Innate immune genes synergize to predict increased risk of chronic disease in hepatitis C virus infection

Megan M. Dring[a], Maria H. Morrison[a], Brian P. McSharry[a], Kieran J. Guinan[a], Richard Hagan[b], Irish HCV Research Consortium[1], Cliona O'Farrelly[c], and Clair M. Gardiner[a,2]

[a]Natural Killer Cell Group, School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland; [b]National Histocompatibility and Immunogenetics Reference Laboratory, Irish Blood Transfusion Service, Dublin 8, Ireland; and [c]Comparative Immunology, School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland

Edited* by Wayne M. Yokoyama, Washington University School of Medicine, St. Louis, MO, and approved February 25, 2011 (received for review November 1, 2010)

Hepatitis C is a common infection with significant morbidity and mortality, and only a minority of patients successfully clear the infection. Identification of factors that influence disease progression in HCV infection is difficult owing to the lack of well-defined patient cohorts. However, recent evidence supports a role for the innate immune system in virus clearance. In this study, we investigated innate immune genes for their contribution to disease progression in a unique cohort of well-controlled HCV-infected patients. The Irish cohort of HCV patients is uniquely homogenous; patients were infected with a single genotype of HCV from contaminated anti-D Ig. We genotyped 543 infected patients, including 247 patients who spontaneously resolved infection, for natural killer (NK) cell-associated killer cell Ig-like receptors (KIR) genes and the recently reported *IL28B (IFNλ3)* SNP. The NK cell gene *KIR2DS3* was significantly increased in patients with chronic infection [odds ratio (OR) 1.90, 95% confidence interval (CI) 1.25–2.90, $P < 0.002$]. The *IL28B* "T" allele was also significantly increased in chronically infected patients (OR 7.38, 95% CI 4.93–11.07, $P < 10^{-8}$). The presence of both markers synergized to significantly increase the risk of chronic infection over either factor alone (OR 20.11, 95% CI 9.05–44.68, $P < 10^{-7}$). In functional experiments, we found that IL28A significantly inhibited IFN-γ production by NK cells. Thus, we demonstrate a functional link between NK cells and type 3 IFN. Our findings may contribute to the development of a prognostic test for HCV and identify therapeutic strategies for the clinical management of HCV-infected patients.

Hepatitis C virus (HCV) is a common infection associated with significant morbidity and mortality worldwide (1). A successful immune response to HCV infection is thought to underlie the spontaneous resolution of infection that is observed in ≈20% of patients (1–3). However, most patients develop chronic infection, often leading to cirrhosis of the liver, hepatic cellular carcinoma, and liver failure (4). Identification of patients who naturally resolve HCV infection is a significant clinical challenge because these patients may not be aware of their infection status owing to mild clinical symptoms. This lack of well-defined cohorts has hindered identification of host immune genetic factors involved in viral persistence and clearance (5), thereby limiting opportunities for development of immunotherapies or immune-based prognostic tests for HCV infection.

Although a role for the adaptive immune response has been well established (6–9), more recent evidence supports a role for the innate immune system in response to HCV infection. This includes detection of viral infection, activation of effector cells, particularly natural killer (NK) cells, and the production of cytokines, of which the type 1 IFNs are particularly important (10, 11). Indeed, the only current treatment for chronic HCV infection is type 1 IFN (usually given in combination with ribavirin). NK cells can directly induce apoptosis of HCV-infected hepatocytes and themselves produce a range of antiviral cytokines (12, 13). Evidence is also accumulating that NK cell depletion or dysfunction may contribute to HCV persistence (14). NK cell activities are regulated in part through cell-surface receptors, including the killer cell–Ig-like receptors (KIR), encoded by a family of genes located on human chromosome 19q13.4. NK cells detect virally infected cells through KIR interactions with HLA class I. Several studies have investigated the role of KIR and HLA in HCV infection, but low sample numbers and heterogeneity of patient cohorts in terms of sex, age, ethnicity, route of infection, and HCV genotype have resulted in conflicting results (15–17). One larger study identified an inhibitory KIR gene, *KIR2DL3*, associated with resolution of HCV infection, and this was dependent on a homozygous HLA class I ligand background (11, 18, 19). More recent evidence to support a role for the innate immune system in HCV has come from a series of reports on SNPs in the *IL28B* gene region that can predict responsiveness of patients with chronic HCV to type 1 IFN treatment (20–22). One of these SNPs, rs12979860 (20), was also significant in predicting spontaneous resolution of HCV infection (23, 24). Although not within a coding region of a gene, SNP rs12979860 is found adjacent to the *IL28B* gene that encodes for a unique type 3 IFN called IFN-λ3. Little is known about the IFNλ family, but evidence is mounting to support a role for them in the immune response to viral infection (25, 26). It is of particular interest that both the NK cell-associated KIR genes and the *IL28B*-associated SNP are found in the same region of the genome. In light of these reports, we investigated the hypothesis that multiple immune-related loci in chromosome region 19q13 are involved in spontaneous clearance of HCV virus.

The patients in the present study were all infected with HCV through contaminated anti-D blood products in Ireland in 1977/8. As such, this cohort is extremely homogenous: all patients are female, fertile, and were aged 16–44 (mean 27.4 ± 5.5) y when infected. They come from a homogenous genetic background (Irish Caucasians) and were all infected through the same route with a similar, low level of inoculum and, importantly from an epidemiological viewpoint, they were all infected with a single source of HCV of defined genotype (genotype 1b) (27). Thus, they represent a uniquely informative cohort for the study of HCV that excludes many of the confounding variables associated with other studies. These women have been extensively studied and have a relatively high rate of resolution (≈45%) (27). They are therefore particularly interesting in the context of defining immunogenetic factors that determine whether an individual will eliminate virus or succumb to a lifetime chronic infection. To test

Author contributions: C.O'F. and C.M.G. designed research; M.M.D., M.H.M., B.P.M., K.J.G., R.H., and the I.H.R.C. performed research; M.M.D., M.H.M., B.P.M., K.J.G., R.H., and C.M.G. analyzed data; and M.M.D., C.O'F., and C.M.G. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]The Irish HCV Research Consortium members are as follows: Gary Courtney, Orla Crosbie, John Crowe, John Hegarty, Dermot Kelleher, Emer Lawlor, John Lee, Susan McKiernan, Frank Murray, Suzanne Norris, Cliona O'Farrelly, and Leila Thornton.

[2]To whom correspondence should be addressed. E-mail: clair.gardiner@tcd.ie.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1016358108/-/DCSupplemental.

our hypothesis, we undertook to investigate the contribution of *KIR*, *HLA* class I, and *IL28B* innate immune genes to resolution of infection in these patients.

Results

KIR2DS3 **Gene Frequency Is Increased in Chronic HCV Infection.** The present study investigated a total of 543 HCV-infected patients, of whom 296 were chronically infected and 247 had resolved infection. All patients were first typed for 14 KIR genes by PCR-SSP (sequence-specific primers) (Table 1). A single activatory gene, *KIR2DS3*, was significantly increased in chronically infected patients (0.314, $n = 92$) compared with those who resolved infection (0.194, $n = 47$, $P = 0.002$). The carrier frequency of the *KIR2DS3* gene in healthy, noninfected, Irish women was found to be 0.233 ($n = 116$), which was similar to the HCV-infected group as a whole (0.256, $n = 543$). Although not reaching statistical significance, the inhibitory gene *KIR2DL5* was also more frequent in chronically infected patients (0.526, $n = 153$) vs. resolvers (0.454, $n = 109$, $P = 0.100$). KIR gene haplotypes are broadly categorized as being either A-type, with a restricted gene content (characterized by *KIR3DL3*, *KIR2DL3*, *KIR2DL1*, *KIR2DL4*, *KIR3DL1*, *KIR2DS4*, and *KIR3DL2*), or B-type, which varies both in number and content of KIR genes (includes additional genes that encode activatory receptors) (28, 29). Because *KIR2DS3* and *KIR2DL5* are both genes associated with B-type KIR haplotypes, this suggested that certain B-haplotypes were increased in frequency in patients with chronic HCV infection.

KIR2DS3 **Predisposes to Chronic HCV Infection in the Presence of *HLA-C2*.** KIR and HLA genes are inherited on different chromosomes, and both ligand and receptor must be present for particular functional interactions to occur (30). We tested whether *KIR2DL3* was associated with resolution of HCV infection on an *HLA-C1* homozygous background, as has been previously reported. HLA class I genes can be stratified according to the ligands provided to KIR molecules. In brief, *C1* refers to *HLA-C* alleles that encode ligands for the KIR2DL3 receptor (including its common allotype, KIR2DL2), and *C2* refers to *HLA-C* alleles that encode ligands for the KIR2DL1 receptor (31, 32). Although not reaching statistical significance, we found a trend in our cohort toward a higher frequency of *KIR2DL3+* on *HLA-C1/C1* background in resolved patients (0.429, $n = 91$) vs. chronically infected patients (0.356, $n = 95$, $P = 0.101$; Table 2), a finding previously reported by Khakoo et al. (18). However, examination of the interaction on receptor and ligand homozygous backgrounds showed no significant effect on resolution of HCV infection (*KIR2DL3/KIR2DL3/C1/C1*: 0.191, $n = 53$ and 0.236, $n = 54$ for chronic and resolved patients, respectively, $P = 0.243$; Table 2). On the basis of the finding that the *KIR2DS3* gene frequency is significantly increased in patients with chronic HCV infection (Table 1) and that multiple KIR genes with different HLA specificities are inherited on haplotypes, and the fact that HLA can profoundly influence the NK cell response, we tested whether the association of *KIR2DS3* with chronic infection was influenced by the *HLA-C* genetic background. When analyzed in terms of HLA class I ligands, *KIR2DS3* was only significantly increased in patients with chronic HCV infection when present on a *HLA-C2*$^+$ genetic background (Table 2).

Table 1. ***KIR2DS3*** **gene is increased in chronic HCV infection**

	Frequencies		Chronic vs. resolvers	
KIR gene	Chronic HCV (*n*)	Resolvers (*n*)	χ^2 (*P*)	OR (95% CI)
KIR2DS2	0.519 (152)	0.469 (115)	1.30 (0.25)	1.22 (0.86–1.74)
KIR2DL3	0.898 (265)	0.918 (223)	0.59 (0.44)	0.79 (0.42–1.49)
KIR2DL2	0.527 (155)	0.486 (118)	0.92 (0.34)	1.18 (0.83–1.68)
KIR2DL5	0.526 (153)	0.454 (109)	2.70 (0.10)	1.33 (0.93–1.91)
KIR2DS3	0.314 (92)	0.194 (47)	9.89 (0.002)*†	1.90 (1.25–2.90)*
KIR2DL1	0.976 (287)	0.959 (235)	1.31 (0.25)	1.76 (0.61–5.21)
KIR3DL1	0.966 (283)	0.979 (235)	0.12 (0.73)	0.84 (0.28–2.45)
KIR3DS1	0.405 (115)	0.389 (91)	0.14 (0.71)	1.07 (0.74–1.55)
KIR2DS5	0.284 (83)	0.321 (79)	0.86 (0.35)	0.84 (0.57–1.24)
KIR2DS1	0.381 (111)	0.395 (96)	0.10 (0.75)	0.94 (0.66–1.36)
KIR2DS4	0.966 (284)	0.963 (237)	0.03 (0.87)	1.08 (0.40–2.93)

Carrier frequency of KIR genes in 296 chronic HCV patients compared with 247 spontaneous resolvers. Differences in frequency distribution between populations were tested for significance by χ^2 test. A positive OR indicates an association with increased risk for chronic HCV.
*Significant value.
†Bonferroni corrected *P* value for multiple comparisons, $P = 0.022$.

Identification of a Specific KIR Haplotype Associated with Chronic HCV Infection. Given that KIR receptors are encoded for by multiple functionally related genes at a single locus, it is likely that the inheritance of a haplotype rather than a single KIR gene is the source of the significant association found. Furthermore, because our results show that *KIR2DS3* and *KIR2DL5* genes are increased in patients with chronic HCV infection and that these are in high linkage disequilibrium (LD) together (D' = 0.89), this suggested KIR B-haplotype involvement (33). The *KIR2DL4* gene has previously been shown to separate KIR genes in the centromeric from the telomeric end of the KIR gene cluster, with LD stronger between the genes within each end (33, 34). Haplotypes were reconstructed using *KIR3DL3*, *KIR2DS2*, *KIR2DL3*, *KIR2DL5*, *KIR2DS3*, and *KIR2DL1* genes, which are all found centromeric of the *KIR2DL4* gene. A total of 25 haplotypes (of which the 12 most frequent are shown in Table 3), accounting for 100% of haplotypes, were generated and used in subsequent analysis. Comparison of patient groups demonstrated that a single centromeric KIR haplotype was significantly increased in patients with chronic infection (0.154, $n = 90.0$) compared with resolved patients (0.091, $n = 44.2$, $P = 0.002$), and as predicted, it contained *KIR2DL5* and *KIR2DS3* genes (Table 3). Analysis of the frequency distribution of reconstructed telomeric gene haplotypes (*KIR2DL4*, *KIR3DL1/S1*, *KIR2DS1*, *KIR2DS4*, and *KIR3DL2*) revealed no differences between the patient groups (Table S1), further supporting the lack of involvement of the telomeric end of the KIR gene locus with chronic HCV infection.

IL28B **Adjacent SNP, rs12979860, Is Associated with Chronic HCV Infection.** All of the HCV study cohort were typed for the rs12989760 SNP (hereafter referred to as *IL28B*). This SNP is defined by either a C or a T nucleotide. When the C and T allele frequencies were compared between the spontaneously resolving and the chronic HCV-infected groups, the T allele was found to be significantly increased in the chronic group [Table 4; 0.134 vs. 0.395, $P < 10^{-8}$; odds ratio (OR) 4.20, 95% confidence interval (CI) 3.05–5.79]. Further analysis showed that both the CT and the TT genotypes were significantly increased in the chronically infected group compared with the spontaneous resolvers, indicating that the *IL28B-T* allele has a dominant effect (Table 4). This conclusion was also supported by analysis showing that *IL28B* genotypes were in Hardy-Weinberg equilibrium (HWE) for the spontaneous resolvers ($P > 0.05$) but were out of HWE, with an excess of heterozygotes, in the chronically infected group. Typing for *IL28B*-associated SNP in a group of healthy Irish controls ($n = 173$) confirmed the genotypes to be in HWE and at a similar frequency to the complete HCV cohort.

Combination of *IL28B-T* and *KIR2DS3* Synergize to Increase the Risk of Developing Chronic HCV Infection. To test for independence of the *KIR2DS3* association, in the light of the much stronger association of the *IL28B* SNP and the fact that both loci are found in the same chromosomal region (19q13), carrier frequencies for *KIR2DS3*

IMMUNOLOGY

Table 2. *KIR2DS3* is associated with chronic HCV infection only in the presence of *HLA-C2*

	Frequencies		Chronic vs. resolvers	
KIR–HLA	Chronic HCV (*n*)	Resolvers (*n*)	χ^2 (*P*)	OR (95% CI)
KIR2DL3⁺ *C1/C1*	0.356 (95)	0.429 (91)	2.68 (0.10)	0.73 (0.50–1.08)
KIR2DL3/KIR2DL3/C1/C1	0.191 (53)	0.236 (54)	1.36 (0.24)	0.77 (0.49–1.20)
KIR2DL2⁺ *C1/C1*	0.179 (50)	0.182 (41)	0.01 (0.93)	0.98 (0.61–1.59)
KIR2DL2/KIR2DL2/C1/C1	0.041 (12)	0.017 (4)	2.70 (0.10)	2.53 (0.75–9.43)
KIR2DS3⁺ *C1/C1*	0.079 (22)	0.060 (14)	0.72 (0.39)	1.35 (0.64–2.86)
KIR2DS3⁺ *C2+*	0.198 (55)	0.098 (23)	9.75 (0.002)*†	2.26 (1.30–3.94)*

Frequency of *KIR2DL3* and *KIR2DL2* genes in combination their HLA C ligand (C1) were compared in 296 chronic HCV patients and 247 spontaneous resolvers, as well as *KIR2DS3* with either *HLA-C1* homozygotes or *HLA-C2* carriers. KIR⁺ indicates carriers for that gene; HLA/HLA indicates a particular HLA genotype; HLA⁺ indicates carriers for that HLA allele; *KIR2DL2/KIR2DL2*, *KIR2DL3/KIR2DL3*, and *C1/C1* indicate homozygosity for that *KIR* gene or HLA type. Calculations as for Table 1.

*Significant value.

†Bonferroni corrected *P* value for multiple comparisons, *P* = 0.012.

were stratified by the presence of the *IL28B-T* allele and found to be significantly associated with the chronic group irrespective of the *IL28B-T* allele. Equally, when stratified by the presence/absence of *KIR2DS3*, the *IL28B-T* allele carriers were still significantly increased in the chronic group (Table S2). Thus, each locus on its own increases the risk of developing chronic HCV infection.

Because these two genes encode for molecules involved in the innate immune response to virus, we tested for the possibility of interaction between these two risk factors. Statistical interaction between *KIR2DS3* and *IL28B-T* was tested for by departure from additivity (35–37). ORs were calculated by multinomial logistic regression, and the presence of both alleles (i.e., *KIR2DS3* carriers and *IL28B-T* carriers) compared with the absence of both (*KIR2DS3* negative and *IL28B-CC*) resulted in a strikingly increased OR in chronic HCV-infected patients (Fig. 1; OR 20.11, 95% CI 9.05–44.68, $P < 10^{-7}$). This synergy was confirmed to be statistically significant, with a Synergy index (S) of 2.43 (95% CI 1.03–5.68) and with the attributable proportion due to interaction (AP) 55.9% (95% CI 20.80–91.00%) (Fig. 1). No additional risk was provided by the presence of HLA-C2 (Table S3). In summary, the presence of both "risk" alleles, *KIR2DS3* and *IL28B-T*, synergizes to significantly increase the risk of chronic HCV infection compared with the presence of either marker alone.

IL28A Inhibits IFN-γ Production by Human NK Cells. Our genetic data demonstrated synergy between NK cell receptors and type 3 IFNs in increasing risk of chronic HCV infection. However, no functional link between NK cells and type 3 IFNs has ever been shown. We therefore carried out experiments to explore a direct link between NK cells and some of the type 3 IFN family of cytokines. Our experiments focused on IFN-γ production by NK cells as it is a key component of the NK cell response to virus. We found that IL28A (and IL28B to a lesser extent) significantly inhibited IFN-γ production by human NK cells (Fig. 2; $P < 0.05$). Although the inhibition was not profound, eight of 11 donors showed decreased production of IFN-γ in response to IL12+IL15 in the presence of IL28A. Furthermore, three individual donors within the group seemed to be particularly sensitive to the effects of IL28A, and in these, IFN-γ production was completely inhibited. Changes in CD69 expression, an antigen expressed by activated lymphocytes, correlated with the cytokine results ($n = 8$). In donors sensitive to IL28A inhibition of IFN-γ production, IL28A also robustly inhibited CD69 expression on NK cells, whereas there was no change in CD69 expression in donors that had low or no inhibition of IFN-γ production in response to IL28A. Our data demonstrate a direct interaction between NK cells and type 3 IFN and suggest that IL28A has some inhibitory effects on NK cell functions and that these are particularly potent in certain individuals. However,

Table 3. Genes contributing to the association with chronic HCV infection are found in the centromeric part of the KIR haplotype

	Centromeric KIR genes						Chronic HCV frequency (2N = 586)	Resolver frequency (2N = 486)	Chronic vs. resolvers	
Haplotype	*KIR3DL3*	*KIR2DS2*	*KIR2DL3/2*	*KIR2DL5*	*KIR2DS3*	*KIR2DL1*			χ^2 (*P*)	OR (95% CI)
1	■		3			■	0.5196	0.5537	1.29 (0.26)	0.87 (0.68–1.12)
2	■		3	■		■	0.1242	0.1321	0.12 (0.73)	0.94 (0.64–1.37)
3*	■	■	2	■	■	■	0.1535*	0.0909*	9.66 (0.002)*	1.82 (1.22–2.72)*
4	■	■	2			■	0.0820	0.0859	0.07 (0.79)	0.94 (0.60–1.49)
5	■	■	2				0.0250	0.0420	2.04 (0.15)	0.61 (0.29–1.27)
6	■	■	2	■		■	0.0256	0.0301	0.27 (0.60)	0.82 (0.38–1.80)
7	■		3	■	■	■	0.0259	0.0209	0.29 (0.59)	1.25 (0.52–3.02)
8	■	■	2	■			0.0158	0.0227	0.77 (0.38)	0.67 (0.25–1.76)
9	■		2			■	0.0061	0.0083	(1.00)	0.83 (0.17–3.95)
10	■		3				0.0062	0.0052	(1.00)	1.11 (0.21–6.24)
11	■		3		■	■	0.0032	0.0028	(1.00)	1.66 (0.12–46.36)
12	■		2	■	■	■	0.0026	0.0020	(1.00)	1.66 (0.12–46.36)

Estimated haplotype frequencies for centromeric KIR genes in chronic HCV patients were compared with those in spontaneous resolvers. Shaded boxes indicate presence of a gene (with paler shading and a "2" for the *KIR2DL2* allele of a *KIR2DL3*), and unshaded boxes indicate absence of a gene on a haplotype. Differences in haplotype distribution between populations were tested for significance by χ^2 test or Fisher exact test where appropriate.

*Significant value.

Table 4. *IL28B* is associated with an increased risk of chronic HCV

IL28B C/T genotype	Frequencies: Chronic HCV (*n*)	Frequencies: Resolvers (*n*)	Chronic vs. resolvers: χ^2 (*P*)	Chronic vs. resolvers: OR (95% CI)
CC	0.296 (87)	0.756 (183)		
CT	0.619 (182)	0.219 (53)		
TT	0.085 (25)	0.025 (6)	112.61 ($<10^{-8}$)*	n/a
C	0.605 (356)	0.866 (419)		
T	0.395 (232)	0.134 (65)	89.78 ($<10^{-8}$)*	4.20 (3.05–5.79)*
CC	0.296 (87)	0.756 (183)		
CT+TT	0.704 (207)	0.244 (59)	112.49 ($<10^{-8}$)*	7.38 (4.93–11.07)*
CC	0.296 (87)	0.756 (183)		
TT	0.085 (25)	0.025 (6)	27.91 (10^{-7})*	8.76 (3.26–24.82)*

Genotype and allele frequency distributions of the *IL28B* SNP (rs12979860) were compared in 294 chronic HCV patients and 242 spontaneous resolvers. Differences in frequency distribution between populations were tested for significance by χ^2 test. A positive OR indicates an association with increased risk for chronic HCV. n/a, test not applicable.
*Significant value.

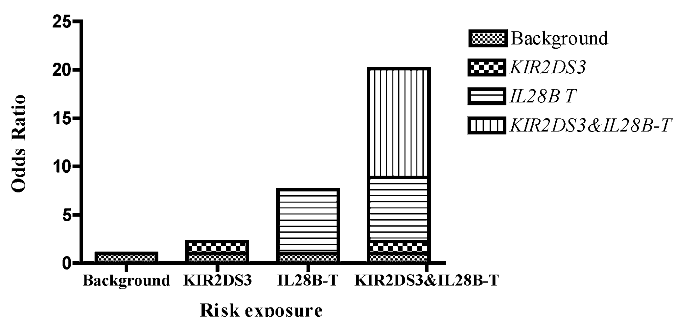
there was no apparent correlation between the IL28B SNP and sensitivity to the effects of either IL28A or IL28B.

Discussion

Multiple factors, many immunological, have been implicated in determining disease outcome in HCV infection (1, 2). Some individuals naturally clear infection, whereas most succumb to chronic disease, resulting in liver cirrhosis and/or hepatic carcinoma, which may require liver transplantation (4). Therefore, identification of factors involved in the persistence of viral infection is important and may lead to new therapeutic interventions, the development of specific prognostic tests, and/or improved treatment management for patients. Although emphasis has previously been placed on the importance of an effective adaptive immune response (6–9), evidence from this study and others (18, 23) highlights a role for the innate immune response in regulating disease progression in HCV infection.

The Irish cohort of HCV-infected patients is extremely important because we can directly compare patients who resolve infection or develop chronic infection. Conventional analysis of individual genes identified *KIR2DS3* as an NK cell-associated KIR gene that was at significantly increased frequency in patients who resolved infection compared with patients who developed chronic HCV infection. Although trends for particular HLA associations were observed (Table S4), no definitive associations were found for HLA class I genes in terms of their provision of ligands for NK cell receptors (*C1*, *C2*, *Bw4*). Importantly, our data also support the previously reported finding of a role for KIR genes in resolving HCV infection in a large independent cohort (18). Although not reaching statistical significance, we found that *KIR2DL3*+ on an *HLA-C1/C1* background in our cohort was increased in patients who resolve infection and conversely, the absence of *KIR2DL3* (defined by *KIR2DL2* allele homozygosity) was associated with an increase in chronic infection on an *HLA-C1* genetic background (OR 2.53, 95% CI 0.75–9.43). Our study provides information for a revised model of the role of NK cells in HCV infection because it identifies a strong influence of a KIR B haplotype (and *HLA-C2*) on the development of chronic infection, whereas previously a beneficial effect of a KIR A haplotype (with *HLA-C1*) was observed (18). Differences in host genetics in terms of the combinations of NK cell receptors and ligands present clearly affect disease outcome.

Within our patient cohort, we found a strong association between the presence of the NK cell-associated *KIR2DS3* and chronic HCV infection. *KIR2DS3* has previously been implicated as a risk factor in different viral infections (38, 39) and as a risk factor for graft-vs.-host disease after stem cell transplant for hematological malignancies (40, 41). However, the little that is known about the biology of the receptor does not support a role for it as a functional receptor. Indeed, the *KIR2DS3* gene itself has been lost from some populations, and KIR2DS3 protein has been shown to have little or no cell-surface expression in transfection experiments (42). Furthermore, no avidity for HLA-C1, HLA-C2, or any other HLA class 1 epitope



Exposure	OR (CI)[a]	P	S (CI)[b]	AP (CI)[c]
KIR2DS3	2.27 (1.28-4.02)	0.003		
IL28B-T	7.59 (4.86-11.87)	$<10^{-7}$		
KIR2DS3&IL28B-T	20.11 (9.05-44.68)	$<10^{-7}$	2.43 (1.03-5.68)	55.9% (20.8-91.0%)

Fig. 1. Presence of both *KIR2DS3* and *IL28B-T* synergize to increase the risk of developing chronic HCV infection. ORs for *KIR2DS3* alone, *IL28B-T* carriers (CT or TT) alone, and *KIR2DS3* with *IL28B-T* compared with neither risk factor present were calculated from multinomial logistic regression and used to calculate S and AP. The contribution of individual and combined risk factors to the ORs is graphically represented in the bar chart. Positive OR indicates an association with increased risk for chronic HCV. [a]Odds ratio (95% confidence interval). [b]Synergy index (95% confidence interval). [c]Attributable proportion for interaction (95% confidence interval).

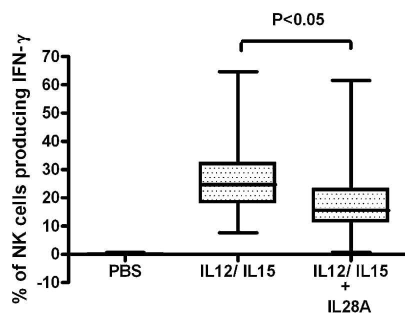


Fig. 2. IL28A inhibits IFN-γ production by human NK cells. Peripheral blood mononuclear cells ($n = 11$) from healthy normal donors were stimulated with PBS or IL12 (30 ng/mL)/IL15 (100 ng/mL) in the presence or absence of IL28A (500 ng/mL). Percentage of $CD56^{+}$ $CD3^{-}$ NK cells expressing IFN-γ as measured by intracellular staining is shown. Horizontal lines indicate the median percentages, and vertical lines indicate the range of values. A paired Student *t* test was used to compare data.

tested was found, and its ligand remains unknown (43). Although it is tempting to speculate a direct role for KIR2DS3 in the immune response to HCV, it is likely that *KIR2DS3* is a marker for a haplotype that is contributing to the development of chronic viral infection. The association between *KIR2DS3* and chronic infection was only seen on an *HLA-C2* genetic background. Because *HLA-C2* is not a ligand for *KIR2DS3* (43), the receptor interacting with HLA-C2 is probably encoded for by a gene in LD with *KIR2DS3* (either within the NK complex or the wider leukocyte receptor complex genomic region). Indeed, our analysis led to identification of a specific *KIR* haplotype in the centromeric haplotype block that is associated with chronic HCV infection in patients. This haplotype also included *KIR2DL2*, *KIR3DL3*, *KIR2DS2*, and *KIR2DL1* genes, some of which have been found to be associated with chronic HCV infection in other studies (16, 18). Indeed, because *KIR2DL1* and *KIR2DL2* both encode receptors that bind to HLA-C2 (32, 44), these are possible candidate genes for explaining the increased risk of chronic infection associated with *KIR2DS3* and *HLA-C2*.

Even more striking than the association with the KIR genes was the association of the *IL28B* SNP as a predictor of developing chronic HCV infection. The presence of a T allele was sufficient to confer an increased risk of chronic infection. These data confirm the previous finding of Thomas et al. (23) in a second large cohort of spontaneous HCV-resolving patients. The statistical strength of the association found between *IL28B-T* and chronic infection is very robust; however, because the SNP is located in the intergenic region between *IL28A* and *IL28B* genes, the biological mechanism behind the genetic association remains to be elucidated. Our data suggested a synergistic interaction between the unique type 3 IFN and NK cells in an antiviral immune response. We investigated this at a functional level and found that IL28A inhibited IFN-γ production by NK cells. It has been shown by numerous studies that NK cells in patients with chronic HCV infection have normal or relatively higher cytotoxic activity (14, 45, 46), leading to the suggestion that functional or activated NK cells may contribute to the chronic persistence of infection in these patients. Indeed, a relative expansion of $CD56^{bright}$ cells, known to produce IFN-γ, has previously been reported for the present cohort of patients (14), and high levels of IFN-γ expression have been reported in the livers of both humans and chimpanzees with chronic HCV infection (47, 48). Attenuation of chronically activated NK cells may therefore be beneficial in the treatment of persistent HCV infection. Our findings support this concept: we have shown that one of these cytokines, IL28A, can significantly inhibit NK cell activation. Further experiments will be required to elucidate the full extent of the functional synergy between NK cells and type 3 IFN in the immune response to virus.

The homogeneous nature of our cohort (all female, all infected with the same genotype of virus) allowed identification of factors involved in the development of chronic HCV infection that might not have been identified in a heterogeneous cohort. From a clinical perspective, we found that the combination of *KIR2DS3* and the *IL28B-T* allele dramatically increased the OR for development of chronic HCV (OR 20.11, 95% CI 9.05–44.68) compared with either risk factor alone (OR 2.27, 95% CI 1.28–4.02 for *KIR2DS3* and OR 7.59, 95% CI 4.86–11.87 for presence of *IL28B-T* allele; S 2.43, 95% CI 1.03–5.68). This was independent of the HLA background of the patients. Although this synergistic response needs to be validated in a heterogeneous HCV patient cohort, these data may contribute to the development of a relatively simple and specific host genotype test for high-risk patients or healthcare workers that will predict the clinical outcome in HCV infection more accurately that any current prognostic indicator. In summary, our data provide a significant advance in terms of understanding the role of the immune system in disease progression during HCV infection. This has clinical potential to lead to new therapeutic interventions and may also contribute to the development of a robust prognostic test for HCV-infected individuals.

Materials and Methods

Patient Cohort. The study population consisted of a well-defined cohort of females who had been inoculated with HCV (genotype 1b)-contaminated anti-D immunoglobin, as described in detail elsewhere (27, 49). On a 17-y follow-up, only 55% of subjects who were antibody-positive had chronic HCV infection (27). Of the 543 patients involved in this study, 296 had developed chronic HCV infection, and 247 had spontaneously resolved infection. Informed written consent was obtained from each patient, and the study received ethical approval from the Research and Ethics Committee at St. Vincent's University Hospital.

Genotyping for KIR Genes and the *IL28B*-Associated SNP rs12979860. DNA was isolated from blood, using the Qiamp DNA blood Mini Kit system (Qiagen, Hilden). The presence or absence of 14 KIR genes (*KIR3DL3*, *KIR2DS2*, *KIR2DL3*, *KIR2DL2*, *KIR2DL5*, *KIR2DS3*, *KIR2DL1*, *KIR2DL4*, *KIR3DL1*, *KIR3DS1*, *KIR2DS5*, *KIR2DS1*, *KIR2DS4*, and *KIR3DL2*) was determined using a PCR-SSP method as described by Vilches et al. (50). The PCR-SSP discriminated between full-length and deleted versions of *KIR2DS4* alleles, and the presence of two bands indicated heterozygosity for these forms (50). Genotyping for the rs12979860 SNP was performed using the ABI *Taqman* allelic discrimination kit (23). For routine quality control purposes, ≈10% of samples were retyped anonymously, and no mismatches were found.

Cell Stimulation and IFN-γ Intracellular Staining for Flow Cytometric Analysis. Peripheral blood mononuclear cells were isolated from venous blood of healthy normal donors by density gradient centrifugation. Cells were stimulated for 18 h at a density of 1.5×10^{6} cells/mL, the last 4 h in the presence of Golgi-Plug (BD Pharmingen); 100 ng/mL rhIL15, 30 ng/mL rhIL12 (Strathmann Biotec), and 500 ng/mL of either IL28A or IL28B (R&D Systems) were used. NK cells were stained and analyzed for intracellular production of IFN-γ as previously described (51).

Statistical Analysis. Genotype, allele, and carrier frequency differences and OR trend tests between populations were tested for significance by direct counting using a χ^2 test, or when sample size was <5 for the contingency table, a Fisher exact test as implemented by EPI-INFO 3.5.1. HWE was estimated using GenePop 4.0 (www.genepop.curtin.edu.au). KIR haplotypes were reconstructed using PHASEv2.1.1 (52, 53). A number of constraints were placed on the haplotype reconstruction on the basis of LD and known haplotype structure within the KIR gene complex (33); these are detailed in *SI Text*.

Differences in haplotype distribution between populations were tested for significance using a χ^2 or Fisher exact test. Statistical interaction between *KIR2DS3* and *IL28B* was evaluated by departure from additivity using the method developed by Andersson et al. (35–37) based on ORs derived from multinomial logistic regression (release 16.0; SPSS Inc.). An S >1 and an AP >0 indicate a significant synergystic interaction.

A paired Student *t* test performed using PRISM software (version 4.0; Graphpad Software Inc.) was used to investigate the effect of either IL28A or IL28B on IFN-γ production by human NK cells, and $P < 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS. We thank all the patients and medical staff who cooperated in this study; Mary Carrington, Carlos Vilches, Andrew Lloyd, and Liz Ryan for helpful comments on the manuscript; and Sean Hegarty and Brian Graham of Irish Blood Transfusion Service for performing additional HLA class I typing. Funding for the project was provided by an Irish Health Research Board Project grant. Additional funding for C.M.G. was provided by a Science Foundation Ireland Principal Investigator grant.

1. Alter HJ, Seeff LB (2000) Recovery, persistence, and sequelae in hepatitis C virus infection: A perspective on long-term outcome. *Semin Liver Dis* 20:17–35.
2. Rehermann B (2009) Hepatitis C virus versus innate and adaptive immune responses: A tale of coevolution and coexistence. *J Clin Invest* 119:1745–1754.
3. Micallef JM, Kaldor JM, Dore GJ (2006) Spontaneous viral clearance following acute hepatitis C infection: A systematic review of longitudinal studies. *J Viral Hepat* 13: 34–41.
4. Shepard CW, Finelli L, Alter MJ (2005) Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis* 5:558–567.
5. Cox AL, et al. (2009) Rare birds in North America: Acute hepatitis C cohorts. *Gastroenterology* 136:26–31.
6. Cooper S, et al. (1999) Analysis of a successful immune response against hepatitis C virus. *Immunity* 10:439–449.
7. Lechner F, et al. (2000) Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* 191:1499–1512.
8. Thimme R, et al. (2002) Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci USA* 99:15661–15668.
9. Thimme R, et al. (2001) Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* 194:1395–1406.
10. Ahlenstiel G, et al. (2010) Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner. *Gastroenterology* 138: 325–335.e1-2.
11. Stegmann KA, et al. (2010) Interferon-alfa-induced tumor necrosis factor-related apoptosis-inducing ligand on natural killer cells is associated with control of hepatitis C virus infection. *Gastroenterology* 138:1885–1887.
12. Golden-Mason L, Rosen HR (2006) Natural killer cells: Primary target for hepatitis C virus immune evasion strategies? *Liver Transpl* 12:363–372.
13. Lodoen MB, Lanier LL (2006) Natural killer cells as an initial defense against pathogens. *Curr Opin Immunol* 18:391–398.
14. Golden-Mason L, et al. (2008) Altered natural killer cell subset distributions in resolved and persistent hepatitis C virus infection following single source exposure. *Gut* 57:1121–1128.
15. Montes-Cano MA, et al. (2005) HLA-C and KIR genes in hepatitis C virus infection. *Hum Immunol* 66:1106–1109.
16. Paladino N, et al. (2007) Increased frequencies of activating natural killer receptors are associated with liver injury in individuals who do not eliminate hepatitis C virus. *Tissue Antigens* 69(Suppl 1):109–111.
17. Rauch A, et al.; Swiss HIV Cohort Study (2007) Influence of inhibitory killer immunoglobulin-like receptors and their HLA-C ligands on resolving hepatitis C virus infection. *Tissue Antigens* 69(Suppl 1):237–240.
18. Khakoo SI, et al. (2004) HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* 305:872–874.
19. Knapp S, et al. (2010) Consistent beneficial effects of killer cell immunoglobulin-like receptor 2DL3 and group 1 human leukocyte antigen-C following exposure to hepatitis C virus. *Hepatology* 51:1168–1175.
20. Ge D, et al. (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 461:399–401.
21. Suppiah V, et al. (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 41:1100–1104.
22. Tanaka Y, et al. (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 41: 1105–1109.
23. Thomas DL, et al. (2009) Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 461:798–801.
24. Tillmann HL, et al. (2010) A polymorphism near IL28B is associated with spontaneous clearance of acute hepatitis C virus and jaundice. *Gastroenterology* 139:1586–1592, 1592.e1.
25. Marcello T, et al. (2006) Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology* 131:1887–1898.
26. Robek MD, Boyd BS, Chisari FV (2005) Lambda interferon inhibits hepatitis B and C virus replication. *J Virol* 79:3851–3854.
27. Kenny-Walsh E; Irish Hepatology Research Group (1999) Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. *N Engl J Med* 340: 1228–1233.
28. Uhrberg M, et al. (1997) Human diversity in killer cell inhibitory receptor genes. *Immunity* 7:753–763.
29. Marsh SG, et al. (2003) Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Hum Immunol* 64:648–654.
30. Parham P (2005) MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol* 5:201–214.
31. Biassoni R, et al. (1997) Role of amino acid position 70 in the binding affinity of p50.1 and p58.1 receptors for HLA-Cw4 molecules. *Eur J Immunol* 27:3095–3099.
32. Winter CC, Gumperz JE, Parham P, Long EO, Wagtmann N (1998) Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *J Immunol* 161:571–577.
33. Middleton D, Meenagh A, Gourraud PA (2007) KIR haplotype content at the allele level in 77 Northern Irish families. *Immunogenetics* 59:145–158.
34. Martin MP, Single RM, Wilson MJ, Trowsdale J, Carrington M (2008) KIR haplotypes defined by segregation analysis in 59 Centre d'Etude Polymorphisme Humain (CEPH) families. *Immunogenetics* 60:767–774.
35. Andersson T, Alfredsson L, Källberg H, Zdravkovic S, Ahlbom A (2005) Calculating measures of biological interaction. *Eur J Epidemiol* 20:575–579.
36. Rothman KJ (1976) The estimation of synergy or antagonism. *Am J Epidemiol* 103: 506–511.
37. Rothman KJ, Greenland S, Walker AM (1980) Concepts of interaction. *Am J Epidemiol* 112:467–470.
38. Zhi-ming L, et al. (2007) Polymorphisms of killer cell immunoglobulin-like receptor gene: Possible association with susceptibility to or clearance of hepatitis B virus infection in Chinese Han population. *Croat Med J* 48:800–806.
39. Wauquier N, Padilla C, Becquart P, Leroy E, Vieillard V (2010) Association of KIR2DS1 and KIR2DS3 with fatal outcome in Ebola virus infection. *Immunogenetics* 62: 767–771.
40. Zhao XY, Huang XJ, Liu KY, Xu LP, Liu DH (2007) Prognosis after unmanipulated HLA-haploidentical blood and marrow transplantation is correlated to the numbers of KIR ligands in recipients. *Eur J Haematol* 78:338–346.
41. McQueen KL, et al. (2007) Donor-recipient combinations of group A and B KIR haplotypes and HLA class I ligand affect the outcome of HLA-matched, sibling donor hematopoietic cell transplantation. *Hum Immunol* 68:309–323.
42. VandenBussche CJ, Mulrooney TJ, Frazier WR, Dakshanamurthy S, Hurley CK (2009) Dramatically reduced surface expression of NK cell receptor KIR2DS3 is attributed to multiple residues throughout the molecule. *Genes Immun* 10:162–173.
43. Moesta AK, et al. (2010) Humans differ from other hominids in lacking an activating NK cell receptor that recognizes the C1 epitope of MHC class I. *J Immunol* 185: 4233–4237.
44. Moesta AK, et al. (2008) Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. *J Immunol* 180:3969–3979.
45. Cheent K, Khakoo SI (2011) Natural killer cells and hepatitis C: Action and reaction. *Gut* 60:268–278.
46. Koziel MJ (2006) NK cells: Natural born killers in the conflict between humans and HCV. *Hepatology* 43:395–397.
47. Baroni GS, et al. (1999) Hepatic stellate cell activation and liver fibrosis are associated with necroinflammatory injury and Th1-like response in chronic hepatitis C. *Liver* 19: 212–219.
48. Major ME, et al. (2004) Hepatitis C virus kinetics and host responses associated with disease and outcome of infection in chimpanzees. *Hepatology* 39:1709–1720.
49. Fanning LJ (2002) The Irish paradigm on the natural progression of hepatitis C virus infection: An investigation in a homogeneous patient population infected with HCV 1b (review). *Int J Mol Med* 9:179–184.
50. Vilches C, Castaño J, Gómez-Lozano N, Estefanía E (2007) Facilitation of KIR genotyping by a PCR-SSP method that amplifies short DNA fragments. *Tissue Antigens* 70:415–422.
51. Athié-Morales V, O'Connor GM, Gardiner CM (2008) Activation of human NK cells by the bacterial pathogen-associated molecular pattern muramyl dipeptide. *J Immunol* 180:4082–4089.
52. Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* 68:978–989.
53. Stephens M, Donnelly P (2003) A comparison of bayesian methods for haplotype reconstruction from population genotype data. *Am J Hum Genet* 73:1162–1169.