Lymphocytes Degranulation in Liver in Hepatitis C Virus Carriers Is Associated With IFNL4 Polymorphisms and ALT Levels

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Background. The polymorphisms of IFNL4 are strongly associated with both spontaneous hepatitis C virus (HCV) clearance and response to peg-IFN-α/ribavirin treatment. To further establish the biological effects of the IFNL4 and rs1297860 variations, we studied the activity of liver immune cells.

Methods. Fresh liver samples were collected from HCV-infected patients before any treatment and from NASH patients as controls. Degranulation activity of each lymphocyte type was assessed by the surface expression of CD107a. IFNL4 polymorphisms and HCV genotypes were determined.

Results. In the liver, frequency of CD107a+ immune cells was significantly higher in HCV patients compared to NASH patients. Higher degranulation activity was observed in lymphocytes of HCV patients with favorable IFNL4 genotypes compared to patients with unfavorable genotypes. Multivariate regression analyses indicated that serum ALT levels were dependent on both Metavir activity score and frequency of CD107a positive NKT cells. The high level of degranulation activity observed before treatment was associated with a high HCV RNA decline at the early stage of peg-IFN-α/ribavirin treatment in patients with favorable genotypes.

Conclusions. These data underline that intrahepatic lymphocyte degranulation activity in HCV-infected patients is associated with IFNL4 polymorphisms and contributes to the clearance of HCV in patients with favorable genotypes under antiviral therapy.

Keywords. interferon lambda; natural killer cells. T lymphocytes; ISG.
were looking for possible open reading frame; recently, Prokunina-Olsson and coworkers [11] identified a new transiently induced region harboring a polymorphism ss469415590 (ΔG or TT alleles). The SNP rs12979860, previously known as IL28B (current name interferon-lambda 3: IFNL3) variant, is located within the first intron of interferon-lambda 4 (IFNL4) and thus is also an IFNL4 polymorphism. For clarity, we refer to these variants as IFNL4 and rs12979860. Most importantly, ss469415590 ΔG is a frameshift variant that creates a coding gene for a novel interferon protein named interferon-lambda 4 (IFNL4). Only patient with ss469415590 ΔG allele may express IFNL4, which is unfavorable for successful pegIFN-α/ribavirin therapy [11]. In addition, experiments on HepG2 cells [11] showed that IFNL4 may induce expression of interferon stimulated genes (ISGs).

Interestingly, it is also known that HCV itself interferes with ISG expression. Briefly, during HCV infection, the immune response allowed the induction of IFN-α. Direct consequence of this IFN production is the establishment of an antiviral state with up-regulation of intracellular ISG expression through the JAK-STAT pathway [12, 13]. In parallel, subsets of suppressor ISGs negatively regulate the IFN pathway. Taken together, these data confirm the key role of ISG expression in the establishment of immune response during HCV infection [14]. In support of this observation, studies on SNP rs1297860 pointed out that chronically infected patients display a significant difference in regard to the ISG expression [15, 16]. Thus, in patients inheriting the favorable CC genotype of rs12979860, the expression of antiviral ISGs is found to be low [16–19], and the expression of suppressor ISGs is high. Interestingly, the fact that IFNL4 induces expression of ISGs [11] suggests that inhibition of IFNL4 might represent a novel therapeutic target for treatment of HCV infection. However, the relationship between rs1297860 and IFNL4 genotype with the host response has not yet been investigated ex vivo in HCV-infected patients. Here, we collected peripheral blood and fresh liver biopsies from HCV-infected patients and analyzed extemporaneously the function of immune cells involved in the antiviral response. We show that sustaining degranulation activity of IHI cells, detected by the CD107a marker is associated with genetic variation of IFNL4 and rs1297860. This may help to explain how upon bi-therapy treatment, patients with IFNL4 favorable TT/TT genotype and rs1297860 favorable CC genotype are more competent to eradicate HCV. Moreover, we have found the association between degranulation activity of IHI cells and serum ALT levels in HCV-infected patients contributing to the understanding of liver immune response during chronic HCV infection.

METHODS

Patients
Eighty-two patients were included in the study and divided into 2 groups according to the cause of chronic liver disease. Group 1, including 72 patients, had histologically proven untreated chronic hepatitis C (Department of gastroenterology and Hepatology, Centre Hospitalo-Universitaire de Grenoble) and underwent liver biopsy as part of their diagnostic evaluation; group 2, including 10 patients with histologically proven nonalcoholic-steato-hepatitis (NASH) and histologic necrosis/activity/steatosis score ≥4 according to Kleiner classification [20] was considered as the control group. We excluded patients with cirrhosis because effector cells and homing signals are substantially altered in fibrotic tissue [21–24]. In group 1, all patients were anti-HCV antibody positive (ELISA 3, Ortho Diagnostic Systems, USA) and HCV-RNA positive in serum as measured by reverse-transcription quantitative polymerase chain reaction (RT-qPCR; AmpliCor HCV, Roche Diagnostic Systems). HCV genotype was determined by Inno-Lipa technology (Innogenetics). ALT serum activity was determined by automated sequential analyser and results were expressed as multiples of normal value [25]. Patient tests were negative for human immunodeficiency virus (HIV) and hepatitis B virus (HBV) infection, and their alcohol consumption was lower than 30 g/day in men and 20 g/day in women. No patients had biochemical markers of autoimmune hepatitis. The study protocol was conformed to the ethical guidelines of the 1975 Helsinki declaration. Patients were enrolled after giving their written informed consent.

Extraction of Intrahepatic Lymphocytes and Peripheral Blood Mononuclear Cells
Liver biopsy specimens were obtained using a 1.5 mm diameter disposable biopsy needles with a length range 10–20 mm. One part of the biopsy was used for histopathological examination, and a second part was collected in 1 mL of appropriate medium and immediately analyzed as described elsewhere [25]. Hepatic cells were then resuspended and immediately stained for FACS analysis. Peripheral blood mononuclear cells (PBMC) were extracted as described elsewhere [25].

Flow Cytometry Analysis and FACS Immunostaining
Lymphocytes phenotyping was performed with LSRII cytometer (BD biosciences) using FCS Express 3 and antibodies: anti-CD45 [HI30] anti-CD3 [UCHT1]); anti CD107a (H3A4) from Biolegend, anti-CD56 (NCAM16.2) from BD Biosciences. Immune cells were first selected using a gate set on forward scatter vs side scatter and then for their CD45 expression. From CD45+ gate, natural killer (NK) cells, NKT and T lymphocytes were distinguished through differential expression of CD56 and CD3 (Supplementary Figure 1) [25]. Finally, degranulation activity of immune cells was monitored using CD107a expression by flow cytometric analysis [26, 27].

IFNL4 and rs1297860 Genotyping
Genomic DNA was extracted from serum of patients using High Pure PCR Template Preparation Kit (Roche) according to manufacturer’s instructions and DNA concentration was measured.
by using the NanoDrop 2000 spectrophotometer (Thermo Scientific). IFNL4 genotyping (ss469415590) was performed by the 5’ nuclease assay with custom-designed TaqManMGB probes (Life Technologies-Invitrogen) and with primer pairs as described by Prokunina-Olsson [11]. Briefly, the amplification mixture consisted of 2.2 μL of 2X master mix (Roche Diagnostics), 5 pmol of each primer, 2.5 pmol of the TaqManMGB probe, and 10 ng of DNA. The thermal cycling conditions were following: initial denaturation at 95°C 10 minutes, PCR cycling (45 cycles) at 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 10 seconds. Data were analyzed by endpoint genotyping LightCyclersoftware 1.5 (Roche Diagnostics). Genotyping of rs1297860 was performed using serum-derived constitutional DNA by the Roche assay kit according to the manufacturer’s instructions.

**Statistical Analysis**

Analyses were performed using SPSS 19.0 software (SPSS Inc, Chicago, IL) as described elsewhere [22] and included the following statistical tests: Mann–Whitney U test, the 2-tailed Spearman’s correlation coefficient and linear univariate and multivariate regression analyses. Two-sided P values < .05 were considered to be significant.

**RESULTS**

The epidemiological, clinical, biochemical, and virological variables as well as the IFNL4 and rs1297860 genotyping for all patients are summarized in **Supplementary Table 1**. Among 72 HCV-infected patients, 56 were infected by HCV-1 genotype (1 [n = 10], 1a [n = 16] and 1b [n = 30]), and 16 by others viral genotypes (2c, 3a, and 4).

**IFNL4 and rs1297860 Genotyping**

As summarized in Table 1, 19 patients (26%) were homozygous for the favorable IFNL4 (TT) haplotype and 53 patients (74%) were bearing unfavorable AG allele. Similar distribution was detected for rs1297860, where unfavorable T allele frequency was 73.0%. Only one out of 72 HCV samples was discordant as regards IFNL4 vs rs1297860 genotyping. The distribution of the unfavorable alleles is close to previous reports for European individuals [10] (Supplementary Table 1). Among NASH patients, 70% were genotyped IFNL4 TT/TT, 30% were ΔG/TT + ΔG/ΔG, and same distribution was detected for rs1297860.

**Association of IFNL4 Polymorphisms With the Activity of Intrahepatic Lymphocytes From HCV-Infected Patients**

First, we investigated the frequency of CD107a+ in 34 fresh paired PBMCs and liver samples from HCV-infected patients. In the overall population, the frequencies of peripheral CD107a+ immune cells were very low (<1%) and statistically different than that observed in the liver (3.3% vs 0.2%-0.4%) and mainly in liver.

**Table 1. Baseline Characteristics and Genotyping Analyses of IFNL4 and rs1297860**

<table>
<thead>
<tr>
<th></th>
<th>HCV All Viral Genotypes (n = 72)</th>
<th>HCV-1 Viral Genotype (n = 56)</th>
<th>NASHa (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, n (%)</td>
<td>41; 57%</td>
<td>32; 57%</td>
<td>6; 60%</td>
</tr>
<tr>
<td>Age (y)b</td>
<td>52.5 ± 10.6</td>
<td>53.0 ± 11.0</td>
<td>59.3 ± 11.7</td>
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<tr>
<td>Viral load (log U/mL)b</td>
<td>5.8 ± 0.8</td>
<td>5.9 ± 0.8</td>
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<tr>
<td>ALTc</td>
<td>1.6 ± 1.7</td>
<td>1.5 ± 1.3</td>
<td>2.2 ± 2.0</td>
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<tr>
<td>Histology</td>
<td>Metaviar activity, n (%)</td>
<td>Metaviar fibrosis, n (%)</td>
<td></td>
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<tr>
<td>Metaviar activity, n (%)</td>
<td></td>
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</tr>
<tr>
<td>HCV</td>
<td>Viral Genotype, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10; 14%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>16; 22%</td>
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<tr>
<td>1b</td>
<td>30; 42%</td>
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<tr>
<td>Others</td>
<td>16; . . .</td>
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<tr>
<td>IFNL4 Polymorphism, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT/TT</td>
<td>19; 26%</td>
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<tr>
<td>ΔG/TT</td>
<td>42; 58%</td>
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<tr>
<td>ΔG/ΔG</td>
<td>11; 15%</td>
<td></td>
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<tr>
<td>With 1 ΔG allele</td>
<td>53; 74%</td>
<td>41; 73%</td>
<td>3; 30%</td>
</tr>
<tr>
<td>rs1297860 Polymorphism, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>20; 28%</td>
<td>16; 29%</td>
<td>7; 70%</td>
</tr>
<tr>
<td>CT</td>
<td>41; 57%</td>
<td>33; 59%</td>
<td>2; 20%</td>
</tr>
<tr>
<td>TT</td>
<td>11; 15%</td>
<td>7; 13%</td>
<td>1; 10%</td>
</tr>
<tr>
<td>With 1T allele</td>
<td>52; 72%</td>
<td>40; 71%</td>
<td>3; 30%</td>
</tr>
</tbody>
</table>

| Abbreviations: HCV, hepatitis C virus; NASH, nonalcohol-steatohepatitis. |
| a NASH patients with NAS > 4. |
| b Results are expressed as mean ± SD. |
| c ALT, Alanine aminotransferase, results are expressed as multiples of normal values. |

Analysis of polymorphisms in patients with histologically proven chronic HCV (n = 72), chronic hepatitis C patients restricted to HCV-1 genotype (n = 56), and patients with histologically proven NASH (n = 10).

**P < .001 in T lymphocytes, 4.6% vs 0.4%-P < .001 in NKT lymphocytes, and 5.1% vs 0.2%-P < .001 in NK cells; Figure 1.**

These data indicated that the degranulation process occurred mainly in liver.

Then we investigated if IFNL4 polymorphisms might affect immune response in HCV-infected patients. Patients displayed highly similar global distribution of T, NKT lymphocytes, and NK cells within the CD45-positive population independently of the IFNL4 or rs1297860 genotype (Supplementary Table 1). However, when we focused on degranulation activity, we observed significant differences in T (5.1% vs 2.7%-P = .001),
NKT (7.3% vs 4.1%-*P* = .004) and NK cells (6.3% vs 4.3%-*P* = .017) of patients with the favorable IFNL4 TT/TT genotype (n = 19) compared to patients with ΔG/TT + ΔG/ΔG genotypes (n = 53; Figure 2A). Thus, unfavorable IFNL4 ΔG/TT + ΔG/ΔG genotypes were associated to weaker intrahepatic lymphocyte degranulation activity, suggesting a dominant role for the ΔG allele in the degranulating activity of liver-infiltrating immune cells. Similar differences were observed in association of rs1297860 polymorphism and degranulation activity of lymphocytes (Supplementary Figure 2A).

In a second step, we included only patients infected by the viral subgenotype HCV-1 (1, 1a, and 1b; n = 56). Interestingly,
patients homozygous for *IFNL4* favorable TT/TT genotype showed again a higher number of T (5.1% vs 3.0%-*P* = .008) and NKT (7.1% vs 4.5%-*P* = .015) CD107a positive lymphocytes than those carrying *IFNL4* ΔG/TT + ΔG/ΔG genotypes (Supplementary Figure 2B), whereas no difference was found for NK cells. It should be noted that in unfavorable genotype, the distribution of IHI cell degranulation activity was more homogeneous and restraint than in the patients inheriting the favorable allele as shown in Figure 2A (eg, standard deviation for T lymphocytes was 1.8 [ΔG/TT + ΔG/ΔG] vs 3.4 [TT/TT]). Similar differences were observed also when we focused on genetic polymorphism at the SNP rs1297860 (Supplementary Figure 2C).

### Activity of Intrahepatic Lymphocytes in Patients With NASH

One puzzling question was to determine if the HCV itself affects the threshold of degranulation activity in IHI cells in HCV-infected patients. Therefore, we chose to analyze the degranulation activity of IHI cells in patients with NASH, who were considered as a control group [20]. The frequency of T, NKT, and NK immune cells degranulation in NASH individuals was significantly lower than frequency observed in HCV-infected patients (0.8% vs 3.2%-*P* = .003, 1.1% vs 4.7%-*P* = .001, and 1.5% vs 4.8%-*P* = .030, respectively; Figure 3B), even though the favorable *IFNL4* TT/TT genotype was found in majority (70%) of NASH patients. Similar differences were also found between control group and HCV-1 infected patients (0.8% vs 3.3%-*P* = .002, 1.1% vs 5.2%-*P* = .001, and 1.5% vs 4.9%-*P* = .021, respectively; Supplementary Figure 2D). These results underline that the presence of the HCV by itself influences the level of degranulation activity in IHI cells.

### Relationship Between Degranulation Activity in Liver, Clinical Parameters, and *IFNL4* Polymorphism

The relationship between sustaining degranulation activity of IHI cells and clinical or biological parameters was investigated in HCV-infected patients. Univariate analysis was conducted with serum ALT levels as the dependant variable and frequencies of CD107a positive IHI cells as independent variables. A regression model indicated that when the frequencies of CD107a positive T, NKT lymphocytes, and NK cells increased...
by 1 U, serum ALT levels increased by 0.114 U/L (P = .020), 0.155 U (P < .001), and 0.065 (P = .0102) respectively (Figure 3A). Then, multivariate analysis was conducted, and regression model showed that only CD107a positive NKT lymphocytes remained independently correlated with serum ALT levels (P < .001) keeping CD107a positive T and NK cells as constant. Interestingly, for a given Metavir activity score, when CD107a positive NKT lymphocytes increased by 1 U, ALT increased by 0.13 IU/L (P < .001) and ALT increased by 1 IU/L when Metavir activity score changed from 1 to 2 (P = .042) and from 1 to 3 (P = .034; Table 2). Taken together, multivariate analysis revealed that (i) the frequency of CD107a positive NKT lymphocytes and serum ALT levels were linked, (ii) as expected, in our cohort of patients, serum ALT levels were associated with Metavir activity score, and (iii) importantly, variations in serum ALT levels cannot be explained only by Metavir activity score but depend also on the frequency of CD107a positive NK T lymphocytes. In addition, when multivariate analysis including IFNL4 polymorphism was conducted, the regression model indicated that when the CD107a positive NKT lymphocytes increased by 1 U, ALT increased by 0.157 IU/L (P = .032) in patients with genotypes 2–6 and by 0.124 (P = .001) in patients with genotype 1 (Figure 3B). On the other hand, no correlation was found between frequencies of CD107a positive IHI cells and viral load or Metavir fibrosis stage.

### Degranulation Activity of Liver Lymphocytes and HCV Viral Load Decline Upon Treatment

Finally, we analyzed the antiviral response to pegIFN-α/ribavirin during the first 4 weeks of treatment, and we investigated the relationship between viral decline and degranulation activity before treatment [28]. Eighteen patients received a treatment by pegIFN-α/ribavirin. The viral load was available at the time of the biopsy and 4 weeks upon treatment administration. As expected, the viral load decline was significantly higher in 5 patients with TT/TT IFNL4 genotype compared to 13 patients with ΔG/TT + ΔG/ΔG genotype (4.0 log UI/mL vs 1.3 log UI/mL-P = .026; Figure 4A). Interestingly, these 5 patients all have a decrease in viral load up to 3 log UI/mL. In addition, higher degranulation activity in T lymphocytes was observed in patients with favorable genotype (TT) compared to patients with unfavorable genotype (7.8% vs 2.3%-P < .001; Figure 4B).

### DISCUSSION

In this work, we investigated the cytolytic potential of immune cells by analyzing expression of CD107a [26, 27, 29, 30]. Our data point out that the degranulation activity of immune cells in the liver are associated with both IFNL4 and rs12979860 genotypes.

It has been established that in HCV-infected patients responding to pegIFN-α/ribavirin treatment, ISG transcription in the liver is minimally induced [17, 19]. Furthermore, rs12979860 CC genotype, linked to good treatment response, was associated to low baseline ISG expression [31]. The scenario, which is consistent with these data, proposes that low baseline ISG expression observed in the good responders renders their IHI cells more sensitive to the biological effect of the IFN-α based treatment [31]. This is further supported by the fact that recently discovered IFNL4, which is active in nonresponders and linked to rs12979860 unfavorable genotype, induces the expression of ISGs [11]. Compiling the features observed for IFNL4 and rs12979860 genotypes, we propose that in patients lacking IFNL4, maintaining of quiescent endogenous ISGs at baseline may facilitate, as observed here, the spontaneous degranulation activity of IHI cells. Inversely, IFNL4 can additively act to increase the levels of ISG, thus contributing to a low degranulation activity in patients with unfavorable genotype.

During HCV infection, liver immunity is confronted to a dual situation: on the one hand, the increase of the cytolytic activity beneficial for the antiviral response but with the risk of liver damage, and on the other hand, the control of the cytolytic activity reducing the risk of damage [32, 33]. Thus, in patients lacking IFNL4, the sustained killing activity might be hampered by anti-inflammatory cytokines contributing to the persistence of HCV infection and limiting liver injuries. This is in agreement with the fact that functions of activated liver lymphocytes in HCV chronically infected patients are multifaceted including both pro-inflammatory and anti-inflammatory cytokines productions toward the establishment of a beneficial equilibrium preventing liver damage but allowing the viral persistence [22, 34]. This statement is further strengthened by the observations that genetic polymorphism at rs12979860 is not associated with necro-inflammation with liver fibrosis progression in the liver of HCV-1 genotype infected patients [35, 36] who correspond actually to the majority of the patients studied herein.

Upon classical bi-therapy treatment, it has been recently reported that ribavirin inhibits the functions of regulatory T cells

| Table 2. Regression Analyses of Serum ALT Levels, Degranulation Activity in the Liver of CD107a Positive NKT Lymphocytes and Metavir Activity Grades in HCV-Infected Patients |
|-----------------|------|---------|
| ALT             | Coef | P > |        |
| CD107a+NKT lymphocytes | 0.182 | 1e-4  |
| Metavir activity score |   |       |
| 2               | 0.441 | 4.2e-2 |
| 3               | 0.715 | 3.4e-2 |
| Cons            | 0.509 | 1.4e-2 |

Abbreviations: ALT, Alanine aminotransferase; HCV, hepatitis C virus.
and reduces the production of IL-10 [37]. The reduction in the secretion of IL-10 would promote an increase of numbers of T and NKT lymphocytes engaged in a process of degranulation, as well as CTL mediated cytotoxicity [15, 32, 33]. This would favor the effectiveness of antiviral response in patients with IFNL4 TT genotype, whereas the combination of standard regimen of pegIFN-α/ribavirin is not efficient in patients expressing IFNL4. Thus, sustained degranulation activity of T and NKT cells can be an asset to achieve the virus clearance under bi-therapy treatment.

Our survey on the frequency of immune cells engaged in degranulation process in human liver by direct ex vivo analyses showed a significant difference in the number of CD107a+ cells in T, NKT lymphocytes, and NK cells between patients with NASH and chronically HCV-infected patients. This observation clearly indicates that the baseline of degranulation activity is augmented in the presence of HCV. Importantly, this enhanced immune mediated activity is independent of the viral genotype. It has been very well documented that a large part of HCV proteins are incorrectly folded and accumulate in infected cells [38], which may lead to process of cell death. Therefore, the increase of degranulation activity of IHI cells during HCV infection is probably related to the fact that immune cells are engaged in the elimination of infected cells that present viral antigens as well as apoptotic cells.

Interestingly, in the same biopsy, the analysis of the frequency of the T, NKT, and NK cells engaged in degranulation process points out that serum ALT levels are linked to the frequency of CD107a positive NKT lymphocytes and that this correlation is also significant in patients with favorable and unfavorable IFNL4 genotype. Furthermore, regression model underlined that immunological parameters such as the frequency of NKT cell degranulation are a parameter contributing to the serum ALT activity. This highlighted the importance of focusing on the functions of immune liver cells in fresh liver biopsy and indicates that the determination of degranulation of immune cells is relevant accounting to the serum ALT levels. Furthermore, our data reinforce the involvement of NKT lymphocytes in the generation of hepatic lesions [18,21,24] and underline that the frequencies of CD107a positive NKT lymphocytes must be maintained to a low level to avoid adverse clinical consequences. These findings may be more important in HCV carriers expressing IFNL4 where the hepatocytes ISG levels remain high and an increased degranulation activity of

Figure 4. Degranulation activity of IHI cells and response to antiviral treatment. A, Box plot showing that decline of viral load from day 0 to week 4 was higher in patients with favorable IFNL4 TT/TG genotype than in those with unfavorable genotypes. B, Box plots showing that in patients bearing IFNL4 TT/TG genotype the degranulation activity of T lymphocytes is higher than in patients with unfavorable genotype. Abbreviations: IHI, intrahepatic immune; NK, natural killer; NKT, natural killer T.
IHI cells could not be balanced enough by a production of protective molecules such as IL-6 as would be required to maintain liver tissue integrity. Finally, one may consider that the serum ALT level mirror immune cell activity of the liver strengthening its importance for the clinical monitoring.

In conclusion, we propose that IFNL4 polymorphisms significantly influence local immune effectors in their function because patients with favorable genotypes appear to be more competent to develop a polarization of their immune cells and to induce and to ensure HCV clearance upon IFN-α/ribavirin treatment. Our data highlight a new link between degranulation activity of IHI cells IFNL4 polymorphisms and ALT levels. These findings further clarify the different behaviors and responses to treatment observed in patients accordingly to their IFNL4 genotypes helping to better guide future strategies for clinical management of HCV-infected patients.

Supplementary Data
Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes
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Contributors. E. J. M.: Study concept and design, analysis and interpretation of data, drafting of the manuscript, and study coordination. Z. M. J.: Acquisition of data, analysis and interpretation of data, and drafting of the manuscript. M. A. T.: Acquisition of data, analysis and interpretation of data, statistical analysis, and drafting of the manuscript. H. M., E. F., N. V. C., X. S. H.: Acquisition of data and technical support. A. M.: Material support. N. S.: Acquisition and interpretation of histology data. M. C.: Critical revision of the manuscript. V. L.: Material support. J. P. Z.: Material support, critical revision of the manuscript. P. N. M.: Critical revision of the manuscript and study supervision.

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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