IFN-λ4 desensitizes the response to IFN-α treatment in chronic hepatitis C through long-term induction of USP18

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The recently discovered interferon lambda 4 (IFN-λ4) is a new member of the human type III interferons which could induce a strong antiviral effect through the JAK–STAT cascade. However, hepatitis C virus (HCV) patients who are capable of expressing IFN-λ4 usually have poor response to IFN-α treatment, and the mechanism behind this paradox remains unknown. Here, we reported that IFN-λ4 desensitized IFN-α-stimulated JAK–STAT signalling. Microarray analysis revealed that IFN-λ4 could induce ubiquitin specific peptidase 18 (USP18), a known inhibitor of the type I IFN signalling pathway, in a more sustained pattern compared with type I interferon induction. Moreover, only HCV genotype 1b but not 2a replicon cells pretreated with IFN-λ4 had an attenuated response to type I IFN treatment, which might be due to the different level of USP18 expression. Consistently, knockdown of USP18 in HCV genotype 1b-containing replicon cells reversed the resistance induced by IFN-λ4 and promoted viral clearance. Finally, IFN-λ4 is also strongly associated with the poor response to IFN-α in a Chinese HCV genotype 1b cohort. In conclusion, these data indicate that IFN-λ4 attenuates the response of HCV genotype 1b to IFN-α therapy and inhibits the JAK–STAT signalling pathway by inducing USP18 expression.

INTRODUCTION

Interferons play critical roles in antiviral responses and have been categorized into three major families: type I (IFN-α, IFN-β, etc.), type II (IFN-γ) and type III (Knight, 1975; Maeda et al., 1980; Rubinstein et al., 1981; Pestka et al., 2004). The type III interferon group (also known as IFN-λ) was believed to comprise three members – IL29, IL28a and IL28b – which are designated as IFN-λ1, 2 and 3, respectively. IFN-λs form a heterodimer receptor complex that consists of the unique IL28Ra and IL10Rb (Kotenko et al., 2003; Sheppard et al., 2003). Although both type I and type III interferons induce similar interferon-stimulated genes (ISGs) through the classic JAK–STAT pathway (Marcello et al., 2006; Zhang et al., 2011; Zhou et al., 2007), IFN-λ receptors are only expressed in specific organs or tissues, suggesting that IFN-λs have different immune functions in vivo (de Weerd & Nguyen, 2012; Doyle et al., 2006; Mahlakov et al., 2015; Mordstein et al., 2010; Sommereyns et al., 2008).

IFN-λs are the dominant IFNs secreted by human hepatocytes infected by hepatitis C virus (HCV) (Park et al., 2012; Thomas et al., 2012). More intriguingly, many studies have shown that some single-nucleotide polymorphism (SNP) variants (rs12979860 and rs8099917) on chromosome 19 near the IFN-λ3 gene are strongly associated with spontaneous
viral clearance and the response to pegylated interferon (PEG-IFN) plus ribavirin therapy. Patients carrying favourable alleles usually have an approximately two fold higher rate of sustained virological response (SVR) compared with those carrying unfavourable alleles or heterozygotes (Suppiah et al., 2009). Although the specific molecular basis underlying the SNP function has not yet been elucidated, the association between the genetic variants and their responses to therapy suggests that IFN-αs may play an important role in HCV pathogenesis (Honda et al., 2010).

Recently, Prokunina-Olsson and colleagues found that the dinucleotide variant ss469415590 (TT or ΔG) was strongly associated with HCV treatment outcomes (Prokunina-Olsson et al., 2013). More interestingly, the ΔG genotype causes a frame shift mutation and leads to the expression of a new interferon-like protein IFN-α4. Results from a previous study have demonstrated that the antiviral functions of IFN-α4 are equivalent to those of other IFN-αs (Hamming et al., 2013). However, the mechanism by which IFN-α4 strongly inhibits viral replication but promotes HCV resistance to IFN-α therapy remains a paradox.

In the IFN-α-based therapy, IFN-α induces hundreds of ISGs to inhibit the entry, replication and release of viruses (Schoggins et al., 2011). Some ISGs can provide negative feedback to the JAK–STAT pathway to desensitize IFN-α stimulation (Hong & Carmichael, 2013; Porritt & Hertzog, 2015). Previous studies have shown that IFN-α and IFN-α could suppress the response to IFN-α in human and mouse hepatocytes through inducing the expression of ubiquitin-specific peptidase 18 (USP18), which directly bound to the receptor of IFN-α (Francois-Newton et al., 2011; Makowska et al., 2011). However, it is still unknown whether IFN-α4 inhibits the IFN-α-induced signal pathway through the same mechanism as other IFN-αs. More importantly, if IFN-α4 inhibits refractoriness, is it crucial for clearing HCV in IFN-α-based therapy?

To answer these questions, first, IFN-α4 was purified from Escherichia coli to test its function and examine its down-stream gene expression profile in hepatocytes. Second, the sub-genomic replicon model of HCV was used to explore the potential role of IFN-α4 in response to HCV treatment in vitro. Finally, the responses of different IFN-α4 genotypes to IFN-α treatment were compared in a Chinese population of HCV patients.

RESULTS

IFN-α4 treatment weakens the response to IFN-α treatment by inhibiting JAK–STAT signalling

Those HCV-infected patients who carry the ss469415590 SNP (ΔG allele, IFN-α4), already have a high level of ISG expression before IFN-α stimulation. To mimic this phenomenon and verify the role of IFN-α4, we stimulated Huh7 cells with 100 ng ml⁻¹ IFN-α4 for 72 h before treating the cells with 50 IU IFN-α ml⁻¹.

In the first set of experiments, we investigated whether IFN-α-induced ISG expression in Huh7 cells was down-regulated in response to IFN-α4 stimulation. Four ISGs (RSAD2, IFITM1, Mx1 and 2', 5'-OAS), which can strongly inhibit HCV reception (Schoggins et al., 2011), were detected in cells both in the presence and absence of IFN-α4 pre-treatment. Pre-treatment with IFN-α4 significantly suppressed the peak expression time and fold enhancement of ISGs’ expression, suggesting that the expression levels of antiviral ISGs in IFN-α4 pre-treated cells were significantly less than those in the control cells (Fig. 1a).

To identify the signalling pathway involved in the IFN-α4 desensitization mechanism in response to IFN-α treatment, Huh7 cells were stimulated with 100 ng IFN-α4 ml⁻¹ for 72 h, and then the cells were treated with 50 IU IFN-α ml⁻¹. The results showed that in the cells that were not pre-treated, IFN-α was able to strongly induce STAT1 and STAT2 phosphorylation, which is critical for IFN-α-mediated anti-HCV activity. However, in the cells that were pre-treated with IFN-α4, IFN-α-induced phosphorylation of STAT1 and STAT2 was significantly suppressed (Fig. 1b, c). This result suggests that IFN-α-stimulated JAK–STAT signalling is inhibited by pre-treatment with IFN-α4.

IFN-α4 induces long-term USP18 expression

To better understand the mechanism by which IFN-α4 induces a refractory state in hepatocytes, an mRNA microarray analysis was performed using Huh7 cells that had been treated with IFN-α4, IFN-α1 and IFN-α. To ensure that the expression levels of the different interferons were similar, the amounts of IFN-α (10 IU ml⁻¹), IFN-α1 (20 ng ml⁻¹) and IFN-α4 (20 ng ml⁻¹) were chosen based on previous reports (Marcello et al., 2006) and preliminary tests. Time points of 6 h and 16 h after stimulation were selected because they represent the peak times of type I- and type III-stimulated ISG induction, respectively. In general, IFN-α4 significantly stimulated the expression of a variety of typical ISGs. At 6 h or 16 h post-stimulation, the numbers of upregulated genes (more than twofold) that were stimulated by IFN-α4 were similar to those in the IFN-α1 treatment group but less than those in the group that was stimulated with IFN-α (Fig. 2a). These results confirmed previous observations that IFN-α4 is a novel member of the type III interferon family (Hamming et al., 2013; Prokunina-Olsson et al., 2013).

Ubiquitin specific peptidase 18 (USP18), also known as ubiquitin-specific protease 43 (UBP43), provides strong negative feedback in the activation of the JAK–STAT pathway by binding to IFNAR2 (Sarasin-Filipowicz et al., 2009). USP18 has been reported to be responsible for the persistent IFN-induced refractory mechanism that occurs following JAK–STAT signalling in mouse and human hepatocytes (Francois-Newton et al., 2011; Sarasin-Filipowicz et al., 2009). The microarray data showed that IFN-α4 induced strong USP18 expression (Fig. 2b). This finding indicated...
Fig. 1. IFN-λ4 induces refractoriness of the IFN-α-stimulated JAK–STAT signalling pathway. (a) Huh7 cells were left untreated (empty circles) or were pre-treated (filled squares) with 100 ng ml$^{-1}$ λ4 for 72 h and then treated with 50 IU ml$^{-1}$ IFN-α every 4 h. Changes in ISG expression were determined by RT-PCR at the indicated time points and normalized to the untreated sample at time point 0. All changes were compared between untreated and pre-treated cells. Results represent the mean values of three biological replicates. Error bars, SD. (**P<0.01; ***P<0.001; ****P<0.0001). (b) Huh7 cells were either left untreated or primed with λ4 (100 ng ml$^{-1}$). After 72 h of stimulation, the cells were re-stimulated for 30 min with IFN-α (50 IU ml$^{-1}$). Cell lysates were analysed using the indicated antibodies. (c) Quantitative analysis of STAT1 and STAT2 phosphorylation. The phosphorylation levels of STAT1 and STAT2 were determined in three experiments and normalized to the levels of β-actin in the cell lysates. Results represent the mean values of three biological replicates. Error bars, SD. (**P<0.01; ***P<0.001).
that USP18 might play an important role in the IFN-λ4-induced refractory state in liver cells. However, in addition to USP18, suppressor of cytokine signalling 1 (SOCS1), SOCS3 and other well-characterized ISGs are also known to negatively regulate IFN-α signalling. Next, the microarray data were verified by real-time PCR, and among all of the well-known negative-feedback ISGs, only USP18 was significantly induced by IFN-λ4 (Fig. 2c).

One of the important signatures of type III interferons is their temporal expression pattern, which is distinctly different from that of type I interferons (Bolen et al., 2014). To better understand time-dependent ISG profiles, the 296 genes that were significantly upregulated (more than twofold) by IFN-λ4 at one or more time points were clustered. These genes were then ranked based on their fold-changes in gene expression between 6 h and 16 h in cells that were treated with IFN-λ4. The genes could be classified into three groups, each representing a distinct temporal pattern of gene expression (Fig. 3a).

The expression patterns of certain representative ISGs were validated in Huh7 cells by real-time (RT)-PCR (Fig. 3b). The genes that were classified into group I were rarely upregulated by IFN-λ4 during the first 6 h, but the degree of upregulation significantly increased after 16 h of stimulation. Group II genes, to which USP18 belongs, were activated immediately following exposure to IFN-α and then returned to baseline

**Fig. 2.** USP18 expression is stimulated by IFN-λ4. (a) Venn diagram of significantly upregulated genes (more than twofold) at 6 h or 16 h after treatment with IFN-α, λ1 or λ4. (b) Box and dot plots of genes’ expression levels that were induced by λ4. (c) Real-time RT-PCR validation of USP18 expression in Huh7 cells. The concentrations of IFN-α, λ1 and λ4 were 10 IU ml⁻¹, 20 ng ml⁻¹ and 20 ng ml⁻¹, respectively. Results represent the mean values of three biological replicates. Error bars, SD, (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).
levels after 6 h. However, in contrast to the group I genes, group II genes exhibited a slower but more sustained response after IFN-\(\lambda_4\) and IFN-\(\lambda_1\) stimulation. The genes that were classified into group III were activated and returned to baseline levels immediately. These data show that when hepatocytes are exposed to IFN-\(\lambda_4\) a sustained dynamic expression of USP18 occurs that is associated with a refractory state in response to IFN-\(\alpha\) treatment.

**IFN-\(\lambda_4\) induced USP18 expression enhanced tolerance in response to IFN-\(\alpha\) treatment for HCV genotype 1b**

To mimic the changes in the viruses that occur in patients carrying an unfavourable allele, Huh7 cells containing the CON1 (HCV genotype 1b) and JFH1 (HCV genotype 2a) sub-genomic replicons were pre-treated with IFN-\(\lambda_4\) for 72 h and then with IFN-\(\alpha\) every 4 h (Fig. 4a). HCV RNA levels were measured during the entire treatment period. During the first 72 h, the expression of both CON1 and JFH1 viral RNAs decreased considerably after the cells were treated with IFN-\(\lambda_4\). However, when the cells were stimulated again with IFN-\(\alpha\), only CON1, but not JFH1, exhibited a weak response (Fig. 4b). The change of non-structural protein 3 (NS3) in those sub-genomic replicons cells also showed similar results (Fig. 4c). To better understand the changes in viral content after IFN-\(\alpha\) treatment, untreated sub-genomic replicons were compared with those that were pre-treated with IFN-\(\lambda_4\). After IFN-\(\alpha\) treatment, the level of viral RNA in the CON1 replicon that was pre-treated with IFN-\(\lambda_4\) did not decrease between 8 and 24 h, in contrast to the control group (Fig. 4d). However, under the same conditions, no differences was observed in the JFH1 replicon between the IFN-\(\lambda_4\) pre-treated sub-genomic replicons and the control group, and both groups responded favourably to IFN-\(\alpha\) treatment (Fig. 4e).

The observed differences in HCV RNA decay rates between the HCV genotype 2a and 1b replicons suggest that these two HCV genotypes have different responses to IFN-\(\alpha\)
Fig. 4. IFN-λ4 induced USP18 expression enhanced tolerance in response to IFN-α treatment for HCV genotype 1b. (a) Diagram showing the experimental design for IFN-λ4 pre-treatment and IFN-α re-treatment in Huh7 cells with the JFH1 (genotype 2a) and CON1 (genotype 1b) sub-genomic replicons. (b) and (c) Changes in the viral RNA (b) and NS3 (c) were determined...
the indicated time points. HCV RNA was normalized to time point 0. Error bars, SD. (d) and (e) Cells were pre-treated or not with 100 ng ml⁻¹ λ4 for 72 h and then re-treated with 50 IU ml⁻¹ IFN-α every 4 h. Changes in the viral RNA were determined by RT-PCR at the indicated time points and normalized to time point 0. All results represent the mean values of three biological replicates. Error bars, SD (P<0.05; **P<0.01). (f) HCV genotype 1b and 2a sub-genomic replicon cells were pre-treated with or without 100 ng ml⁻¹ λ4 for 72 h and then treated with 50 IU ml⁻¹ IFN-α every 4 h. Cell lysates were analysed using the USP18, MX1 and β-actin antibodies. (g) HCV genotype 1b and 2a sub-genomic replicon cells were treated with 5 nM Daclatasvir (BMS-790052) for 1 week. Then viral RNA and NS3 were determined to make sure virus were cleared. (h) Huh7 cells and HCV cleared sub-genomic replicon cells were pre-treated with or without 100 ng ml⁻¹ λ4 for 72 h and then treated with 50 IU ml⁻¹ IFN-α every 4 h. Cell lysates were analysed using the USP18, MX1 and β-actin antibodies.

The viral clearance ability of IFN-α is reduced when the JAK–STAT signalling pathway is blocked by IFN-α in HCV CON1 sub-genomic replicon cells. To determine whether the antiviral activity of IFN-α would be reversed after silencing USP18 expression, USP18 expression was knocked down in HCV CON1 sub-genomic replicon cells that were stimulated with IFN-α. Interestingly, USP18-silenced replicon cells almost completely recovered their sensitivity to IFN-α treatment and exhibited a rapid decrease in the HCV RNA content compared with Si-NC replicon cells (Fig. 5e). These results indicate that the knockdown of USP18 can reverse IFN-α-induced refractoriness and promote viral clearance.

IFN-α is associated with a slower decrease in the viral titre in HCV genotype 1b-infected patients

To verify that IFN-α induced tolerance in response to IFN-α treatment is HCV genotype-specific, 154 Chinese HCV patients were recruited and received standard IFN-α plus ribavirin therapy for 48 weeks. Ss469415590 was 100 % disequilibrium-linked with rs12979860 in all of the patients (data not shown). Due to the limited number of available patients, a homozygous carrier of the ΔG allele was not identified. In the early phase of viral kinetics, the rapid virological response (RVR) rate was significantly different between TT and TT/ΔG patients who were infected with HCV genotype 1b (Table 1). This result is consistent with the rs12979860 predicted viral clearance in HCV genotype 1b-infected patients (Ge et al., 2009). Moreover, TT patients, particularly the genotype 1b-infected patients, had a more dramatic viral decline compared with the TT/ΔG patients (Fig. 6a). The patients who were infected with HCV genotype 2a exhibited a smaller difference between these two SNP genotypes (Fig. 6b). Several previous reports have also presented similar results (Kawaoka et al., 2011; Sarrazin et al., 2011). These data indicate that IFN-α is associated with a slower decrease in the viral titre in HCV genotype 1b-infected patients.

DISCUSSION

Two previous studies have reported that the dinucleotide variant ss469415590(ΔG) can induce the expression of the novel protein IFN-α4, which exhibits interferon-like activity by binding to type III interferon receptors (Hamming et al., 2013; Prokunina-Olsson et al., 2013). The present study provides a more comprehensive analysis of the role of IFN-α4 in HCV-infected patients. First, IFN-α4 induced refractoriness in the IFN-α-stimulated JAK–STAT signalling pathway in hepatocytes by upregulating USP18 expression. Second, refractoriness induced by IFN-α4 is crucial for the
clearance of genotype 1b HCV in IFN-α based therapy. To our knowledge, for the first time these data reveal the role of IFN-α4 in the poor response to IFN-α therapy.

Given that type I and type III IFNs are generally considered to be antiviral cytokines, it is interesting that IFN-α4 is associated with a poor response to type I IFN treatment in patients with HCV. In the present study, we investigated the role of IFN-α4 in HCV-infected Chinese patients. Additionally, to our knowledge, this was the first study to use an HCV sub-genomic replicon model to verify the effects of IFN-α4 in an in vitro system. However, more interestingly, IFN-α4 does not affect the IFN-α response in HCV genotype 2a replicons because of the different expression levels of USP18. This finding indicates that the viruses themselves

Fig. 5. USP18 knockdown reverses IFN-α4-induced refractoriness and promotes viral clearance. (a) HCV genotype 1b sub-genomic replicon cells were transfected with USP18-targeting small interfering RNA (siRNA) (Si-USP18). Twenty-four hours after transfection, the cells were either left untreated or primed with α4 (100 ng ml⁻¹). After 72 h of stimulation, the cells were re-stimulated for 30 min with IFN-α (50 IU ml⁻¹). Cell lysates were analysed using the USP18 antibody. (b) Quantitative analysis of USP18 level in knockdown cells. The levels of USP18 were determined in three experiments and normalized to the levels of β-actin in the cell lysates. Results represent the mean values of three biological replicates. Error bars, SD. (c) Lysates from USP18 knockdown cells were analysed using p-STAT1, STAT1, p-STAT2 and STAT2 antibodies. (d) Quantitative analysis of p-STAT1 and p-STAT2 levels in USP18 knockdown cells. (e) After knockdown of USP18 expression, CON1 sub-genomic replicons were pre-treated with 100 ng ml⁻¹β4 for 72 h and then treated with 50 IU ml⁻¹ IFN-α every 4 h. Changes in the viral RNA were determined by RT-PCR at the indicated time points and normalized to untreated cells. All changes were compared between Si-NC and Si-USP18 cells. Results represent the mean values of three biological replicates. Error bars, so, (**P<0.01; ***P<0.001; ****P<0.0001).
may also contribute to the different responses to IFN-α treatment. Unfortunately, we cannot get enough liver biopsy samples from different genotypes of HCV-infected patients to confirm this phenomenon. Thus, it will be intriguing to confirm this phenomenon in vivo and elucidate the mechanisms by which different HCV genotypes lead to different expression levels of USP18 and IFN-α responses in future investigations.

Results from many previous studies have indicated that there is an association between IL28B SNPs and high pre-treatment levels of hepatic ISG (Honda et al., 2010; Lau et al., 2013; Sarasin-Filipowicz et al., 2008). In this scenario, the presence of IFN-λ4 may result in the persistent stimulation of ISGs, including many negative regulators in the IFN pathway, ultimately leading to a poor response to type I interferon therapy. However, our data indicated that IFN-λ4 induced similar ISGs to IFN-λ1 and has analogous capabilities to induce tolerance in response to IFN-α treatment for HCV genotype 1b (Fig. S1, available in the online Supplementary Material). That implies that, besides IFN-λ4, other IFN-λs also play a role in the poor response to type I interferon therapy. Results from some previous studies indicated that both IFN-λ1 and IFN-λ4 can be induced in hepatocytes by HCV infection in vitro (Marukian et al., 2011; Prokunina-Olsson et al., 2013) and in vivo (Amanzada et al., 2013; Park et al., 2012; Thomas et al., 2012), but which one plays the key role is still unknown. To resolve this question, it is necessary to confirm which kind of IFN-λ has a significant difference in liver and serum between those patients who do or do not respond to type I interferon therapy.

Microarray data from Huh7 cells that were stimulated with IFN-λ4 versus IFN-α and IFN-λ1 revealed that among all of the well-known negative regulators of the IFN-α pathway, only USP18 was significantly upregulated. Additionally, USP18 expression, which is induced by IFN-α, weakened the response to IFN-α treatment in a mouse model (Sarasin-Filipowicz et al., 2009). Thus, knockdown of USP18 reversed the IFN-λ4-induced refractoriness of the JAK–STAT signalling pathway and the promoted viral clearance. These results suggest that knockdown of USP18 before IFN-α therapy in non-responsive patients may be a new strategy for interferon-based treatment.

In conclusion, the IFN-λ4-induced expression of USP18 strongly impaired viral clearance in HCV genotype 1b but not 2a infections. This finding indicates that in HCV-

Table 1. Genotypes, viral kinetics and treatment outcomes

<table>
<thead>
<tr>
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<th>All (n=154)</th>
<th>HCV Gen-1b</th>
<th>HCV Gen-2a</th>
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<tr>
<td></td>
<td>(TT/TT (n=81)</td>
<td>TT/AG (n=15)</td>
<td>P value</td>
</tr>
<tr>
<td>Male:female, (n)</td>
<td>119:35</td>
<td>63:18</td>
<td>13:2</td>
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<tr>
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<td>49.5±7.3</td>
<td>50.4±6.9</td>
<td>49.7±10.6</td>
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<td>Body mass index (BMI) (kg m⁻²)</td>
<td>24.3±3.3</td>
<td>24.4±3.6</td>
<td>24.5±2.5</td>
</tr>
<tr>
<td>0 Week HCV RNA (log10 IU ml⁻¹)</td>
<td>6.05±0.78</td>
<td>6.13±0.91</td>
<td>6.31±0.48</td>
</tr>
<tr>
<td>HCV decline from baseline at week 4 (log10 IU ml⁻¹)</td>
<td>4.24±1.80</td>
<td>3.91±1.51</td>
<td>1.28±0.76</td>
</tr>
<tr>
<td>RVR: non-RVR, (n)</td>
<td>78 (51 %): 76 (49 %)</td>
<td>27:54</td>
<td>0:15</td>
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Fig. 6. IFN-λ4 is associated with a slower decrease in viral titre in patients with HCV genotype 1 infection after IFN-α therapy. HCV RNA levels [log10 international units (IU) ml⁻¹] in patients treated with IFN-α and ribavirin were determined at the indicated time points. (a) Patients infected with HCV genotype 1b (TT group, n=81; TT/AG group, n=15). Error bars, SEM. (b) Patients infected with HCV genotype 2a (TT group, n=48; TT/AG group, n=10). Error bars, SEM.
infected patients, host and viral factors function together to determine the outcome of the treatment. Elucidation of the mechanism underlying this phenomenon could provide new insights into interferon-based treatment of viral infection.

**METHODS**

**Patient population.** This study was approved by the ethics committee of the First Hospital of Jilin University and was registered with ClinicalTrials.gov (ID : NCT01760148). Written informed consent was obtained from all participants. The subjects consisted of 154 treatment-naïve patients with chronic HCV infection and baseline HCV RNA levels > 10 000 IU ml⁻¹ (Table 1). All subjects tested negative for HBsAg, HBeAb and HIV Ab, and none of the subjects had serological or histological evidence of liver disease other than HCV. Demographic data, including age, gender and body mass index (BMI), were collected. Patients were treated with unmodified IFN plus ribavirin for 48 weeks. Throughout the treatment period, five million units of IFN-α2b were administered subcutaneously every other day at the clinic. Ribavirin was administered at a concentration of 15 mg · kg⁻¹ · day⁻¹ in two divided doses. Serum HCV RNA levels were measured using the COBAS AmpliPrep/COBAS TaqMan assay (Roche Molecular Diagnostics) with a lower quantitation limit of 15 IU ml⁻¹.

**IL28B genotyping.** DNA was isolated from blood samples using a QIaamp DNA mini kit (Qiagen) and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The rs12979860 and rs469415590 SNPs were measured concomitantly by sequencing the PCR-amplified 715-base-pair fragment using the following primers: forward, 5'-GGGCTTATCCGATAAGGCTA-3'; reverse, 5'-TCCCCACTC TCTCCTCCACTG-3'.

**Cell culture and transfection.** Huh7 cells were purchased from ATCC and cultured in Dulbecco’s modified Eagle's medium (DMEM) containing 10% FBS (Gibco) at 37°C in a CO₂ incubator. JFH1 subgenomic replicon cells were constructed by transfecting G418-selectable sub-genomic transcripts into Huh7 cells (He et al., 2012; Kato et al., 2003). CON1 sub-genomic replicon cells were a kindly gift from Professor Ralf Bartenschlager (Krieger et al., 2001). Lipofectamine LTX reagent (Invitrogen) was used for small interfering RNA (siRNA) transfection following the manufacturer’s protocol.

**Reagents.** Recombinant human IFN-α-2b and IL29 were purchased from ebioscience. Anti-phospho-STAT1 (Tyr701), phospho-STAT2 (Tyr689), STAT1, STAT2 and USP18 antibodies were obtained from Cell Signalling. HCV NS3 antibody (8G-2) was obtained from Abcam. Anti-β-actin (clone AC-40) antibodies were purchased from Sigma. siRNAs for USP18 were ordered from GenePharma, Shanghai. Daclatasvir (BMS-790052) was purchased from Selleck.

**Recombinant protein expression, purification and refolding.** IFN-α4 and A1 expression cDNAs were synthesized by recursive PCR based on sequences available in the NCBI database. IFN-α4 cDNA with the first 24 aa truncated at the N terminus was cloned into pET30a(+), which contains a 6xHis tag and an enterokinase protease cleavage site at the N terminus. Rosetta (DE3) E. coli cells were transformed with the plasmids and grown at 37°C in Luria–Bertani medium containing 100 µg ampicillin ml⁻¹ and 30 µg chloramphenicol ml⁻¹ with continuous shaking until an OD₆₀₀ of 0.6–0.8 was achieved. Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside, and the cultures were incubated for another 6 h at 37°C. IFN-α4 was refolded and purified as previously described (Dellgren et al., 2009; Hamming et al., 2013).

**Microarray and mRNA expression analysis.** Huh7 cells were seeded into six-well plates at a density of 6×10⁵ cells per well and cultured overnight before treatment with either 10 U IFN-α1 ml⁻¹ or 20 ng IL29 or IFN-α4 ml⁻¹. For the microarray analysis, RNA was isolated from Huh7 cells using TRIzol Reagent (Invitrogen) at 0, 6 and 16 h. The purified RNA (1 µg) was quantified and reverse-transcribed using the ReverTraAce qPCR RT Kit (TOYOBO). The aminolytically-RNA (arRNA) probes labelled with NHS-Cy5 (Amersham) were hybridized at 50°C for 16 h to the Human Whole Genome OneArray Version 4.3 (Phalanx-Biotek Group), scanned with an Axon 4000B Scanner (Molecular Devices) and analysed with GenePix software (Molecular Devices). Array data are available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE83085. The expression levels of the mRNA transcripts were calculated relative to the expression of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) using the formula 2⁻ΔΔCt. The primers used in the experiments are listed in Table S1.

**Statistical analysis.** Student’s t-test was used to compare the means of three biological replicates of each experimental group in bar and curve graphs. Error bars indicate SD.

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