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08 May 2024

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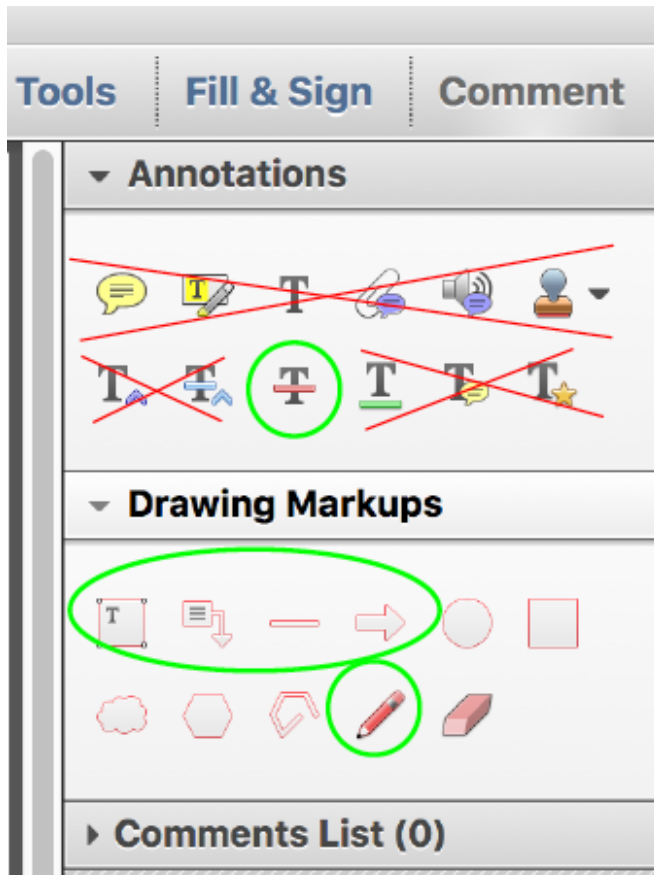
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IFN-λ3, not IFN-λ4, likely mediates *IFNL3–IFNL4* haplotype–dependent hepatic inflammation and fibrosis

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Q2 Genetic variation in the *IFNL3–IFNL4* (interferon-λ3–interferon-λ4) region is associated with hepatic inflammation and fibrosis[1–4]. Whether IFN-λ3 or IFN-λ4 protein drives this association is not known. We demonstrate that hepatic inflammation, fibrosis stage, fibrosis progression rate, hepatic infiltration of immune cells, IFN-λ3 expression, and serum sCD163 levels (a marker of activated macrophages) are greater in individuals with the *IFNL3–IFNL4* risk haplotype that does not produce IFN-λ4, but produces IFN-λ3. No difference in these features was observed according to genotype at rs117648444, which encodes a substitution at position 70 of the IFN-λ4 protein and reduces IFN-λ4 activity, or between patients encoding functionally defective IFN-λ4 (IFN-λ4–Ser70) in comparison to those encoding Q3 fully active IFN-λ4–Pro70. The two proposed functional variants (rs368234815 and rs4803217)[5,6] were not superior to the discovery SNP rs12979860 with respect to liver Q4 inflammation or fibrosis phenotype. IFN-λ3 rather than IFN-λ4 likely mediates *IFNL3–IFNL4* haplotype–dependent hepatic inflammation and fibrosis.

Tissue injury leads to an inflammatory host response that, if sustained, results in excess extracellular matrix deposition and fibrosis[7]. Liver disease is a useful generic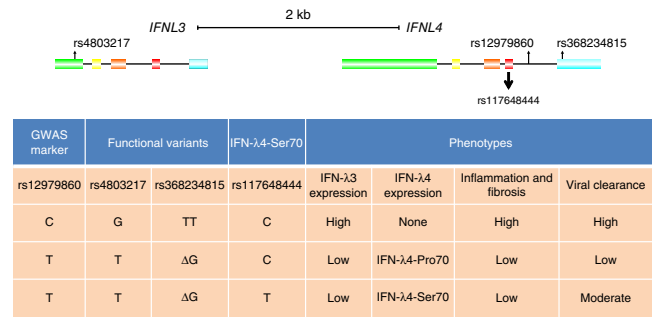 model of tissue inflammation and fibrosis, with most liver-related adverse outcomes being a consequence of fibrosis[7].

The interferon family has a pivotal role in defense against a wide variety of pathogens, and inflammation constitutes an essential part of this response[8]. In the liver, type III interferons (IFN-λ) have a primary antiviral role[9,10] and constitute the dominant interferon subclass produced during hepatitis C virus (HCV) infection[11,12]. There is now unequivocal genetic and functional evidence that IFN-λ has a substantial influence on hepatic inflammation and fibrosis risk, with multiple reports demonstrating that SNPs in the *IFNL3–IFNL4* region, originally discovered through genome-wide association studies (GWAS) as a predictor of HCV clearance[13–16], are a strong predictor of risk[1–4]. IFN-λ levels are elevated in patients with chronic liver disease[17] and correlate with inflammation and fibrosis markers[4,18]. IFN-λ also directly activates a positive feedback inflammatory loop in cell culture models of HCV[19]. Further, whereas expression of type I and II interferon receptors is impaired during HCV infection and in advanced liver disease, IFN-λ receptor expression is unaltered[20,21].

IFN-λ4 is the newest member of the IFN-λ family, with rs368234815 in the *IFNL4* gene upstream of *IFNL3* controlling the generation of IFN-λ4 protein[5]. The rs368234815[ΔG] allele in high linkage disequilibrium (LD) with rs12979860[T] is characterized by expression of IFN-λ4 and is implicated in reduced HCV clearance[5] (**Fig. 1**). The same allele is associated with lower baseline and inducible

GWAS marker	Functional variants		IFN-λ4-Ser70	Phenotypes			
rs12979860	rs4803217	rs368234815	rs117648444	IFN-λ3 expression	IFN-λ4 expression	Inflammation and fibrosis	Viral clearance
C	G	TT	C	High	None	High	High
T	T	ΔG	C	Low	IFN-λ4-Pro70	Low	Low
T	T	ΔG	T	Low	IFN-λ4-Ser70	Low	Moderate

Q27 **Figure 1** Schematic of the *IFNL3* and *IFNL4* genes and genetic variants.

expression of *IFNL3* mRNA in humans (liver biopsies, serum, and peripheral blood mononuclear cells (PBMCs))[14,15,22–24], *in vitro*[25,26], and in chimeric mice[27], although two reports have failed to demonstrate this[13,28]. However, IFN-λ3 protein levels are consistently lower with these alleles[23,25,29–31], including *in vitro*. One study has suggested that IFN-λ3 levels are a better predictor of viral clearance than SNPs in the *IFNL3–IFNL4* region[26]; previous studies are summarized in **Supplementary Table 1**.

Whether IFN-λ4 or IFN-λ3 activity drives the functional association with hepatic inflammation and fibrosis[1–3] is unknown. Addressing this question is challenging, owing to extremely low (and often undetectable) hepatic IFN-λ4 expression[32–34] and because the Q5 *IFNL4* region is absent from rat and mouse genomes. Recently, on the basis of the co-inheritance pattern of a substitution in *IFNL4* at rs117648444 and rs368234815 genotype, we provided a clue suggesting that genetic information can be used to test, in a human context, the role of IFN-λ4 activity in mediating hepatic inflammation and, thereby, fibrosis[35].

We tested for association of IFN-λ4 and IFN-λ3 activity with hepatic inflammation and fibrosis in a well-characterized cohort of Q6 1,923 Caucasian HCV-infected patients. In addition to rs12979860, we assessed *IFNL4* rs117648444 and the two potential functional SNPs *IFNL4* rs368234815 and rs4803217 in the 3′ UTR of *IFNL3* that influences *IFNL3* mRNA stability[6,31]. The *IFNL3* and *IFNL4* genes and genetic variants are schematically presented in **Figure 1**. The association analysis was carried out for three pairwise combinations of phenotype status: significant hepatic inflammation (cases) versus mild inflammation (controls), significant hepatic fibrosis (cases) versus mild fibrosis (controls), and fast fibrosis progression rate (FPR) (cases) versus slow FPR (controls). Histological definitions and patient phenotypes are provided in the Online Methods, and clinical characteristics are shown in **Supplementary Table 2**. The characteristics of the FPR subcohort with a known duration of infection ($n = 1,003$) were generally similar among subjects included and not included in the subanalysis. The genotype distributions of *IFNL3* and *IFNL4* variants (rs4803217, rs368234815, rs117648444, and rs12979860) were in Hardy–Weinberg equilibrium (**Supplementary Table 2**); LD values between variants are displayed in **Supplementary Table 3** and were consistent with previous reports[5,6,36].

On the basis of the strong LD between rs4803217 and rs368234815 ($r^2 = 0.93$) and LD of these SNPs with rs12979860 ($r^2 = 0.93$ and 0.96, respectively), association tests for the functional variants gave results similar to those for rs12979860 and showed strong association with the three phenotypes: significant inflammation ($P < 4.7 \times 10^{-14}$, for all three SNPs), significant fibrosis ($P < 2.6 \times 10^{-6}$, for Q7 all three SNPs), and fast FPR ($P < 5.5 \times 10^{-4}$, for all three SNPs). Associations remained significant after adjustment for sex, age, body

Table 1 Association test results for *IFNL3* and *IFNL4* variants

Genotype	Significant inflammation				Significant fibrosis				Fast FPR			
	Unadjusted OR	P value	Adjusted OR	P value	Unadjusted OR	P value	Adjusted OR	P value	Unadjusted OR	P value	Adjusted OR	P value
rs12979860[CC]	1.94 (1.48–2.55)	1.6×10^{-17}	1.92 (1.49–2.47)	1.3×10^{-16}	1.73 (1.25–2.39)	1.3×10^{-7}	1.70 (1.37–2.1)	1.1×10^{-6}	1.74 (1.34–2.26)	1.3×10^{-5}	1.72 (1.31–2.25)	7×10^{-5}
rs368234815[TT]	1.90 (1.45–2.49)	2.6×10^{-17}	1.92 (1.51–2.50)	1.1×10^{-16}	1.68 (1.35–2.06)	5.5×10^{-7}	1.63 (1.32–2.02)	5×10^{-6}	1.61 (1.24–2.09)	1.5×10^{-4}	1.59 (1.22–2.08)	3.3×10^{-4}
rs4803217[GG]	1.80 (1.37–2.35)	4.7×10^{-14}	1.79 (1.39–2.30)	5.5×10^{-12}	1.56 (1.29–1.89)	2.6×10^{-6}	1.54 (1.19–1.80)	5.2×10^{-5}	1.54 (1.22–2.1)	5.5×10^{-4}	1.53 (1.19–2.007)	6.4×10^{-4}
rs117648444[GA/AA]	1.10 (0.85–1.43)	0.4	1.16 (0.90–1.50)	0.2	1.09 (0.78–1.52)	0.6	1.19 (0.93–1.52)	0.1	1.02 (0.75–1.39)	0.8	1.02 (0.75–1.39)	0.8

OR, odds ratio; FPR, fibrosis progression rate. Odds ratios are provided with 95% confidence intervals in parentheses. Liver biopsy data are according to Metavir score. Cases with inflammation stage ≥A2 were classified as having significant inflammation, and cases with fibrosis stage ≥F2 were classified as having significant fibrosis. FPR was calculated by taking the ratio between the fibrosis stage and the estimated disease duration (in years); FPR greater than or equal to the median rate (0.0714 fibrosis units/year) was used as the cutoff for fast FPR. Adjusted odds ratios were adjusted for clinical variables (sex, age, BMI, recruitment center, liver enzymes, diabetes mellitus, HCV genotype, and alcohol intake).
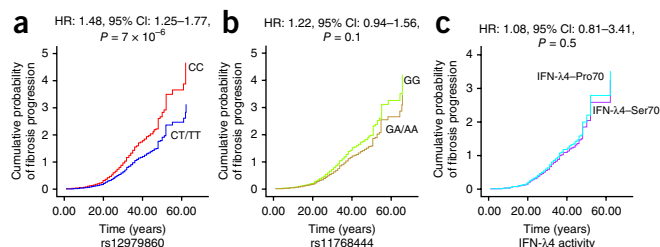
Q34 Q35

Figure 2 Univariate Cox regression analysis of association of *IFNL3* and *IFNL4* variants or IFN-λ4 activity with fibrosis progression. (**a–c**) Univariate Cox regression analysis of the effect of rs12979860 (**a**), rs117648444 (**b**), and IFN-λ4 activity (**c**) on the cumulative probability of progression to significant fibrosis (≥F2) in 1,003 patients with a known duration of HCV infection. Data for SNPs rs12979860 and rs117648444 are shown for a dominant model. IFN-λ4 activity analysis is based on comparison of patients producing a fully functional IFN-λ4 protein (IFN-λ4–Pro70) or both IFN-λ4–Pro70 and IFN-λ4–Ser70 to those producing only functionally defective IFN-λ4–Ser70. Similar results were observed in multivariate Cox regression analysis after adjusting for clinical covariates (age, sex, BMI, HCV genotype, recruitment center, and liver enzymes). HR, hazards ratio; CI, confidence interval.

mass index (BMI), recruitment center, liver enzymes, diabetes mellitus, HCV genotype, and alcohol intake (**Table 1**). For confirmation, fibrosis progression was assessed by Cox proportional-hazards model to address the concern that FPR might not be constant[37]; similar findings were obtained (**Fig. 2**).

In contrast, we observed no association of rs117648444 with any of the three phenotypes (**Table 1**). rs117648444 was also not associated with an increased hazard of progression to significant fibrosis (**Fig. 2**). Similar results were obtained in linear regression (considering the phenotypes as continuous variables) (estimate = 0.034, *P* = 0.2 and estimate = 0.019, *P* = 0.5 for inflammation and fibrosis, respectively) and when adopting other genetic models (additive or recessive).

We next tested for association of IFN-λ4 activity with inflammation and fibrosis phenotypes based on co-inheritance of rs368234815 and rs117648444. The combination frequency distribution (**Supplementary Table 4**) and associated haplotype (**Supplementary Table 5**) and diplotypes (**Supplementary Table 6**) were concordant with earlier reports[5,35]. There were four haplotypes: TT/G (60%), which does not produce IFN-λ4; ΔG/G (29%), which produces IFN-λ4-Pro70; ΔG/A (10%), which produces functionally defective IFN-λ4-Ser70; and TT/A (0.6%), which does not produce IFN-λ4. The diplotypes were classified according to their ability to produce fully active or functionally defective IFN-λ4: those producing (i) no IFN-λ4 (*n* = 688; 35.8%), (ii) IFN-λ4–Pro70 (only IFN-λ4–Pro70 or both IFN-λ4–Pro70 and IFN-λ4–Ser70 (*n* = 961; 50%), and (iii) exclusively functionally defective IFN-λ4–Ser70 (*n* = 274; 14.2%). Group

(ii) comprised 854 patients (88.9%) producing only IFN-λ4–Pro70 and 107 (11.1%) producing both IFN-λ4–Pro70 and IFN-λ4–Ser70.

No association of IFN-λ4 activity with clinical, viral, or biochemical covariates was found. We analyzed inflammation and fibrosis phenotypes of patients producing IFN-λ4–Ser70 alone versus those producing both IFN-λ4–Pro70 and IFN-λ4–Ser70 versus those not producing IFN-λ4. As expected, patients not producing IFN-λ4 (but producing increased amounts of IFN-λ3) had higher odds for all inflammation and fibrosis phenotypes when compared to patients producing any IFN-λ4 (**Table 2**). In analysis excluding subjects not producing IFN-λ4 (to specifically assess the role of the p.Pro70Ser substitution), there was no difference between those producing fully active IFN-λ4 (IFN-λ4–Pro70 alone or both IFN-λ4–Pro70 and IFN-λ4–Ser70) and those producing defective IFN-λ4–Ser70 (**Table 2**). This was confirmed using Cox proportional-hazards models (**Fig. 2**). Similar results were observed when the 107 patients producing both IFN-λ4–Pro70 and IFN-λ4–Ser70 were excluded. Although an analysis based on repeat biopsies in large cohorts with a wide time interval between biopsies would have been ideal, this was not possible given ethical and logistic concerns and is a limitation of the present study.

Collectively, these data suggest that, in contrast to viral clearance, IFN-λ4 activity as measured by the relative effects of the Pro70- and Ser70-encoding alleles, does not contribute to hepatic inflammation or fibrosis phenotypes. For the former, virus-triggered weak hepatic expression of IFN-λ4 (which is inefficiently secreted and not abundant)[32–34] likely induces baseline interferon-stimulated gene expression and inhibits further induction of these genes through institution of interferon-based therapy. However, this is less likely to sustain a proinflammatory state. IFN-λ4 has antiviral and proinflammatory effects *in vitro*[32,34,38], making it unlikely that it has anti-inflammatory effects.

A crucial role has been established for infiltrating immune cells in the progression of hepatic inflammation and fibrosis[39]. Inflammatory cytokines released from these cells perpetuate inflammation as well as activating hepatic stellate cells[39]. Therefore, to gain insights into how the risk haplotype associates with inflammation, we interrogated the profiles of T cell and macrophage markers (CD3, CD8, and CD163) that are known to be relevant to inflammation and fibrosis[39] in the liver lobules and portal areas of 90 patients segregated according to *IFNL3–IFNL4* haplotype and IFN-λ4 activity (characteristics are given in **Supplementary Table 7**). Patients with the risk haplotype had increased numbers of CD3+CD8+ T cells and CD163+ macrophages (**Fig. 3**). In contrast, no difference in immune cell numbers was noted according to IFN-λ4 activity (IFN-λ4–Pro70 versus IFN-λ4–Ser70) or according to *IFNL4* rs117648444 genotype. Consistent with these findings, in a subset of 506 patients (characteristics summarized in **Supplementary Table 8**) who matched the overall cohort with respect to age, sex, and BMI, the levels of sCD163, a

Table 2 Association between IFN-λ4 activity and hepatic inflammation, fibrosis, and fibrosis progression

IFN-l4 activity	Significant inflammation			Significant fibrosis			Fast FPR		
	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value
No IFN-λ4	Reference			Reference			Reference		
IFN-λ4–Ser70 alone	0.462	0.34–0.63	5×10^{-7}	0.708	0.535–0.939	0.01	0.702	0.5–0.92	0.01
IFN-λ4–Pro70 or IFN-λ4–Pro70 + IFN-λ4–Ser70	0.419	0.34–0.52	1.4×10^{-12}	0.623	0.444–0.783	0.006	0.651	0.492–0.893	0.01
IFN-λ4–Pro70 and IFN-λ4–Pro70 + IFN-λ4–Ser70	Reference			Reference			Reference		
IFN-λ4-Ser70 alone	0.874	0.648–1.17	0.3	0.839	0.642–1.09	0.2	0.87	0.51–0.93	0.4

OR, odds ratio; FPR, fibrosis progression rate. Odds ratios are provided with 95% confidence intervals. Analysis of association with IFN-λ4 activity is based on comparison of patients producing a fully functional IFN-λ4 protein (IFN-λ4–Pro70) or both IFN-λ4–Pro70 and IFN-λ4–Ser70 with those producing only functionally defective (IFN-λ4–Ser70) protein. Liver biopsy data are according to Metavir score. Cases with inflammation stage ≥A2 were classified as having significant inflammation, and those with fibrosis stage ≥F2 were classified as having significant fibrosis. FPR was calculated by taking the ratio between the fibrosis stage and the estimated disease duration (in years); FPR greater than or equal to the median rate (0.0714 fibrosis units/year) was used as the cutoff to define fast FPR.

macrophage activation marker associated with hepatic inflammation and fibrosis in chronic hepatitis C[40], were higher in subjects with the rs12979860 risk genotype (**Fig. 3**); this association remained significant in multivariate analysis (estimate = 0.092, standard error (SE) = 0.009;

P = 0.03). Similar results were observed for rs368234815 and rs4803217 (**Supplementary Fig. 1**). There was no difference according to rs117648444 genotype (**Supplementary Fig. 1**) or IFN-λ4 activity (**Fig. 3**).
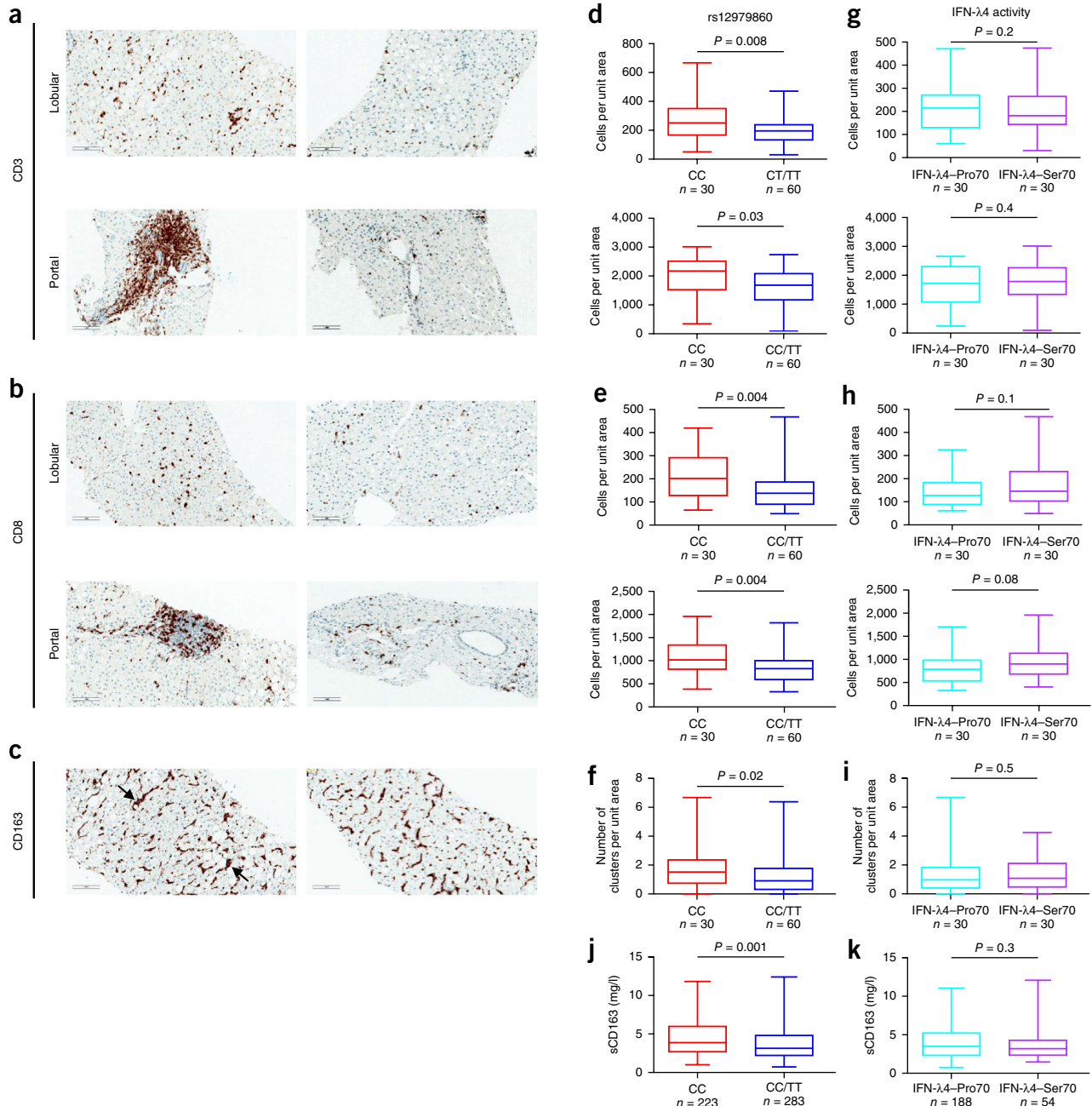

Figure 3 Correlation of *IFNL3* and *IFNL4* variants and inflammatory cells in liver biopsy specimens with serum sCD163 levels. (**a–i**) Immunohistochemical staining of liver biopsy specimens. Inflammatory cells are more numerous in the portal tracts and liver lobules of patients with the CC genotype at rs12979860 than in patients with the CT/TT genotype, while there was no difference according to IFN-λ4 activity. (**a–c**) Representative images are shown (original magnifications, 200×). (**d–i**) The *x* axis shows genotype at rs12979860 using the dominant model of inheritance (CC = 30, CT/TT = 60) (**d–f**) or IFN-λ4 activity based on comparison of patients producing a fully functional IFN-λ4 protein (IFN-λ4–Pro70) or both IFN-λ4–Pro70 and IFN-λ4–Ser70 as compared to those producing only functionally defective IFN-λ4–Ser70 protein (Pro70 = 30, Ser70 = 30) (**g–i**). The *y* axis shows the numbers of cells per unit area. We tested the difference in the median values among the genotypes using two-tailed Mann–Whitney *U* tests performed with a false discovery rate (FDR) procedure for generating corrected *P* values to adjust for multiple testing. (**j,k**) Association between rs12979860 genotype (**j**) and IFN-λ4 activity (**k**) and serum sCD163 levels in 506 patients with chronic hepatitis C. The *x* axis shows genotype at rs12979860 (CC = 223, CT/TT = 283) (**j**) or IFN-λ4 activity (Pro70 = 188, Ser70 = 54) (**k**), and the *y* axis shows sCD163 expression level (mg/ml) by ELISA. The number of samples tested in each group is shown in parentheses. Each group is shown as a box plot, and the median values are shown as thick horizontal lines. The box covers the 25th to 75th percentiles. We plotted the box plots using GraphPad Prism 7.
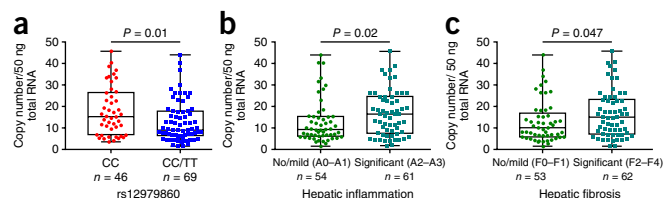
Figure 4 Absolute quantification of *IFNL3* mRNA by ddPCR in human liver. (**a–c**) The expression of *IFNL3* was measured as number of copies/50 ng of total RNA in liver biopsy extracts from patients with chronic hepatitis C (*n* = 115 samples) by ddPCR relative to rs12979860 genotype (**a**), hepatic inflammation (**b**), and liver fibrosis (**c**). The *x* axis shows rs12979860 genotype dichotomized as CC (*n* = 46) and CT/TT (*n* = 69) (**a**), hepatic inflammation dichotomized as absent/mild (A0–A1) (*n* = 54) or significant (A2–A3) (*n* = 61) (**b**), and hepatic fibrosis dichotomized as absent/mild (F0–F1) (*n* = 53) or significant (F2–F4) (*n* = 62) (**c**), and the *y* axis shows hepatic *IFNL3* expression as number of copies/50 ng of total RNA. The number of independent samples tested in each group is shown in parentheses. Each group is shown as a box plot, and the median values are shown as thick horizontal lines. The box covers the 25th to 75th percentiles. We tested the difference in the median values between the genotypes using two-tailed Mann–Whitney *U* tests. We plotted the box plots using GraphPad Prism 7.

Immune cells are major producers of IFN-λ3 (ref. 25); in contrast, IFN-λ4 production has not been demonstrated in these cells. Hence, greater immune cell infiltration in the livers of patients with the risk haplotype should result in higher production of IFN-λ3 by these as well as other cell types in liver. To evaluate this hypothesis, we quantified absolute levels of *IFNL3* transcript by ultrasensitive droplet digital PCR (ddPCR) in 115 liver biopsies from patients with HCV. As expected, subjects with the rs12979860 risk genotype had significantly higher *IFNL3* hepatic expression, consistent with earlier reports[14,15,22–24]. Notably, *IFNL3* expression was elevated in patients with significant inflammation and fibrosis when compared to patients with no or mild inflammation and fibrosis, respectively (**Fig. 4**). This suggests that the IFN-λ3 produced in greater amounts in patients with the risk haplotype (who are also non-producers of IFN-λ4) is likely to mediate the association with hepatic inflammation and fibrosis.

In conclusion, IFN-λ3, but not IFN-λ4, is likely to be the major IFN-λ subclass mediating hepatic inflammation and fibrosis progression in patients with chronic hepatitis C. The effect is likely through increased inflammatory cell infiltration in the liver.

METHODS
Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS
M.E. and J.G. conceived the research. Enrollment of patients, clinical phenotype and data collation, sample acquisition and DNA preparation, sCD163 measurement, ddPCR, and critical analysis were performed by M.E., A.M., T.B., K.T., W.L.I., G.J.D., D.S., H.G., M.L.A., R.H., E.B., U.S., A.R., D.R.B., M.W., L.M., W.C., S.R., J.F., J.N., M.W.D., C.L., E.P., M.R.-G., and J.G. Genotyping was performed by K.S.K. Histological analysis of tissues and scoring were conducted by D.M. and H.M. The manuscript was principally written and revised by M.E. and J.G. All authors critically reviewed the manuscript for important intellectual content and approved the final submitted manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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The International Liver Disease Genetics Consortium (ILDGC):

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EDITORIAL SUMMARY
AOP: Jacob George and colleagues examine whether the association of the *INFNL3–IFNL4* region with hepatic inflammation and fibrosis is mediated by IFN-λ3 or IFN-λ4. They find greater hepatic inflammation, fibrosis progression rate and hepatic infiltration of immune cells in individuals with the risk haplotype that produces IFN-λ3 but not IFN-λ4.

ONLINE METHODS

Patient cohort. The study comprised 1,923 consecutive Caucasian Europeans with chronic hepatitis C from the International Liver Disease Genetics Consortium (ILDGC). All patients with serum positivity for HCV RNA and of European descent at the participating centers who had a liver biopsy with scoring for fibrosis stage and disease activity before treatment between 1999 and 2011 were included if they had available genomic DNA. Patients were excluded if they had evidence of other concomitant liver diseases, including co-infection by HIV or hepatitis B virus (HBV), autoimmune hepatitis, Wilson's disease, α-1 antitrypsin deficiency, or hemochromatosis, by standard tests.

Ethics approval was obtained from the Human Research Ethics Committees of the Sydney West Local Health District and the University of Sydney. All other sites had ethics approval from their respective ethics committees. Written informed consent for genetic testing was obtained from all participants.

Clinical and laboratory assessment. The following data were collected at the time of liver biopsy from all patients: sex, age, ancestry, recruitment center, alcohol intake, BMI, and findings from routine laboratory tests. BMI was calculated as weight divided by the square of height (kg/m^2).

Methods to estimate the duration of infection. Fibrosis progression was examined in 1,003 patients with chronic hepatitis C who had a reliable estimated duration of infection as previously reported[2]. Briefly, for subjects with a history of injection drug use ($n = 663$), the time of infection was estimated using the reported "first year of injection." For patients with a history of blood transfusion ($n = 244$), the onset of infection was assumed to be the year of transfusion. For patients with a history of occupational exposure ($n = 96$), the onset of infection was assumed to be the first year of needle stick exposure. The duration of infection was calculated by subtracting estimated age at the time of infection from age at biopsy.

Genotyping. Genotyping for the rs12979860 and rs368234815 SNPs was undertaken using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems). Genotyping for rs4803217 and rs117648444 was contracted to the Australian Genome Research Facility (AGRF; Queensland, Australia). Samples were genotyped using the Sequenom MassARRAY system and iPLEX Gold chemistry. Part of the genotyping for these SNPs ($n = 1,130$) was undertaken using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems) and the Competitive Allele-Specific PCR (KASP) system (LGC Genomics), respectively. Genotyping was performed on the StepOne RT system, and results were analyzed with StepOne software (Applied Biosystems). All genotyping was undertaken with blinding to clinical variables.

Liver histopathology. Liver histopathology was scored according to METAVIR[41]. Fibrosis was staged from F0 (no fibrosis) to F4 (cirrhosis). Necroinflammation (A) was graded as A0 (absent), A1 (mild), A2 (moderate), or A3 (severe). All biopsies had a minimum of 11 portal tracts, and inadequate biopsies were excluded. The agreement among pathologists was studied previously and was good for METAVIR staging ($\kappa = 77.5$) using κ statistics[42].

Immunohistochemistry. Immunohistochemistry was undertaken in a well-characterized subcohort with liver biopsy sections available for analysis ($n = 90$). Patients with additional risk factors for liver fibrosis other than chronic hepatitis C, that is, those with diabetes, significant or heavy alcohol intake (>50 g/d), or moderate or severe steatosis, were excluded. Sections (4 μm thick) were cut from formalin-fixed, paraffin-embedded (FFPE) specimens, and staining was performed on a Ventana Roche BenchMark ULTRA Slide Staining System according to standard protocols. Slides were deparaffinized in EZprep (Ventana) and washed with Ventana reaction buffer, and HIER was performed with Ventana ultraCC1 at 95–100 °C for between 32 and 64 min, according to specific protocols. Slides were incubated at 36 °C with primary antibodies against CD3 and CD8 (Ventana; prediluted) and against CD163 (CellMarque; 1:50 and 1:1500 dilutions, respectively). Detection was carried out using Ventana optiview (CD163) and Ventana ultraview (CD3 and CD8) with DAB as the chromogen. Slides underwent scanning and digital

microscopy using the Aperio ScanScope System (Aperio). For each biopsy and for each cell marker, a portion of the tissue was measured, the area of included portal tracts and fibrous tissue was measured, and the corresponding liver immune cell content was manually quantified in portal tracts and in lobular parenchyma, all using Aperio ImageScope software. Inflammatory cell density in portal areas/areas of fibrous stroma and in lobular parenchymal regions of the liver was then calculated (cells/mm²). Clusters of CD163+ cells were regarded as foci with two or more CD163+ confluent cells with identifiable nuclei. Singly distributed macrophages were not counted. Individual CD163+ cells could not be distinguished in portal tracts, and reliable counting was not possible.

Determination of plasma sCD163 levels by sandwich ELISA. Plasma sCD163 levels were measured by ELISA using a BEP-2000 ELISA analyzer (Dade Behring). Measurements were performed in duplicate[40]. The limit of detection (lowest standard) was 6.25 μg/l.

RNA extraction and cDNA synthesis. Total RNA was isolated from snap-frozen liver biopsies using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions from 115 patients who underwent liver biopsy at Westmead Hospital, Sydney. Inclusion criteria were availability of stored liver tissue and no additional risk factors for hepatic fibrosis, that is, diabetes, obesity (BMI >30 kg/m^2), or significant alcohol intake (>50 g/d). RNA quality and concentration were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA was prepared using qscript (Quanta Biosciences) on a MasterCycler Gradient 5331 (Eppendorf).

Droplet digital PCR. Absolute quantification of *IFNL3* transcripts was performed by ddPCR analysis according to the manufacturer's instructions. Briefly, reactions were performed in a 20-μl reaction volume that consisted of 10 μl of QX200 ddPCR EvaGreen Supermix (Bio-Rad), 5 μl of gene-specific primer, and 5 μl of cDNA sample. Droplet formation was carried out using a QX100 droplet generator (Bio-Rad). Each sample was partitioned into an emulsion of approximately 20,000 uniformly sized nanoliter droplets. The droplet emulsion was transferred to a 96-well plate (Eppendorf) and heat-sealed at 180 °C for 5 s with foil. Thermal cycling was performed using the C1000 Touch Thermal Cycler (Bio-Rad) according to the manufacturer's cycling conditions. Each plate was incubated at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min, with a final 10-min incubation at 98 °C. At least two negative-control wells with no cDNA template were included in every run. After PCR, the RNA content of the droplets was quantified using the QX200 Droplet Reader (Bio-Rad). ddPCR data were analyzed using QuantaSoft software. The fractions of positive and negative droplets were determined, and data were fitted to Poisson statistics and the background was corrected on the basis of the data for the no-template control. Absolute transcript levels were initially presented as copies per microliter and converted to copies per nanogram of RNA on the basis of the input amount of RNA.

Sample size calculation and linkage disequilibrium. We examined the LD of *IFNL3* and *IFNL4* variants (rs12979860, rs4803217, rs368234815, and rs117648444) by calculating the correlation coefficient (r^2) using Haploview software (version 4.2)[43]. Using the CaTS power calculator for genetic association studies[44] and assuming a minor allele frequency (MAF) for rs117648444 of 0.11, our cohort had 97% power for the dominant genetic model and 94% power for the additive genetic model for liver inflammation. For analysis, the *IFNL3* and *IFNL4* variants (rs12979860, rs4803217, rs368234815, and rs117648444) were coded in a dominant genetic model, which best fit the data and as previously shown[2,35].

Statistical analysis. Results are expressed as mean ± s.d. or as median and range or number (percentage) of patients. Student's *t* test or non-parametric tests, i.e., Wilcoxon–Mann–Whitney *U* tests or Kruskal–Wallis tests, were used to compare quantitative data, as appropriate. χ^2 tests and Fisher exact tests were used for comparison of frequency data and to evaluate the relationships between groups. All tests were two-tailed, and *P* values <0.05 were considered significant.

Multivariate logistic regression analysis with backward elimination was undertaken to test independent associations of the *IFNL3* and *IFNL4* variants

with (i) inflammation, (ii) fibrosis, and (iii) fibrosis progression. Because of the high LD among *IFNL3* and *IFNL4* variants, multiple multivariate models were adopted incorporating biologically relevant covariates associated with risk of liver disease progression (sex, age, BMI, recruitment center, liver enzymes, diabetes mellitus, HCV genotype, and alcohol intake). HCV RNA levels were log transformed before entry into the model. Hepatic inflammation was dichotomized as mild (METAVIR score A0–A1) or significant (METAVIR score A2–A3), and fibrosis was dichotomized as mild (METAVIR stage F0–F1) or significant (METAVIR stage F2–F4). FPR was calculated by taking the ratio between the fibrosis stage and the estimated disease duration (in years)[2]. Patients were stratified into two groups according to the median rate (0.0714 fibrosis units/year), which was used as a cutoff. Final models were assessed using the Hosmer–Lemeshow goodness-of-fit and Pearson goodness-of-fit tests. Results are expressed as β values ± s.e.m. or odds ratios with 95% confidence intervals, as appropriate.

We used Cox regression analysis to model the time taken for significant fibrosis (≥F2) to develop. For this analysis, after checking the normality of the quantified variables, appropriate logarithmic transformations were made. We considered estimated age at infection as the starting point and the first liver biopsy showing significant fibrosis (failure time) or the last liver biopsy showing an absence of significant fibrosis in the absence of treatment (censored time) as the end point. A Cox proportional-hazards regression model was fitted, and covariates were considered significant if $P < 0.05$. Multivariate-adjusted analyses used sex, age, BMI, and recruitment center as covariates.

Multivariable regression modeling with backward elimination was undertaken to test independent associations with serum sCD163 levels. Analysis was adjusted for age, sex, and severity of liver disease. Final models were assessed using Hosmer–Lemeshow goodness-of-fit and Pearson goodness-of-fit tests.

For immunohistochemistry analysis, non-parametric tests were used for statistical analysis. An FDR correction was applied to all P values to adjust for multiple testing using PROC MULTTEST in SAS.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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