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Distinct and overlapping genomic profiles and antiviral effects of Interferon- λ and - α on HCV-infected and non-infected hepatoma cells

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Abstract

Recently, several SNPs in the region of the *IL28B* (IFN- λ) gene have been associated with spontaneous clearance of hepatitis C virus (HCV) and enhanced cure rates for IFN- α -based therapies, suggesting a potential correlation between IFN- λ and the ability to clear HCV. To understand the mechanism of IFN- λ 's as compared to IFN- α 's antiviral activity, we performed a comprehensive analysis of their anti-HCV effects, whole genome transcriptome profiling with validation, and signaling of IFN- α and IFN- λ using J6/JFH-1 and Huh7.5 cells *in vitro*. IFN- λ and IFN- α exhibited comparable anti-HCV activity and gene expression profiles in Huh7.5 cells. While the majority of genes induced by IFN- α and IFN- λ were similar, IFN- λ exhibits profound, but delayed kinetics of ISG induction, while IFN- α induced more rapid induction of ISGs. Furthermore, the increased induction of ISG expression by IFN- λ correlated with up-regulation of IFN- λ receptor (IL-28RA) expression and more prolonged activation of the Jak-STAT signaling pathway. The findings from our comparative analysis of IFN- α and IFN- λ in HCV-infected and non-infected cells support the clinical use of IFN- λ as a potential alternative to IFN- α in the treatment of chronic hepatitis C.

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Statement of Interest

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Keywords

Huh7.5 cells; hepatitis C; IFN- α ; IL28B; JFH-1

INTRODUCTION

Chronic Hepatitis C affects approximately 160 million people worldwide (1). Current standard treatment for chronic hepatitis C includes pegylated interferon α , a Type 1 interferon (IFN), in combination with ribavirin. While effective in curing about 46–76% of patients (2) depending on HCV genotype, this treatment often is associated with numerous serious adverse events.

Recently, several SNPs in the region of interferon-lambda-3 gene (IFN- λ), also referred to as IL-28B, have been associated with spontaneous clearance of HCV and enhanced cure rates for IFN- α (IFN- α)-based therapy (3–6). The exact mechanism associated with this effect is presently unknown, however great interest has been generated by these findings, likely due to the fact that IFN- λ encodes for a Type 3 Interferon, which shares similar biological properties with IFN- α , a Type 1 IFN. IFN- λ utilizes a heterodimeric receptor consisting of IFN- λ R1 and IL-10R2, while Interferon α (IFN- α), uses the IFN- α / β receptor complex formed by two subchains, IFN- α R1 and IFN- α R2. However, both IFN- α and IFN- λ signal via the Jak-STAT pathway, leading to the phosphorylation of STAT1 and STAT2, formation of the ISGF3 transcription factor complexes and subsequent binding to IFN-stimulated response elements (ISRE) in the promoters of many IFN-stimulated genes (ISG) (7, 8). Additionally, previous studies using in vitro HCV culture systems have shown that both IFN- α and IFN- λ inhibit HCV viral replication (9, 10). Presently, clinical trials are being performed to evaluate the safety, tolerability and antiviral efficacy of recombinant human IFN- λ in chronic hepatitis C patients.

Although IFN- α and IFN- λ share a number of biological properties, they are also quite distinct in many other characteristics. In this regard, as compared to IFN- α receptors, which are widely distributed, IFN- λ receptor distribution is limited to hepatocytes in the liver, B cells in the peripheral blood, and epithelial cells (10, 11). This limited receptor distribution might be the reason that early clinical trials of IFN- λ in humans have reported fewer adverse events and hematologic effects as compared to IFN- α treated patients (12, 13). Despite the improved side effect profile and potential clinical utility, the role of IFN- λ in the control of HCV infection has not yet been well defined. The mechanisms of IFN- λ in the control and pathogenesis of chronic hepatitis C is presently unknown.

In this study we compared the antiviral activity of IFN- α and IFN- λ in HCV-infected hepatoma cells. We also compared the ability of IFN- α and IFN- λ to regulate ISG expression in HCV-infected and non-infected hepatoma cells. In this regard, we compared the antiviral effect, gene expression profiles, and signaling induced by IFN- α and IFN- λ in HCV-infected and non-infected Huh7.5 cells.

MATERIALS AND METHODS

HCV infectious clone, Cell line and culture conditions

The Huh7.5 HCC cell line was provided by Apath LLC (Saint Louis, MO, USA). Cells were cultured in DMEM medium (Invitrogen Corporation Inc, Carlsbad, CA, USA) and supplemented with 10 % FBS and Penicillin (100 U mL⁻¹) / Streptomycin (100ug mL⁻¹) at 37°C in a 5% CO₂ containing incubator. The infectious plasmid clone for the HCV J6/JFH1 infectious cell culture system was provided by Apath LLC. Transfection and subsequent

infection of Huh7.5 cells using this plasmid was performed as described elsewhere (14, 15). IFN- α and IFN- λ (R & D Systems Inc. Minneapolis, MN) were added to continuous culture of HCV-infected or non-infected Huh7.5 cells at various concentrations. Concentrations of IFN- α (100 IU mL⁻¹) or IFN- λ (100 ng mL⁻¹) were used for all subsequent experiments because they showed similar antiviral efficacy at 72 hours.

Real Time PCR for HCV copy number

Culture supernatants from HCV-infected cells were collected 48 hours after treatment with IFN- α (100 IU mL⁻¹) or IFN- λ (100 ng mL⁻¹), HCV viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen), and HCV copy numbers were determined using a quantitative real-time PCR.

DNA microarray analysis

In order to compare gene expression profiles induced by IFN- α and IFN- λ we performed comparative DNA microarray analyses. J6/JFH-1 infected and non-infected Huh7.5 cells untreated or treated with Interferon- α (100 IU mL⁻¹) or λ (100ng mL⁻¹) for one hour, were used for DNA microarray analysis. Total RNA was extracted using the RNAQUEOUS-4PCR KIT (APPLIED BIOSYSTEM INC.). The labeled RNA synthesis and hybridization to the Affymetrix U133A human microarray consisting of probes encompassing over 22,000 genes, were performed according to the manufacturer's recommended protocol (Genechip 3'IVT express kit). Gene expression values (log2) were determined by GC-Robust Multi-Array algorithm (Wu ZJ and Irizarry R: <http://www.bioconductor.org>) followed by a Loess normalization using an R package (<http://www.elwood9.net/spike>). Using a SAM algorithm (16), differentially expressed genes from 32 separate experiments were identified between HCV-infected and non-infected Huh7.5 cells and grouped using supervised partitioning clustering.

Validation of DNA Microarray data using qPCR

In order to understand the differential kinetic effects of IFN- α and IFN- λ on select gene expression profiles we performed multiplex PCR on IFN- α and IFN- λ treated Huh7.5 cells at various time points. Total RNA was isolated from J6/JFH-1 infected and non-infected Huh7.5 cells treated with Interferon- α (100 IU mL⁻¹) or λ (100ng mL⁻¹) for one, four and 24 hours. Two micrograms of total RNA was reverse transcribed to cDNA using the manufacturer's recommended protocol (High-capacity cDNA Archive kit from ABI). Ten nanograms of converted cDNA was added to each well of 96 well TaqMan array plates. Each well of the TaqMan array plate contains triplicate wells of primers and probes of three different house keeping genes and 24 selected genes of interest with potential biological relevance. Expression of twenty-four ISGs were validated using the ABI 7500 real-time PCR system on a custom designed multiplex platform. Each gene's expression data was normalized to GAPDH expression levels.

Flow cytometry

We next investigated whether HCV-induced alterations in early signal transduction events could explain the differences in gene expression between HCV-infected and non-infected cells treated with IFN- λ . In this regard, we assessed the levels of IFN- λ receptor (IFN- λ R1, also known as IL28RA) expression in HCV-infected and non-infected Huh7.5 cells.

The expression of IFN- λ R1 on HCV-infected and non-infected Huh7.5 cells was estimated using surface staining with IFN- λ R1 PE - (R&D Systems Inc, Minneapolis, MN, USA). Briefly, cells were washed with 5mM EDTA containing PBS, and then incubated with fresh cold 5mM EDTA containing PBS for 10 minutes. The detached cells were then washed with

cold PBS once, and resuspended in binding buffer. Cells were incubated with anti-human IFN- λ R1 PE, or anti-human CD81-APC or both antibodies respectively. FACS analysis was performed on a FACS Canto bioanalyzer (BD Biosciences San Jose, CA, USA). The FlowJo software (©Tree Star Inc, Ashland, OR, USA) was used to analyze the data.

Western Blot for IFN- α or IFN- λ -inducible STAT Phosphorylation

To determine if HCV-infection of Huh7.5 cells results in a qualitative change in IFN- α and/or IFN- λ signaling, HCV-infected and non-infected control cells were stimulated with IFN- α (100 IU mL⁻¹) or IFN- λ (100 ng mL⁻¹) for one hour, four hours or 24 hours at 37°C. After stimulation, cells were washed with cold PBS and then whole cell lysate was prepared using radioimmunoprecipitation assay (RIPA) buffer containing phosphatase inhibitor cocktail (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein concentration was measured using a BCA protein assay (Pierce). Thirty micrograms of total cellular protein were separated on 4–12% SDS-PAGE gels, and then western blotting analyses were performed using antibodies specific for tyrosine-phosphorylated STAT-1 or STAT2, (Cell Signaling Technology, Danvers, MA, USA). Signals on the membranes were detected using HRP-conjugated secondary antibodies (GE-Healthcare, Piscataway, NJ, USA) with the ECL-Plus kit (GE-Healthcare). The levels of total STAT1 and STAT2 were measured by re-probing the blots with anti-STAT1 and anti-STAT2 antibodies, respectively. To quantify the western blot band intensities, we scanned the blots using the Odyssey imaging system and software program from LI-COR Biosciences, Inc. (Lincoln, NE, USA). Each value represents the mean $\pm$ SD of triplicate determinations.

Statistical Analysis

Analysis of variance with Tukey's multiple comparison test was used to compare means of the independent data. Validated genes were compared using paired t-tests with the Bonferroni adjustment for multiple testing. Mean intensity of bands on Western blot as well as IFN- λ R1 chain expression were compared using t-tests.

RESULTS

Anti-viral effect of IFN- α and IFN- λ

IFN- λ induced similar levels of HCV viral suppression in J6/JFH-1 infected Huh7.5 cells at the doses of 40 ng mL⁻¹, 100 ng mL⁻¹ and 250 ng mL⁻¹. Overall, IFN- α exhibited more suppression of HCV replication than IFN- λ (100 ng mL⁻¹) at 24 hours ($54 \pm 8\%$ vs. $32 \pm 7\%$ IFN- α and IFN- λ respectively, $p < 0.05$) and similar levels of suppression at 72 hours ($69 \pm 4\%$ vs. $85 \pm 8\%$, IFN- α and IFN- λ respectively, $p > 0.05$) (Figure 1A). Therefore, both IFN- α and IFN- λ induced profound and sustained suppression of HCV replication in Huh7.5 cells.

Differential Transcription profiles of IFN- α and IFN- λ in HCV-infected and non-infected Huh7.5 cells

293 differentially expressed genes were identified. The corresponding genes were grouped using supervised partitioning clustering. Functional annotation analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Figure 1B).

As expected, IFN- λ demonstrated a similar pattern of gene expression to that induced by IFN- α (275 out of 293 differentially expressed genes). The gene expression profiles can be summarized as follows based on the distinct functional and biologically relevant classification of genes (Table 1). The first set of genes includes genes induced by both IFN- α and IFN- λ , most of which belong to the ISG family. As shown in Figure 2A, expression

of *MX1* and *OAS1* (representative validated genes) was induced by IFN- α and IFN- λ to similar levels (* $p < 0.05$ for IFN- α and IFN- λ vs. untreated cells). A second, but distinct group of genes were preferentially regulated by IFN- α as compared to IFN- λ . As shown in Figure 2B, IFN- α up-regulated the expression of *IL32* while down-regulating *CXCL5* gene expression (* $p < 0.05$ for IFN- α treated cells vs. untreated controls). A third distinct group of genes were modulated to a larger degree by IFN- λ as compared to IFN- α . As shown in Figure 2C, IFN- λ up-regulated expression of a few distinct ISGs such as *OASL* and *IFI35* more strongly than IFN- α (# $p < 0.05$ and * $p < 0.02$, for IFN- λ and IFN- α treated vs. untreated cells respectively). These differentially regulated genes suggest a potential distinct role for IFN- λ in the host antiviral defense response to HCV.

Detailed profile of time dependent kinetics of IFN-stimulated gene expression by IFN- α and IFN- λ

qPCR of 24 genes induced by IFN- α and IFN- λ treated Huh7.5 cells was performed at one, four and 24 hours (Figure 3A–C). IFN- λ treatment resulted in a later peak of ISG expression (24 hours) ($p < 0.05$) (Figure 3D). These results demonstrate that IFN- α and IFN- λ induce ISG expression with distinct kinetics. These different temporal patterns of ISG expression may explain the distinct gene expression profiles observed with the DNA microarray analysis.

Validation of IFN- λ induced genes in HCV-infected and non-infected Huh7.5 cells

In order to determine if HCV infection alters IFN- λ mediated gene expression profiles we compared DNA microarray data from HCV-infected and non-infected Huh7.5 cells treated with or without IFN- λ . The gene expression profiles are summarized Table 1. The first set of genes were inducible by IFN- λ in both HCV-infected and non-infected cells. As shown in Figure 4A, most of these genes belong to the ISG family, such as *IFIT1* and *IFIT3* (representative validated genes), and were induced by IFN- λ in both HCV-infected and non-infected cells. The second group of genes are up-regulated by IFN- λ more in HCV-infected than non-infected cells. A distinct group of ISGs, such as *ISG20* and *OAS3* were induced more strongly in HCV-infected cells than in non-infected cells (Figure 4B). Surprisingly, HCV infection did not have any positive or negative effect on the transcription profile induced by IFN- α in this cell culture system (data not shown). Finally, a third group of genes were induced selectively by IFN- λ in HCV-infected cells. As seen in Figure 4C, *IL18*, an NK cell stimulatory cytokine, and *CCL4* were upregulated by IFN- λ exclusively in HCV-infected cells. Overall, these results suggest that enhanced IFN- λ signaling induces expression of a larger set of ISGs in HCV-infected cells as compared to non-infected cells.

HCV infection enhances IFN- λ signaling by up-regulating IFNAR1 expression and prolonging Jak-STAT signaling in Huh7.5 cells

As shown in Figure 5A a larger proportion of HCV-infected as compared to non-infected cells expressed the IFN- λ R1 chain ($8 \pm 2.1\%$ HCV non-infected vs. $23 \pm 3.3\%$ HCV-infected cells; $p < 0.001$). In addition mean fluorescence intensity values were higher in HCV-infected than in non-infected cells (42 ± 9.8 vs. 86 ± 12.4 ; $p < 0.001$), indicating an increased density of IFN- λ R1 on HCV-infected cells.

Additionally we found that IFN- α induced rapid activation of STAT1 and STAT2. Although the magnitude of STAT1 and STAT2 activity induced by IFN- λ was less than that induced by IFN- α , the levels of tyrosine-phosphorylated STAT1 and STAT2 declined more slowly in IFN- λ treated cells. There were no difference in the relative levels of STAT1 and STAT2 activity induced by either IFN- α or IFN- λ in HCV-infected versus non-infected cells (Figure 5B and 5C). These findings are consistent with our previous data regarding the kinetics of ISG expression in IFN- α versus and IFN- λ treated Huh7.5 cells (Figure 3D).

DISCUSSION

IFN- λ and IFN- α exhibit comparable anti-HCV activity and gene expression profiles in the Huh7.5 cell culture system. The differential expression of genes induced by IFN- λ and IFN- α is likely due to the different kinetics of ISG gene induction by these two cytokines. In this regard, IFN- λ induced strong, but delayed kinetics of ISG induction, while IFN- α induced more rapid expression of ISGs. Furthermore, HCV infection enhanced IFN- λ , but not IFN- α -mediated induction of ISGs, by inducing increased IFN- λ receptor (IL28RA) expression levels on Huh7.5 cells and more prolonged activation of the Jak-STAT signaling pathway. These results define a unique virus/host interaction that supports a role for IFN- λ as a potential new anti-HCV agent.

The primary goal of our study was to perform a comparative analysis of the anti-HCV effects, whole genome transcriptome profiles and signaling induced by IFN- α versus IFN- λ in HCV-infected and non-infected Huh7.5 cells. Previous studies have suggested that IFN- α and IFN- λ activate the same Jak-STAT signaling pathway and probably have similar biological responses even though they signal through distinct cell surface receptors (7, 9). Others have suggested that the biological functions of IFN- λ are more restricted than those induced by IFN- α because IFN- λ receptors are largely restricted to epithelial cell types, whereas IFN- α receptors are ubiquitously expressed (11). In this regard, it could be speculated that IFN- λ may induce a narrower range of side effects than IFN- α . IFN- λ may be a preferred choice for treatment of chronic viral infections, such as HCV, because of the more tissue restricted expression of IFN- λ receptors (2, 17). However, understanding the mechanisms of the antiviral effect and other biological effects of both cytokines are of paramount significance in furthering the development of IFN- λ as a potential therapeutic agent for treating HCV.

In this study, we found that IFN- λ is as effective as IFN- α in suppressing HCV replication in vitro using J6/JFH-1 virus infected Huh7.5 cells. Additionally, when we examined the gene expression profiles induced by IFN- α and IFN- λ on HCV-infected and non-infected Huh7.5 cells, we found that both cytokines induce similar overlapping effects on gene expression. Most genes were induced by IFN- λ and IFN- α to a similar extent in J6/JFH-1 non-infected Huh7.5 cells. However, there were small, yet distinct clusters of genes that were differentially induced by IFN- λ and IFN- α , particularly in J6/JFH-1 infected Huh7.5 cells. Since both cytokines activate the same intracellular signaling pathway, this difference in gene expression could be explained either by a difference in the kinetics of Jak-STAT signaling or a complementary effect of HCV replication in Huh7.5 cells, which in turn leads to differential ISG induction by IFN- λ . In this regard, our data from qPCR experiments demonstrated that IFN- λ induces a distinct kinetic profile of ISG expression than that induced by IFN- α . We observed a more rapid ISG induction pattern for IFN- α as has been previously noted by others using HaCaT cells as targets (18). In contrast, IFN- λ induced a more delayed but profound ISG induction pattern in Huh7.5 cells. These data could explain the DNA microarray data showing relatively higher ISG expression by IFN- α as the gene expression profiling was performed immediately (one hour) after cytokine treatment. Notably, IFN- α but not IFN- λ induced expression of *IL32*, a novel proinflammatory cytokine. The biological significance of the induction of this cytokine is not clear, however, it is possible that this cytokine may contribute to some of the well characterized inflammatory responses often associated with IFN- α therapy in chronic hepatitis C patients.

Another group of genes that was identified in this study were preferentially induced by IFN- λ in HCV-infected but not non-infected cells. While it is plausible that HCV infection of Huh7.5 cells may have resulted in the induction of these genes, which were also induced by IFN- λ , a similar response was not observed in IFN- α treated HCV-infected Huh7.5 cells.

Another possible explanation for this observation might be that HCV infection of Huh7.5 cells somehow augments IFN- λ signaling. Previous studies have demonstrated that HCV infection has no such effect on IFN- α signaling (19), however data are currently lacking regarding the potential effects of HCV replication on IFN- λ signaling.

Since IFN- λ and IFN- α have distinct cell surface receptors, we investigated whether HCV-infection modulates the expression of either one of these receptors to promote IFN- λ signal transduction. Our data clearly demonstrate that HCV infection upregulated the expression of the IFN- λ specific receptor, IFN λ R1 (IL28RA) on the surface of Huh7.5 cells. Since modulation of receptor expression itself is often not an indication of the efficiencies of signaling (20) we investigated whether the Jak-STAT phosphorylation pathway was also modulated by HCV infection. Although the magnitude of STAT1 and STAT2 activity induced by IFN- λ was less than that induced by IFN- α , the levels of tyrosine-phosphorylated STAT1 and STAT2 declined more slowly in IFN- λ -treated than in IFN- α -treated cells. These findings provide further support for our conclusion that IFN- λ induces more prolonged activation of STAT1 and STAT2 than IFN- α in both HCV-infected and non-infected Huh7.5 cells, confirming augmentation of IFN λ R1 signaling by HCV infection and bringing to question the potential differential role of HCV non-structural proteins in the inhibition of IFN- α as compared to IFN- λ induced signaling (21, 22). Some of the candidate genes that were expressed as a result of this unique interaction may have a critical biological function as is the case with *IL18*, which can specifically recruit and activate NK cells that play a key role in viral suppression.

Our results offer proof of principle for the preferential role of IFN- λ in the treatment of chronic hepatitis C infection, however differences in gene expression may be seen when using other cell culture lineages (23) or in the HCV genotypes. Early ongoing clinical trials of recombinant human IFN- λ in patients with chronic HCV infection will determine whether our *in vitro* findings have any direct clinical significance.

In summary, we demonstrate a novel relationship between hepatitis C virus infection and IFN responsiveness using a well-defined HCV continuous culture system. Although IFN- α and IFN- λ share a common signaling pathway, and highly similar gene expression profiles, there are some interesting differences between the IFN- α and IFN- λ regarding their effects on the kinetics of Jak-STAT signaling, ISG induction and susceptibility to HCV-infection mediated changes in cell surface receptors and intracellular signaling, which may be of clinical significance. Augmentation of IFN- λ signaling by HCV infection could be exploited in clinical trials using IFN- λ rather than IFN- α , which may aid in more effective clearance of HCV from hepatocytes, yet with fewer side effects (12), given the more narrow tissue distribution of IFN- λ receptors.

CONCLUSIONS

IFN- λ and IFN- α induce very similar transcription profiles in HCV-infected and non-infected Huh7.5 cells. HCV infection suppresses Type 1 IFN signaling in Huh7.5 cells, thereby circumventing IFN-mediated suppressive effects, however our data suggest a complementary role for HCV replication on IFN- λ signaling. IFN- λ , but not IFN- α , induces expression of a small group of genes, including *IL-18*, that may be important in NK cell activation in the liver and aid in the clearance of HCV. Kinetics of the IFN- λ -mediated antiviral effect suggests a delayed but more sustained antiviral effect on HCV replication. Our data support a role for IFN- λ as a viable alternative to IFN- α as a therapeutic agent to treat chronic HCV.

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Figure 1. Comparison of IFN-α and IFN-λ

A. Suppression of HCV replication by IFN-α and IFN-λ. Overall IFN-α (100 IU mL⁻¹) and IFN-λ at 40ng mL⁻¹, 100ng mL⁻¹, 250ng mL⁻¹ had a similar effect on HCV suppression. Overall, IFN-α exhibited more suppression of HCV replication than IFN-λ (100 ng mL⁻¹) at 24 hours ($54 \pm 8\%$ vs. $32 \pm 7\%$ IFN-α and IFN-λ respectively, $p < 0.05$) and similar levels of suppression at 72 hours ($69 \pm 4\%$ vs. $85 \pm 8\%$, IFN-α and IFN-λ respectively, $p > 0.05$)

B. Differential Gene Expression Profiles of IFN-α and IFN-λ

DNA microarray analysis was performed using total RNA isolated from HCV infected or non-infected Huh7.5 cells in the presence or absence of IFN-α or IFN-λ as described in the Materials and Methods. Differences in relative levels of gene expression (Z-score) are indicated in color, where red indicates up-regulation and green indicates down-regulation relative to that of corresponding gene expression in controls (HCV uninfected, untreated Huh7.5 cells). Supervised partitioning was used to classify the genes into seven distinct clusters based on differential expression between the six groups. Cluster 1 are genes upregulated more by IFN-λ than IFN-α in infected and non-infected cells. Cluster 2 are genes upregulated more by IFN-λ than IFN-α in non-infected cells. Cluster 3 are genes downregulated more by IFN-α than IFN-λ in infected and non-infected cells. Cluster 4 are genes upregulated equally by IFN-α and IFN-λ in infected and non-infected cells. Finally, Cluster 5 are genes upregulated more by IFN-α than IFN-λ in infected cells.

Figure 2. Validation of select genes differentially induced by IFN-α and IFN-λ

Validation of DNA microarray analysis was performed by qPCR for selected genes as described in the Materials and Methods. Representative data of all validated genes.

Figure A: *MX1* and *OAS1* are upregulated by both IFN-α and IFN-λ (*p<0.05 compared to untreated cells).

Figure B: *IL32* and *CXCL5* are modulated by IFN-α only (*p<0.05 compared to untreated cells).

Figure C: *OASL* and *IFI35* are upregulated by IFN-λ more than by IFN-α. (*p<0.02 and #p<0.05 compared to untreated cells respectively)

Figure 3. Kinetics of differential gene expression by IFN- α and IFN- λ

Differential kinetics of ISG expression in IFN- α or IFN- λ (at one, four and 24 hours) treated Huh7.5 cells were performed using a custom made multiplex qPCR as described in the Materials and Methods. In order to understand the differential kinetic effect of IFN- α and IFN- λ on gene expression profiles we performed multiplex PCR on IFN- α and IFN- λ treated Huh7.5 cells of 24 ISGs at one, four and 24 hours (Figure 3A–C respectively). IFN- λ treatment resulted in a later induction of ISGs (24 hours) of a greater magnitude from baseline (Figure 3D) (* $p < 0.05$), suggesting a differential kinetic effect of ISGs induced by IFN- λ and IFN- α .

Figure 4. Validation of select genes differentially induced by IFN-λ in HCV infected and uninfected cells

Validation of DNA microarray analysis was performed by qPCR for selected genes regulated by IFN-λ in HCV-infected and non-infected Huh7.5 cells as described in the Materials and Methods. Representative data of all validated genes.

A: *IFIT1* and *IFIT3* are upregulated in HCV infected and non-infected cells by IFN-λ (* $p < 0.05$ compared to untreated cells).

B: *ISG20* and *OAS3* are upregulated by IFN-λ more in HCV infected cells than in non-infected cells (* $p < 0.01$ and # $p < 0.05$ compared to untreated cells respectively).

C: *IL18* and *CCL4* are upregulated by IFN-λ in HCV infected cells only (* $p < 0.05$ compared to untreated cells).

Figure 5. Effect of HCV infection on IFN- λ Receptor Expression and Cell Signaling

The cell surface expression of IFN λ -R1 was measured on HCV infected and uninfected Huh7.5 cells as described in the Materials and Methods.

A: HCV infection alone increases the number of cells expressing the IFN- λ receptor ($8 \pm 2.1\%$ HCV uninfected vs. $23 \pm 3.3\%$ HCV infected cells; $*p < 0.001$) as well as IFN- λ receptor density (as measured by mean fluorescence intensity, $(42 \pm 9.8$ vs. 86 ± 12.4 ; $*p < 0.001$), in infected vs. non-infected cells).

B: Comparison of Western blot intensities representing phosphorylated (p) STAT-1 and pSTAT-2 in HCV uninfected cells showed higher levels of pSTAT after 1 and 4 hours of treatment with IFN- α ($p < 0.05$). However, at 24 hours, higher levels were seen in IFN- λ treated cells ($p < 0.05$), indicating a later, more sustained effect of IFN- λ on cell signaling.

C: Comparison of Western blot intensities representing phosphorylated (p) STAT-1 and pSTAT-2 in HCV infected cells showed higher levels of pSTAT after 1 and 4 hours of treatment with IFN- α ($*p < 0.05$). However, at 24 hours, higher levels were seen in IFN- λ treated cells ($*p < 0.05$), again indicating a later, more sustained effect of IFN- λ on cell signaling.

Table 1

Symbol	Gene Name	Function	Modulated by IFN- α vs. IFN- λ	Modulated by IFN- λ in HCV infected vs. non- infected cells
CD46	CD46 molecule, complement regulatory protein	Inhibitory complement receptor	IFN- α	No change
CXCL5	Chemokine (C-X-C motif) ligand 5	Chemoattractant	IFN- α	No change
IL32	Interleukin 32	Proinflammatory cytokine	IFN- α	No change
MT2A	Metallothionein 2A	Protects against oxidative stress	IFN- α	No change
OAS3	2'-5'-oligoadenylate synthetase 3	IFN stimulated gene	IFN- α	Infected > Uninfected Cells
CXCL10	Chemokine (C-X-C motif) ligand 10	Chemoattractant	IFN- λ	Infected > Uninfected Cells
IFI35	IFN-induced protein 35	IFN stimulated gene	IFN- λ	Infected > Uninfected Cells
IFI44	IFN-induced protein 44	IFN stimulated gene	IFN- λ	Infected > Uninfected Cells
OASL	2'-5'-oligoadenylate synthetase-like	IFN stimulated gene	IFN- λ	Infected > Uninfected Cells
STAT2	Signal transducer and activator of transcription 2	IFN stimulated gene	IFN- λ	Infected > Uninfected Cells
IFI44L	IFN-induced protein 44-like	IFN stimulated gene	IFN- λ	Infected=Uninfected
IRF1	IFN regulatory factor 1	IFN stimulated gene	IFN- α and IFN- λ	No change
USP18	Ubiquitin specific peptidase 18	IFN stimulated gene	IFN- α and IFN- λ	No change
EIF2AK2	Eukaryotic translation initiation factor 2- α kinase 2	IFN stimulated gene	IFN- α and IFN- λ	Infected > Uninfected Cells
IFIH1	IFN induced with helicase C domain 1	IFN stimulated gene	IFN- α and IFN- λ	Infected > Uninfected Cells
IFIT2	IFN-induced protein with tetratricopeptide repeats 2	IFN stimulated gene	IFN- α and IFN- λ	Infected > Uninfected Cells
IFIT3	IFN-induced protein with tetratricopeptide repeats 3	IFN stimulated gene	IFN- α and IFN- λ	Infected > Uninfected Cells
ISG20	IFN stimulated exonuclease gene	IFN stimulated gene	IFN- α and IFN- λ	Infected > Uninfected Cells
IFI27	IFN, α -inducible protein 27	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
IFI6	IFN, α -inducible protein 6	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
IFIT1	IFN-induced protein with tetratricopeptide repeats 1	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
IFIT5	IFN-induced protein with tetratricopeptide repeats 5	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
IFITM1	IFN induced transmembrane protein 1	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
IFITM2	IFN induced transmembrane protein 2	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
IFITM3	IFN induced transmembrane protein 3	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
IRF9	IFN regulatory factor 9	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
ISG15	ISG15 ubiquitin-like modifier	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
MX1	Myxovirus (influenza virus) resistance 1	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
PLSCR1	Phospholipid scramblase 1	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected

Symbol	Gene Name	Function	Modulated by IFN- α vs. IFN- λ	Modulated by IFN- λ in HCV infected vs. non- infected cells
SP110	SP110 nuclear body protein	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
STAT1	Signal transducer and activator of transcription 1	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
SP100	SP100 nuclear antigen	IFN stimulated gene	IFN- α and IFN- λ	Infected > Uninfected Cells
OAS1	2',5'-oligoadenylate synthetase	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
OAS2	2'-5'-oligoadenylate synthetase	IFN stimulated gene	IFN- α and IFN- λ	Infected=Uninfected
CCL4	Chemokine (C-C motif) ligand 4	IFN stimulated gene	IFN- α and IFN- λ	Infected only
CD55	CD55 molecule, decay accelerating	Blocks MAC formation	IFN- α and IFN- λ	Infected only
IL18	Interleukin 18 (interferon-gamma)	NK cell stimulating cytokine	IFN- λ only	Infected only
IL8	Interleukin 8	Proinflammatory cytokine	IFN- α and IFN- λ	Infected only