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Abstract
Hepatitis C virus (HCV) infections are the major cause of chronic liver disease, cirrhosis and hepatocellular carcinoma worldwide. Both spontaneous and treatment-induced clearance of HCV depend on genetic variation within the interferon-lambda locus, but until now no clear causal relationship has been established. Here we demonstrate that an amino-acid substitution in the IFNλ4 protein changing a proline at position 70 to a serine (P70S) substantially alters its antiviral activity. Patients harbouring the impaired IFNλ4-S70 variant display lower interferon-stimulated gene (ISG) expression levels, better treatment response rates and better spontaneous clearance rates, compared with patients coding for the fully active IFNλ4-P70 variant. Altogether, these data provide evidence supporting a role for the active IFNλ4 protein as the driver of high hepatic ISG expression as well as the cause of poor HCV clearance.

Reference

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Reduced IFNλ4 activity is associated with improved HCV clearance and reduced expression of interferon-stimulated genes

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Hepatitis C virus (HCV) infections are the major cause of chronic liver disease, cirrhosis and hepatocellular carcinoma worldwide. Both spontaneous and treatment-induced clearance of HCV depend on genetic variation within the interferon-lambda locus, but until now no clear causal relationship has been established. Here we demonstrate that an amino-acid substitution in the IFNλ4 protein changing a proline at position 70 to a serine (P70S) substantially alters its antiviral activity. Patients harbouring the impaired IFNλ4-S70 variant display lower interferon-stimulated gene (ISG) expression levels, better treatment response rates and better spontaneous clearance rates, compared with patients coding for the fully active IFNλ4-P70 variant. Altogether, these data provide evidence supporting a role for the active IFNλ4 protein as the driver of high hepatic ISG expression as well as the cause of poor HCV clearance.
We expressed the mature form of both variants in *Escherichia coli*, purified them (Fig. 1a) and tested their activity. The IFN4-A S70 variant induced significantly lower levels of both MX1 and IFIT1 transcription in human hepatocellular carcinoma cells (HepG2; Fig. 1b). Next, a dose–response experiment was performed in human embryonic kidney cells (HEK293) transfected with an IFN-responsive firefly luciferase reporter construct, a *Renilla* luciferase construct for normalization and a construct expressing the IFNLR1 receptor chain (Fig. 1c). We obtained half-maximal effective concentration (EC50) values of 4.0 ng ml−1 for IFN4-A-P70 and 9.5 ng ml−1 for IFN4-A-S70. This difference was significant using 99% confidence intervals (Supplementary Table 1). Finally, we performed an antiviral assay using *encephalomyocarditis virus* (EMCV) infection in HepG2 cells (Fig. 1d; Supplementary Table 1): Here we saw a significant shift in the (EC50) values from 0.2 ng ml−1 for IFN4-A-P70 to 1.4 ng ml−1 for IFN4-A-S70. Thus, the IFN4-A-S70 variant is substantially less active than the IFN4-A-P70 variant.

*rs117648444* is an independent predictor of HCV clearance. If the active IFN4-A protein is the causative agent responsible for the poor HCV clearance in patients, then one would expect the P70S substitution (*rs117648444*) to influence the ability of patients to clear HCV. Thus, we evaluated the role of the *rs117648444* (IFN4-A-S70, MAF = 0.11) and *rs368234815*G (expressing IFN4-A, MAF = 0.35) polymorphisms in HCV clearance in a cohort of 574 chronically infected patients who underwent pegIFNα/ribavirin treatment, as well as in 122 patients with spontaneous clearance (Table 1; Supplementary Tables 2 and 3). We analysed the independent contribution of each polymorphism, by including both of them in the same logistic regression model. As expected, *rs368234815*G was associated with a lower SVR rate (odds ratio (OR) = 0.23, 95% confidence interval (CI) 0.15–0.35, P = 1.5E-11, dominant mode of inheritance), while *rs117648444*G (IFN4-A-S70) was associated with a higher SVR rate (OR = 1.66, 95% CI 1.08–2.55, P = 0.02). Thus, both *rs117648444*G and *rs368234815*G are independent predictors of treatment-induced HCV clearance. A similar trend was observed for spontaneous clearance (OR = 0.21, 95% CI 0.13–0.34, P = 4.4E-10 for *rs368234815*G, OR = 1.63, 95% CI 0.88–3.02, P = 0.12 for *rs117648444*G; Table 1).

**Stratification of patients according to IFN4-A functionality.**

A haplotype analysis was performed to understand the co-inheritance pattern of *rs368234815* and *rs117648444*, which would help to clarify the role of the activity of IFN4-A in HCV clearance. This analysis showed that 95% of the chromosomes are composed of three main haplotypes: TT G (65%), which is predicted not to produce IFN4-A, AG G (22%), which is predicted to produce the IFN4-A-P70 variant, and AG A, which is predicted to express the IFN4-A-S70 variant (11%; Supplementary Table 4). The frequency distribution in our study population is in accordance with previously published data.15,18 The fourth haplotype TT A, which does not express IFN4-A, was found in only 0.5% of the chromosomes, and is grouped with the TT G haplotype in the subsequent analyses. The AG G haplotype (IFN4-A-P70, R = 0.74) was in linkage disequilibrium (LD) with the SNP rs8099917, which emerged as the top hit for spontaneous or treatment-induced HCV clearance in three out of four initial hepatitis C genome-wide association studies (GWAS; Table 2).

The haplotypic combinations produced eight diplotypes (Table 3), which can be classified into three main functional groups according to their ability to produce IFN4-A-P70 or IFN4-A-S70. The first group consists of patients not producing IFN4-A (N = 283). The second group contains diplotypes that
exclusively code for IFNλ4-S70 (N = 114). The third group consists of those that give rise to the fully functional IFNλ4-P70 (N = 299). This group is composed of 270 samples with the TT G/ΔG G and the ΔG G/ΔG G diplotype producing only the IFNλ4-P70 variant, and 29 samples with the ΔG A/ΔG G diplotype producing both variants of IFNλ4.

IFNλ4-S70 associates with a better clearance rate of HCV. We analysed the response to treatment in patients producing IFNλ4-S70 alone and those producing IFNλ4-P70 alone grouped with IFNλ4-P70/S70 compared with those not producing IFNλ4. Patients not producing IFNλ4 had an overall SVR rate of 81% after treatment with pegIFNα2b/ribavirin compared with only 47% among those who produced the IFNλ4-P70 or P70/S70 variants (OR = 0.21, 95% CI 0.14–0.33, P = 8.3E–13; Table 1; Fig. 2a). We then excluded patients not producing IFNλ4 to specifically assess the role of the P70S substitution. Interestingly, patients who produced exclusively the IFNλ4-S70 had a much higher response rate (69%) compared with those producing the IFNλ4-P70 or P70/S70 variants (OR = 2.52, 95% CI 1.55–4.13, P = 0.0002). This clearly shows that the chance of clearing the virus is highest in the group of patients not expressing IFNλ4, slightly lower in those expressing the impaired IFNλ4-S70 variant and the worst in those expressing the fully active IFNλ4-P70 form. The differences in SVR rates between all three groups were significant. This agrees well with the biochemical data, which show that IFNλ4-S70 has an impaired but still clearly measurable activity. These associations were still significant in a multivariate model after adjustment for relevant covariates (Table 1). The association was similar, although less significant, when the analysis was restricted to patients infected with HCV genotype 1 (Supplementary Table 5).

Roughly, a quarter of all HCV-infected individuals spontaneously clear the HCV infection, mainly during the acute phase of the infection by mounting an efficient immune response to the virus21. We investigated whether the level of IFNλ4 activity was associated with the spontaneous clearance. Similarly to what was observed for the treatment-induced clearance, the proportion of patients with spontaneous clearance was 29% among patients unable to produce IFNλ4, 7% among those producing the IFNλ4-P70 or P70/S70 variants and 15% among those producing exclusively IFNλ4-S70 (Table 1; Fig. 2b).

IFNλ4-P70 is associated with high ISG expression. We have previously shown that the quantification of the expression of four ISGs (IFI27, ISG15, RSAD2 and HTATIP2) in liver biopsy samples allows one to predict the response to pegIFNα2b/ribavirin treatment10. This 4-gene classifier was obtained through a multivariate analysis using decision tree ensembles in the form of a random forest classifier and is expressed as the probability of obtaining a sustained virological response (pSVR). The pSVR value contains the collective information from the four genes measured and is inversely correlated with ISG expression in the liver10. In the present work, the 4-gene classifier was used as a surrogate marker for the overall ISG expression in 104 liver biopsies from patients with chronic hepatitis C. The biopsies were then genotyped for the IFNλ4 variants and classified into the three groups corresponding to the genomic analysis described above. In an analysis including all viral genotypes, the median...
Discussion

IFN-β was restricted to patients infected with HCV genotype 1 (Fig. 3b). The results did not change when the analysis groups 1 and 3, but not between 1 and 2, was statistically significant (Fig. 3a). Therefore, prolonged IFN-β signalling caused by the IFNL4 protein is obviously inhibitory to subsequent treatment with pegIFNα/ribavirin. Interestingly, previous work has observed an association between both SNP rs12979860 (ref. 14) (situated in the IFNL4 intronic region) and rs368234815 (ref. 13) (the ΔG allele determining IFNL4 expression) and the rate of spontaneous clearance of HCV. We observed a clear correlation between the activity of the IFNL4 protein and the hepatic ISG expression level.

Discussion

Because IFN-β, but not the functionally defective IFNL4-A-S70, is associated with a strong induction of ISG expression (and therefore a low pSVR), we conclude that IFNL4 is the major component of the molecular mechanisms responsible for hepatic ISG expression in patients with HCV infections. Sustained signalling through the IFNL4 receptor can inhibit subsequent responses to type I IFN through the induction of the USP18 protein. Therefore, prolonged IFNL4 signalling caused by the IFNL4 protein is obviously inhibitory to subsequent treatment with pegIFNα/ribavirin. Interestingly, previous work has observed an association between both SNP rs12979860 (ref. 14) (situated in the IFNL4 intronic region) and rs368234815 (ref. 13) (the ΔG allele determining IFNL4 expression) and the rate of spontaneous clearance of HCV. We observed a clear correlation between the activity of IFNL4 and the spontaneous clearance rate. Patients producing the impaired IFNL4-A-S70 had a 15% chance of spontaneous clearance compared with a 7% clearance rate among

Table 1 | Association between IFNL4 rs368234815 and rs117648444 polymorphisms and spontaneous and treatment-induced HCV clearance.

<table>
<thead>
<tr>
<th>Response to treatment (N = 574)</th>
<th>NR</th>
<th>SVR</th>
<th>Proportion SVR</th>
<th>Univariate models</th>
<th>Multivariate models*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual contribution of both polymorphisms</td>
<td></td>
<td></td>
<td></td>
<td>0.23 (0.15–0.35)</td>
<td>1.5E – 11</td>
</tr>
<tr>
<td>rs368234815 TT/G or -G/G versus TT/TT</td>
<td>39</td>
<td>161</td>
<td>0.81</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>rs1176484444 G/A or A/A versus G/G</td>
<td>147</td>
<td>130</td>
<td>0.47</td>
<td>1.66 (1.08–2.55)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

| Contribution of IFNL4-A-S70 alone and IFNL4-P70 alone grouped with IFNL4-A-P70/S70 compared with that of no IFNL4 |  |  |  | 0.16 (0.09–0.28) | 3.2E – 10 |
| No IFNL4 | 39 | 161 | 0.81 | Reference | Reference |
| IFNL4-A-S70 alone | 30 | 67 | 0.69 | 0.54 (0.31–0.94) | 0.03 |
| IFNL4-P70 and P70/S70 | 147 | 130 | 0.47 | 0.21 (0.14–0.33) | 8.3E – 13 |

| Contribution of IFNL4-A-S70 alone compared with that of IFNL4-A-P70/S70 after exclusion of no IFNL4 |  |  |  | 0.61 (0.29–1.28) | 0.19 |
| IFNL4-A-S70 alone | 30 | 67 | 0.69 | 2.52 (1.55–4.13) | 0.0002 |
| IFNL4-P70 and P70/S70 | 147 | 130 | 0.47 | Reference | Reference |

<table>
<thead>
<tr>
<th>Spontaneous clearance (N = 696)</th>
<th>CHI</th>
<th>SC</th>
<th>Proportion clearance</th>
<th>Univariate models</th>
<th>Multivariate models*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual contribution of both polymorphisms</td>
<td></td>
<td></td>
<td></td>
<td>0.21 (0.13–0.34)</td>
<td>4.4E – 10</td>
</tr>
<tr>
<td>rs368234815 TT/G or -G/G versus TT/TT</td>
<td>200</td>
<td>83</td>
<td>0.29</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>rs1176484444 G/A or A/A versus G/G</td>
<td>277</td>
<td>22</td>
<td>0.07</td>
<td>1.63 (0.88–3.02)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

| Contribution of IFNL4-A-S70 alone and IFNL4-P70 alone grouped with IFNL4-A-P70/S70 compared with that of no IFNL4 |  |  |  | 0.20 (0.12–0.33) | 2.4E – 10 |
| No IFNL4 | 200 | 83 | 0.29 | Reference | Reference |
| IFNL4-A-S70 alone | 97 | 17 | 0.15 | 0.42 (0.24–0.75) | 0.003 |
| IFNL4-P70 and P70/S70 | 277 | 22 | 0.07 | 0.19 (0.12–0.32) | 1.3E – 10 |

| Contribution of IFNL4-A-S70 alone compared with that of IFNL4-A-P70/S70 after exclusion of no IFNL4 |  |  |  | 0.41 (0.23–0.74) | 0.003 |
| IFNL4-A-S70 alone | 97 | 17 | 0.15 | 2.21 (1.12–4.33) | 0.02 |
| IFNL4-P70 and P70/S70 | 277 | 22 | 0.07 | Reference | Reference |

Table 2 | Linkage disequilibrium between different IFNL4 polymorphisms.

<table>
<thead>
<tr>
<th>rs8099917</th>
<th>rs12979860</th>
<th>rs368234815</th>
<th>rs1176484444</th>
<th>TT G (no IFNL4)</th>
<th>ΔG G (IFNL4-A4-P70)</th>
<th>ΔG A (IFNL4-A4-S70)</th>
<th>TT A (no IFNL4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs8099917</td>
<td>0.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs12979860</td>
<td>0.45</td>
<td>0.37</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs368234815</td>
<td>0.44</td>
<td>0.92</td>
<td>0.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1176484444</td>
<td>0.03</td>
<td>0.21</td>
<td>0.19</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT G (no IFNL4)</td>
<td>0.43</td>
<td>0.93</td>
<td>0.98</td>
<td>0.23</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔG G (IFNL4-A4-P70)</td>
<td>0.74</td>
<td>0.53</td>
<td>0.60</td>
<td>0.04</td>
<td>0.59</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>ΔG A (IFNL4-A4-S70)</td>
<td>0.03</td>
<td>0.21</td>
<td>0.22</td>
<td>0.95</td>
<td>0.22</td>
<td>0.04</td>
<td>0.11</td>
</tr>
<tr>
<td>TT A (no IFNL4)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
<td>0.01</td>
<td>0.01</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*Multivariate models for spontaneous clearance are adjusted for age, sex and HCV risk. Multivariate models for response to treatment are adjusted for age, sex, fibrosis stage and viral genotype. Multivariate models include a smaller number of treated patients (N = 404) due to missing covariates.
were significantly different from each other (Dunn’s multiple comparison test).

Overall ISG expression in liver biopsies was assessed with the 4-gene classifier calculating the probability of a sustained virological response for pegIFNα/ribavirin treatments. A high pSVR corresponds to low overall ISGs expression in the liver and vice versa. The biopsies were grouped according to their IFNL4 diplotype as indicated. Shown are results including (a) sustained virological response after treatment with pegIFNα/ribavirin or (b) spontaneous clearance. P values were obtained by logistic regression. N = number of patients in a given group.

Fig. 3 | Association of IFNL4 genotypes with ISG expression in liver biopsies. Overall ISG expression in liver biopsies was assessed with the 4-gene classifier calculating the probability of a sustained virological response for pegIFNα/ribavirin treatments. A high pSVR corresponds to low overall ISGs expression in the liver and vice versa. The biopsies were grouped according to their IFNL4 diplotype as indicated. Shown are results including (a) all HCV genotypes and (b) HCV genotype 1 only. Statistical analysis with Kruskal–Wallis revealed significant overall differences of the median pSVR of the three groups for all genotypes and for genotype 1 only, with \( P = 0.0058 \) and \( P = 0.026 \), respectively. Between groups, only the groups ‘no IFNL4’ and ‘IFNL4 P70’ were significantly different from each other (Dunn’s multiple comparison test). N = number of samples per group.

Table 3 | IFNL4 haplotype combination and associated clearance phenotypes.

<table>
<thead>
<tr>
<th>Diplootype</th>
<th>( N )</th>
<th>Proportion of patients</th>
<th>IFNL4 production</th>
<th>SVR</th>
<th>% SVR</th>
<th>CHI</th>
<th>SC</th>
<th>% SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT G/TT G</td>
<td>277</td>
<td>0.398</td>
<td>–</td>
<td>–</td>
<td>39</td>
<td>156</td>
<td>0.800</td>
<td>195</td>
</tr>
<tr>
<td>TT G/TG A</td>
<td>231</td>
<td>0.332</td>
<td>–</td>
<td>–</td>
<td>111</td>
<td>105</td>
<td>0.486</td>
<td>216</td>
</tr>
<tr>
<td>TT G/TG A</td>
<td>110</td>
<td>0.158</td>
<td>–</td>
<td>–</td>
<td>30</td>
<td>64</td>
<td>0.681</td>
<td>94</td>
</tr>
<tr>
<td>( \Delta G ) G/( \Delta G ) G</td>
<td>39</td>
<td>0.056</td>
<td>–</td>
<td>–</td>
<td>17</td>
<td>17</td>
<td>0.500</td>
<td>34</td>
</tr>
<tr>
<td>( \Delta G ) A/( \Delta G ) G</td>
<td>29</td>
<td>0.042</td>
<td>–</td>
<td>–</td>
<td>19</td>
<td>8</td>
<td>0.296</td>
<td>27</td>
</tr>
<tr>
<td>TT G/TG A</td>
<td>5</td>
<td>0.007</td>
<td>–</td>
<td>–</td>
<td>0</td>
<td>4</td>
<td>1.000</td>
<td>4</td>
</tr>
<tr>
<td>( \Delta G ) A/( \Delta G ) A</td>
<td>4</td>
<td>0.006</td>
<td>–</td>
<td>–</td>
<td>0</td>
<td>3</td>
<td>1.000</td>
<td>3</td>
</tr>
<tr>
<td>TT A/TG A</td>
<td>1</td>
<td>0.001</td>
<td>–</td>
<td>–</td>
<td>0</td>
<td>1</td>
<td>NA</td>
<td>1</td>
</tr>
</tbody>
</table>

CHI, chronic infection; IFNL4, interferon-lambda; NA, not applicable; SC, spontaneous clearance; SVR, sustained viral response.

*Diplotypes are composed of rs368234815, which can be either TT or \( \Delta G \), and rs117648444, which can be either G or A.
replication, which in turn leads to an inefficient adaptive immune response, and that this is the base of poor HCV clearance. This is supported by a recent study showing an association between the rs368234815 ΔG allele (IFNL4 expression) and activity level of T cells. Furthermore, our findings are supported by a recent paper, showing that patients carrying the IFNL4-S70-coding allele exhibit a faster decline in HCV RNA levels at week 4 of treatment.

Combining rs368234815 and rs117648444 polymorphisms, it is possible to stratify patients into three groups, with the highest cure rates in patients not producing IFNL4, intermediate cure rates among those producing the IFNL4-S70 variant and the lowest cure rates among those producing the IFNL4-P70 variant. Furthermore, there is an excellent agreement between the predicted response rate (pSVR) obtained by measuring ISG expression in liver biopsies and the experimentally observed SVR, once patients are stratified according to the three groups defined above. In our cohort, 26% of the patients being able to express IFNL4 harbour only the IFNL4-S70 variant, and thus have a prognosis that is almost as good as those not producing IFNL4. Thus, by combining both polymorphisms, it is possible to substantially improve the accuracy of the predicted response rate before treatment. In addition, we can clarify a discrepancy that has puzzled the field. The rs368234815 polymorphism that determines the ability to express IFNL4 is not in LD with rs8099917, which was found as a good SVR predictor in three out of four of the initial GWAS studies. This was unexpected and did initially argue against the IFNL4 protein as the causative agent; however, it is now clear that the minor allele of rs8099917 specifically tags the haplotype producing the IFNL4-P70 variant, which we have shown to be associated with the poorest clearance rates.

One may argue that the use of genetic markers may become obsolete with the advent of direct-acting antiviral agents (DAAs), due to their high cure rates. Nevertheless, the role of IFNL3/IFNL4 polymorphisms has been convincingly demonstrated in the first generation DAA treatment regimes. The SOUND-C2 trial assessing the combination of faldaprevir and deleobuvir with or without ribavirin showed a significantly higher SVR rates for patients homozygous for the favourable allele of rs12979860 (CC), clearly demonstrating a role for IFNL4 genotype in IFN-free DAA treatment regimens. Thus, in mechanistic term, the role of the IFNL4 genotype is not restricted to IFN-based therapies, but do influence DAA-based treatments. However, recent development in DAA combination therapies using drugs with a substantially improved potency has pushed SVR above 90% for all patients, regardless of their IFNL4 genotype. With cure rates, this high level of the genotype will have little impact on patients’ choices of treatment. However, there are several clinical as well as scientific reasons to pursue a deeper understanding of how IFNL4 genotype impact viral clearance. Several recent studies show that rs368234815 influences the response kinetics to DAA and, however, the role of rs117648444 in DAA treatment has not yet been investigated. This suggests that patients not expressing IFNL4 can receive a shortened DAA treatment and possibly also reduced doses. There will be both obvious benefits for patients and substantial cost reduction by a targeted shortening of therapy in patients with favourable IFNL4 genotype. Our study clearly shows that the decision should be based on a dual genotyping of rs117648444 and rs368234815.

The expression of active IFNL4 influences both IFN-based and IFN-free treatment of chronic HCV infections, as well as strongly influences the spontaneous clearance rate. These observations point towards a fundamental role of active IFNL4 in facilitating and maintaining a chronic HCV infection. A recent study showed that rs368234815 also influenced susceptibility to cytomegalovirus retinitis among HIV-infected patients, suggesting that the IFNL4 protein might play detrimental role in several different chronic viral infections. Thus, we believe that the activity of IFNL4 could play an important role in multiple chronic viral infections by a mechanism similar to that observed in hepatitis C.

**Methods**

**Protein expression and purification.** BL21 (DE3) E. coli cells transformed with the plasmids were grown at 37 °C in Luria Bertani medium containing 100 µg ml⁻¹ ampicillin and 100 µl ml⁻¹ antifoam A (Sigma-Aldrich, catalogue number A5633) under continuous shaking until an OD₆₀₀ of 0.8–1. Protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside and incubating for another 4 h at 37 °C. Refolding and purification were performed as previously described. The His-tag was removed by the tobacco etch virus (TEV) protease cleavage after the refolding was completed. The TEV was removed during the subsequent cation exchange chromatography using a HiTrap SP FF column. The TEV cleavage was omitted for IFNL3. It is worth noting that the yield of IFNL4 protein is low with 1–2 mg of purified protein from 121 E. coli culture. We did not note any consistent differences in the expression levels between IFNL4-P70 and IFNL4-S70.

**Plasmids.** The IFNL4-S70 constructs were generated by site-directed mutagenesis using PhiUltra II Fusion HS DNA polymerase according to the manufacturer’s instructions (Agilent). For the mammalian cell expression, pEF2-IFNL4-S70-FLAG was generated on pEF2-IFNL4-P70-FLAG template using forward 5'-CCGCGGAG ATGTCCTCCTGGGCG-3' and reverse 5'-GGCCAGAGGATCTCTCCGGG-3' primers. For the expression in E. coli, pET15b-IFNL4-S70 was generated on pET15b-IFNL4-P70 construct using forward 5'-GCTGCTGAGAGGCGAAT CAGACGCGGAGCAG-3' and reverse 5'-CGTCCGCTGTGATTCCGCTGT GCAGACGGC-3' primers. The following PCR programme was used: (1) 95 °C for 30 s; (2) 16 cycles of 95 °C for 30 s, 65 °C for 1 min and 72 °C for 4 min; and (3) 72 °C for 10 min.

**Cell culture.** Unless otherwise stated, all cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), which was supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. Cells were purchased from the German Collection of Microorganisms and Cell Cultures, DSMZ (HeP2 DSMZ-No: ACC-180; HEK293 DSMZ-No. ACC-305).

**Activity assay in HEK293 cells.** HEK293 cells were seeded at a density of 7.5 × 10⁵ cells per well in a 48-well plate and left to rest for 24 h. After 24 h, the cells were transfected with the pEF2 plasmid encoding IFNL4, firstly luciferase under the control of the Mx1 promoter and Renilla luciferase under the control of the β-actin promoter. Eighteen hours post-transfection, cells were induced in quadruplicates with eight dilutions of IFNL4-P70 and IFNL4-S70 in the concentration range covering 0.006–100 ng ml⁻¹ for 24 h. After 24 h, the cells were washed with PBS and lysed with Passive Lysis Buffer (Promega). The lysates were then centrifuged at 4 °C for 10,000 × g at 4 °C. The clear supernatants were used for the measurement of luciferase activity (Dual-Luciferase Reporter Assay System, Promega). EC₅₀ efficacy and exponential slope values were estimated in GraphPad prism version 5.04 by fitting a sigmoidal equation to the plot. The equation used for fitting was: firefly/Renilla ratio = baseline + (efficacy – baseline)/(1 + (10^\text{cell damping - 3.5} × \text{slope})). Values for baseline were restrained to zero.

**Real-time quantitative PCR.** HepG2 cells were seeded at a density of 2 × 10⁴ cells per well in 12-well plates, and after 24 h, fresh media were added with the indicated IFNs in quadruplicates. After 4 h of incubation, cells were lysed and RNA was purified using an extraction kit (Omega) according to the manufacturer’s instructions. Complementary DNA synthesis was performed with 1 µg RNA using RevertAid reverse transcriptase and random hexamer primer according to the manufacturer’s instructions (Thermo Scientific). The cDNA obtained from cells was quantified by real-time quantitative PCR using SYBR Green I (Roche) on Roche LightCycler 480 II. The following primers were used: GAPDH, forward 5'-GAGACATGTGTTGCAAGCTCA-3' and reverse 5'-GGTGTCACCAATCTTCT-3'; ACT-3, forward 5'-GCTCTCCGGTGGTCTGTTACA-3' and reverse 5'-GGCCATCTGGGCGTCTG-3'; 18S, forward 5'-ACTACAGCCGCGTTGCTT GAAA-3' and reverse 5'-CGGCTAAGGATACGACAGG-3'. The cycling parameters were: 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 12 s. The crossing points of the amplification curves were determined using the second derivate method on the Roche 480 LightCycler software 1.5 (Roche). The level of mRNA was normalized against the GAPDH mRNA content. The mean of the quadruplicates of the untreated control was used to calculate the fold induction for the other samples.

**Antiviral assay.** HepG2 cells were seeded at a concentration of 1.8 × 10⁴ cells per well in 96-well plates and incubated with 20 dilutions of IFNL4-P70 or IFNL4-S70.
in the concentration range covering 0.008–200 ng ml\(^{-1}\) for 24 h. The cells were then infected with EMCV (VR-1298 strain) at a multiplicity of infection of 0.45. After 24 h, MITT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma Aldrich) was added at a concentration of 0.8 mg ml\(^{-1}\) in DMEM, and the cells were incubated for additional 4 h. The cells were dissolved in dimethyl sulfoxide (Sigma-Aldrich). MTT conversion was determined by measurement of the absorbance at 570 nm and subtracting the background absorbance of the relevant culture at 650 nm, using the following equation: absorbance of the IFN wells (Abs) = (Abs – VC/CC × VC) × 100 to allow activity comparisons between plates. EC\(_{50}\) efficacy and exponential slope values were estimated as described in the section entitled ‘Activity assay in HEK293 cells’.

**Liver biopsies.** Liver biopsies from patients (n = 104) were obtained in the outpatient clinic of the Division of Gastroenterology and Hepatology, University Hospital Basel, Switzerland. Parts of the biopsy material that were not needed for routine histopathological use were frozen at −80°C for future use in genetic studies and available DNA were collected from the Swiss Hepatitis C Genome-wide association Study (Lausanne), Comitato etico cantonale (Lugano) and by the Ethical committee of Giovanni Rotondo, Italy. The study was approved by the Ethics Committee of each hospital.

Total RNA and genomic DNA were isolated from liver biopsies using Trizol reagent (Invitrogen, Basel, Switzerland) and DNeasy Blood & Tissue Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer’s instructions. A fragment of 850 base pair covering rs8099917 and rs11764844 was amplified with the Expand High Fidelity PCR System (Roche) using forward 5′-ACCTGGTTGTTG TGGCTGGCCCTTC-3′ and reverse 5′-GGAGCAGAGGGCGGTAGAGG-3′ primers. The product was purified on a column using NucleoSpin Gel and PCR clean-up (Macherey-Nagel AG, Oensingen, Switzerland) according to the manufacturer’s instructions and then sequenced (Microsynth AG, Balgach, Switzerland). The pSV2 vector was calculated using the 4-classification system as described previously\(^{10}\). Statistical analysis was performed using Prism (GraphPad software. La Jolla, CA, USA).

**Genetic association study.** Samples from patients with written informed consent for genetic studies and available DNA were collected from the Swiss Hepatitis C Cohort Study (SCCS), a multicenter study of HCV-infected patients enrolled at eight major Swiss hospitals and their local affiliated centres, as described previously\(^{16,25}\), and from the Liver Unit of the Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy. The study was approved by the Ethics Committee of each SCCS center (Ethikkommission beider Basel EKBB, Basel), Ethikkommission des Kantons St Gallen (St Gallen), Comité intercantonal d’éthique (Neuchâtel), Comité d’étique du département de Médecine (Geneva), Department Innere Medizin Ethisches Komitee (Zurich), Commission d’éthique de la recherche clinique (Lausanne), Comitato etico cantonale (Lugano) and by the Ethical committee of ‘Casa Sollievo della Sofferenza’. The study included Caucasian patients with spontaneous HCV clearance (defined as presence of anti-HCV antibodies but undetectable HCV RNA without previous antiviral treatment) and those who had chronic infection with HCV genotypes 1, 2, 3 or 4 and were assessable for response to therapy with pegIFN and ribavirin, that is, who received ≥80% of the recommended dose of each drug. Polymorphisms in the IFNL3/4 region (rs12979860, rs862834815, rs11764844 and rs80999917) were extracted from a GWAS-generated data set\(^{14}\) or genotyped by TaqMan (Applied Biosystems, Rotkreuz, Switzerland) or by the Competitive Allele Specific PCR (KASP) system (LG Genomics, Herts, UK; http://www.lggenomics.com/kaschallenge). Haplotypes were inferred using PHASE software, version 2.1 (University of Washington, available through Express Licensing Program, Seattle, WA, USA; http://stephenslab.uchicago.edu/software.html). Statistical analyses were performed using Stata (version 13.1, StataCorp LP, College Station, TX, USA). LD was assessed using the programs PLWLD implemented in Stata. The association of polymorphisms with response to treatment and spontaneous clearance was performed by univariate and multivariate logistic regression. The multivariate models for spontaneous clearance are adjusted for age, sex and HCV risk. The multivariate models for response to treatment are adjusted age, sex, fibrosis stage and viral genotype.

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Author contributions


Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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