	PARP9	Poly (ADP-ribose) polymerase family, member 9	2.39	2.46	2.94
M87503	IRF9	Interferon regulatory factor 9	2.61	2.59	2.85
AK297137	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	1.90	2.36	2.79
AK290655	EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	2.47	2.45	2.77
BX648758	PARP14	Poly (ADP-ribose) polymerase family, member 14	2.07	2.25	2.66
BC132786	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	1.83	2.17	2.59
AF445355	SAMD9	Sterile alpha motif domain containing 9	2.07	2.08	2.56
	DDX60L	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like	1.63	1.92	2.39
BC014896	USP18| USP41	Ubiquitin-specific peptidase 18/ubiquitin-specific peptidase 41	1.52	1.78	2.11
AB044545	OAS3	2′-5′-oligoadenylate synthetase 3, 100kDa	1.44	1.57	2.10
BC010954	CXCL10	Chemokine (C-X-C motif) ligand 10	0.76	1.66	1.99
BC014896	USP18	Ubiquitin-specific peptidase 18	1.33	1.55	1.99
AL832618	IFI44L	Interferon-induced protein 44-like	0.58	1.31	1.95

We also assessed whether IFN-λ3 and IFN-α in combination could produce additive or synergistic effects on antiviral activity. In previous studies, additive antiviral activity against HCV was reported with the combination of IFN-λ1 and IFN-α [9, 10]. However, there have been no previous reports on the combined effects of IFN-λ3 and IFN-α. In this study, the focus was on IFN-λ3, because IFN-λ3 is suspected to be the key molecule, mediating the effect of SNPs in the IL-28B gene region on the anti-HCV response to IFN-α. As shown in Fig. 3 and Table S1, synergistic induction of anti-HCV activity occurred in both the OR6/C-5B and Huh7.5/JFH-1 HCV replicon systems. Synergy was demonstrated by the combination index values (Table S1).

Although it has been reported that the upregulated genes induced by IFN-λ are similar to those induced by IFN-α [9, 14–16], there have been no previous reports on

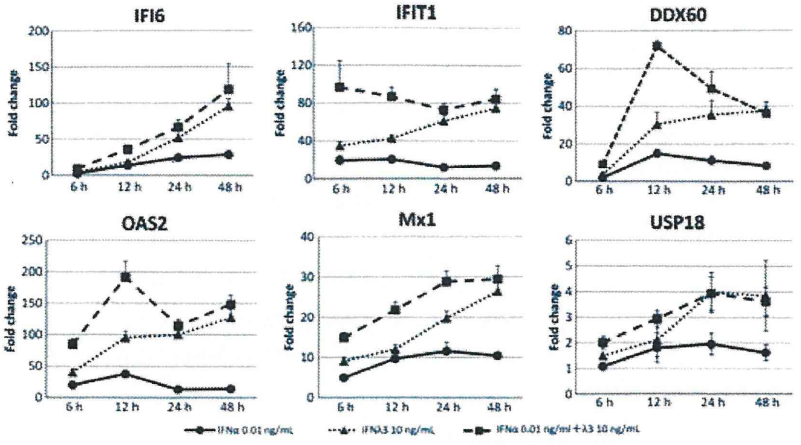

Fig. 5 Time course of ISG expression induced by 0.01 ng/mL IFN-α, 10 ng/mL IFN-λ3 or 0.01 ng/mL IFN-α plus 10 ng/mL IFN-λ3. Expression of the ISGs – IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18 – in OR6/ORN/C-5B/KE cells treated 6, 12, 24 and 48 h were determined by qRT-PCR. Results are presented as the relative fold induction. Symbols show the mean value of triplicate wells; error bars show the SD. *Solid lines* represent 0.01 ng/mL IFN-α alone, whereas *fine dashed lines* show 10 ng/mL IFN-λ3 alone, and *coarse dashed lines* show the combination of the 2 cytokines.

the genes induced by IFN-α and IFN-λ in combination. In cDNA microarray analysis, as demonstrated in Table 1, the most strongly upregulated genes induced by IFN-α/IFN-λ3 alone or in combination were almost identical, and most of them were ISGs. As no genes showed upregulation specific to IFN-λ3, we speculate that IFN-α and IFN-λ3 share a similar antiviral intracellular mechanism at the molecular level.

Unexpectedly in microarray analyses, synergistic upregulation of ISGs was not observed. In the same manner, TaqMan real-time RT-PCR analysis showed that the combination of IFN-α and IFN-λ3 did not upregulate ISGs synergistically (Fig. 5). In addition to cDNA microarray analysis, ISRE reporter assays were performed to determine the activation of components of the JAK-STAT pathway common to both type I and III IFNs. As shown in Fig. 4, each IFN upregulated ISRE activity, and the combination of IFN-λ3 and IFN-α did not synergistically enhance ISRE activity either.

Meanwhile, the peak time of the induction of ISG expression differs for IFN-α and IFN-λ1 [9, 17]; peak gene expression occurs earlier with IFN-α than with IFN-λ1. In our study, we confirmed that the peak induction of gene expression occurred later (24 h) and lasted longer (24–48 h) with IFN-λ3 than with IFN-α (12 h). Importantly, gene expression appeared early (12 h) and was prolonged (48 h) by the combination of both IFNs. Similarly to the peak time difference between IFN-α and IFN-λ3 seem for ISG expression, a time-dependent increase in ISRE activation was observed with the combination of both IFNs. While the precise mechanism remains to be clarified, differential regulation of the time-dependent induction of ISG gene expression could be one of the mechanisms underlying the synergistic antiviral effect. One of the molecules contributing to time-dependent ISG upregulation is the ISG known as ubiquitin-specific peptidase 18 (USP18), which has been reported to bind to IFNAR2 and inhibit the interaction of Jak1 with its receptor, thereby preventing IFN-α signalling while leaving IFN-λ signalling unaffected [18, 19]. Actually, expression of USP18 is specifically upregulated with IFN-λ3 in this study as shown in Fig. 5. If the ISGs upregulated by IFN-α are downregulated by USP18, it is plausible that the expression of genes induced by IFN-α decreases early, while expression of genes induced by IFN-λ lasts longer.

A number of clinical studies have confirmed that SNPs around the IL-28B gene are associated with the response to PEG-IFN and RBV therapy, and as previously indicated, various investigations have been performed to clarify the underlying mechanism. Specifically, increased IL-28B mRNA expression in PBMC [2, 3], high serum concentrations of IFN-λ1 (IL-29) [20], low expression of ISGs in the liver prior to IFN treatment [8, 21] and high upregulation of ISG expression by IFN treatment [8, 22] were found in subjects with IL-28B SNP genotypes associated with SVR (rs12979860 CC and rs8099917 TT). Although the functional role of IFN-λ3 still needs to be investigated more thoroughly, if IFN-λ3 expression change is the essential difference in determining the clinical treatment response to PEG-IFN and RBV therapy and if its expression is decreased in patients with the specific IL-28B genotype, which is associated with non-SVR, it is possible that exogenous administration of IFN-λ3 might improve IFN-α-induced viral clearance and that such treatment would be beneficial for patients with the IFN-resistant IL-28B genotype.

In present study, the OR6-cultured cells harboured the rs8099917 TT genotype, and recombinant IFN-λ3 (IL-

28B) protein used in the experiment was derived from cells with the rs8099917 TT genotype (data not shown). Therefore, the viral responses and/or cellular gene expression change in cells and/or proteins with different IL-28B genotypes *in vitro* should be determined in future studies.

In conclusion, we demonstrated that IFN-α and IFN-λ3 synergistically enhance anti-HCV activity *in vitro*. Although the ISGs upregulated by IFN-α and IFN-λ3 were similar, differences in time-dependent upregulation of these genes, especially prolonged ISGs expression by IFN-λ3, might contribute to their synergistic antiviral activity.

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1: IFN-α and IFN-λs inhibit HCV core protein secretion. JFH1-infected Huh7.5.1 cells were incubated with various concentrations of IFN-α and IFN-λ1, -λ2, -λ3. After 48 h of treatment, HCV core protein in the medium was measured. Symbols show the mean value of triplicate wells; error bars show the SD. *: $p < 0.05$ vs. control (IFN 0ng/ml).

Figure S2: The dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium assay was performed after OR6/ORN/C-5B/KE cells were cultured with various concentrations of (A) IFN-α, (B) IFN-λ1, (C) IFN-λ2, (D) IFN-λ3 and (E) combination of IFN-α and IFN-λ3 for 48 h. Symbols show the mean value of triplicate wells; error bars show the SD. *: $p < 0.05$ vs. control (IFN 0ng/ml).

Figure S3: The dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium assay was performed after Huh7.5.1/JFH-1 cells were cultured with various concentrations of (A) IFN-α, (B) IFN-λ1, (C) IFN-λ2, (D) IFN-λ3 and (E) combination of IFN-α and IFN-λ3 for 48 h. Symbols show the mean value of triplicate wells; error bars show the SD. *: $p < 0.05$ vs. control (IFN 0ng/ml).

Table S1: Combination index after 48hr stimulation by CalucSyn.

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