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# HCV-specific cellular immune responses in subjects exposed to but uninfected by HCV

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# Interleukin 12B gene polymorphism and apparent resistance to hepatitis C virus infection

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## Introduction

Hepatitis C virus (HCV) infection represents a major public health problem worldwide, with an estimated 180 million infected individuals [1]. HCV is a blood-borne virus, with the vast majority of new infections in developed countries arising as a consequence of intravenous drug use [2,3]. Chronic infection with persisting viraemia is the norm, with only a minority of infected individuals clearing detectable HCV spontaneously [4]. Anti-viral treatment with pegylated interferon and ribavirin can clear virus in more than 50% of chronically infected cases [5,6]. Both spontaneous and treatment-related resolution of infection have been shown to be mediated by cellular immune responses, particularly T helper (Th) cell responses of Th1 type [7–10]. The genetic influence over the generation of these advantageous immune responses has been much studied [11,12]. Interleukin (IL)-12 is a heterodimer of p35 and p40 subunits and is a key cytokine in promoting anti-viral Th1 responses. The gene encoding IL-12 p40 (IL12B) is polymorphic and located on

## Summary

Cellular immunity with interferon gamma production could have a role in protection from hepatitis C virus (HCV). Interleukin (IL)-12 is a key cytokine in promoting such anti-viral T helper 1 (Th1) responses. We hypothesized that a genetic background able to promote cellular responses may be associated with apparent protection from infection and have investigated the distribution of the functional 1188A/C polymorphism of IL-12B in HCV exposed but uninfected cases. The frequency of the high IL-12-producing C allele was determined by restriction enzyme genotyping in 76 exposed–uninfected individuals and 105 healthy controls. Overall, the C allele was found in 27.6% of exposed–uninfected cases compared with 16.7% of healthy controls [ $\chi^2 = 6.3$ ,  $P = 0.02$ , odds ratio (OR) = 1.9, 95% confidence interval (CI) = 1.1–3.2]. CC genotype was found in 10.5% of exposed–uninfected cases compared with 0.9% controls ( $\chi^2 = 9.3$ ,  $P = 0.01$ , OR = 12, 95% CI = 1.5–100). Individuals at high risk of HCV infection yet who remain uninfected may be resistant in some way to infection. In our cohort of exposed–uninfected cases a genetic background of enhanced IL-12 production was associated with apparent resistance to HCV infection. This lends support to a central role for cellular immune responses in protecting from infection.

**Keywords:** cytokine, hepatitis C virus, polymorphism, protection from infection

5q31–33 [13] and a functional single nucleotide polymorphism (A/C) of the 3' untranslated region (3'UTR) at position 1188 has been identified recently [14]. The variant C allele of the 1188A/C polymorphism has been associated with enhanced IL-12 production [15]. This polymorphism is associated with susceptibility to multiple sclerosis and insulin-dependent diabetes mellitus [16,17]. Our group and others have investigated the A/C polymorphism in patients with HCV infection with the high IL-12-producing C allele found to be associated both with spontaneous viral clearance [18] and treatment response [19]. Genetically determined factors able to confer protection from HCV infection are of great interest, but are challenging to identify. We have identified a cohort of long-term intravenous injection drug users who remain uninfected by HCV despite multiple episodes of exposure risk. We have termed this group 'exposed–uninfected'. We have studied the IL-12 B 3'UTR (A/C) polymorphism in this group and have compared results with those found in both healthy controls and our previously reported cases with established HCV infection.

**Table 1.** Exposed–uninfected injection drug users.

Exposed–uninfected subjects	
Number	76
Male : female	67:9
Shared needles and/or other injection equipment	76 (100%)
Shared needle with known HCV infected case	24 (31.6%)
Age (years) – mean $\pm$ s.d.	32.3 $\pm$ 6
Age at first drug use – mean $\pm$ s.d. (range)	19.7 $\pm$ 4.6 (15–32 years)
Duration of drug use mean $\pm$ s.d. (range)	7.1 $\pm$ 6.2 (0.1–26 years)
Still injecting	26 (34.2%)
Multiple injections each week	73 (96.1%)
Total estimated injection episodes – median (range)	4368 (156–28 400)

The exposed–uninfected cases were identified from a number of settings including needle exchange centres, drug rehabilitation facilities and local prisons. Risk behaviour was assessed by means of a questionnaire detailing injection drug use history. HCV, hepatitis C virus; s.d., standard deviation.

## Methods

This study was approved by the local research ethics committee and written informed consent was obtained from all subjects.

## Subjects

*Hepatitis C virus exposed–uninfected cases.* We have identified a total of 76 cases, 67 male and nine female. All cases were considered to be at very high risk of acquiring HCV infection from injection drug use, but despite this tested both HCV antibody- and HCV RNA-negative. All cases had an extensive history of injection drug use and sharing of injecting equipment, including needles, syringes, filters and spoons, used during the injection process. Many cases were known to have shared needles with other cases of known HCV infection. Mean duration of injection drug use was more than 7 years. One case who had injected only briefly was included because of their history of sharing needles repeatedly with a known HCV-infected case. The exposed–uninfected cases were identified from a number of settings including needle exchange centres, drug rehabilitation facilities and local prisons. Risk behaviour was assessed by means of a questionnaire detailing injection drug use history and the findings are summarized in Table 1.

## Comparison groups

*Healthy controls.* The control samples ( $n = 105$ ) used in this study were ethnically matched, unselected Caucasian (49 males, 56 females) cord blood samples taken as repre-

sentative of the general population for genetic studies. The cord blood was collected sequentially after normal obstetric delivery from the Obstetric Department, Derriford Hospital (Plymouth, UK).

*Hepatitis C-infected cases.* Our previously reported cohort of HCV antibody-positive cases was used for comparison [18]. These were divided into spontaneously resolved cases who remained HCV antibody-positive but were repeatedly HCV RNA-negative without previous antiviral therapy ( $n = 72$ ) and chronically infected cases with detectable HCV RNA ( $n = 123$ ).

## Virological testing

The presence or absence of HCV antibodies was determined by third-generation assay (Abbott IMx, Abbott Diagnostics, Maidenhead, UK). The presence or absence of HCV RNA was determined by a commercially available assay (Amplicor, Roche, Basel, Switzerland).

## Genetic studies

*Restriction fragment length polymorphism.* Genomic DNA was extracted from whole blood using a salt precipitation method and the polymorphism studied using polymerase chain reaction (PCR) and restriction enzyme (Taq-1) digestion. The primers were as follows: forward: 5'-CTG ATC CAG GAT GAA AAT TTG G-3; reverse: 5'-CCC ATG GCA ACT TGA GAG CTG G-3'.

Polymerase chain reaction was performed using a PTC-200 Thermocycler (MT-Research, Dunmow, Essex, UK). Cycles involved a starter step at high temperature of 94°C for 4 min followed by 35 cycles with the following cycle profile: denaturation at 94°C for 30 s, annealing temperature 55°C for 1 min and elongation at 72°C for 30 s and a final elongation at 72°C for 5 min. The PCR product [227 base pairs (bp)] was digested by the restriction enzyme *Taq-1* (Promega, Southampton, UK) at 65°C for 120 min. *Taq-1* recognizes the sequence of 5'-T/CGA-3' or 3' AGC/T5'. The presence of the C allele produces two fragments of 156 and 71 bp, which were separated on agarose gel (2%) and visualized under UV light.

## Data analysis

The frequency of alleles and genotypes was compared between the control and patient populations using a  $2 \times 2$  contingency test and  $\chi^2$  with Yates' correction where appropriate. *P*-values of  $< 0.05$  were considered to be significant. Odds ratios (OR) with 95% confidence intervals (CI) were calculated using the statistical program *SPSS*, version 15 (SPSS Inc., Chicago, IL, USA). The sample size of 76 exposed but uninfected individuals and 105 healthy controls gives a power of 80% for detecting a difference of 10% based on a  $\chi^2$  test at the 0.05 significance level.

**Table 2.** The frequency of genotypes and alleles of the 3' untranslated region (1188A/C) polymorphism on interleukin (IL-12B) gene.

	Genotype			Allele	
	AA (%)	AC (%)	CC (%)	A (%)	C (%)
Exposed-uninfected					
<i>n</i> = 76	42	26	8*	110	42†
(%)	55.3	34.2	10.5	72.4	27.6
HCV RNA positive					
<i>n</i> = 123	81	39	3	201	45
(%)	65.9	31.7	2.4	81.7	18.3
HCV RNA negative					
<i>n</i> = 72	36	33	3	105	39
(%)	50	45.8	4.2	72.9	27.1
Healthy controls					
<i>n</i> = 105	71	33	1	175	35
(%)	67.7	31.4	0.9	83.3	16.7

\*The CC genotype was significantly more frequent in exposed-uninfected cases compared with both hepatitis C virus (HCV) RNA-positive patients and controls [ $\chi^2 = 6.5$ ,  $P = 0.04$ , odds ratio (OR) = 4.7 and  $\chi^2 = 9.3$ ,  $P = 0.01$ , OR = 12 respectively]. †The C allele was significantly more frequent in exposed-uninfected cases compared with HCV RNA-positive patients and controls ( $\chi^2 = 4.8$ ,  $P = 0.03$ , OR = 1.7 and  $\chi^2 = 6.3$ ,  $P = 0.02$ , OR = 1.9 respectively).

## Results

Polymerase chain reaction-based restriction fragment length polymorphism analysis revealed three genotypes: AA, AC and CC. The genotype distribution in the healthy controls was in Hardy-Weinberg equilibrium, confirming the relevance of the sample as a control group (AA = 67.7%, AC = 31.4% and CC = 0.9%) (Table 2). The frequency of the CC genotype was increased significantly in the exposed-uninfected cases compared with both healthy controls (10.5% *versus* 0.9%,  $\chi^2 = 9.3$ ,  $P = 0.01$ , OR = 12, 95% CI = 1.5–100) and patients with chronic HCV infection (10.5% *versus* 2.4%,  $\chi^2 = 6.5$ ,  $P = 0.04$ , OR = 4.7, 95% CI = 1.2–18.3), while it did not show any significant difference with patients who resolved the virus [10.5% *versus* 4.2%,  $\chi^2 = 2.2$ ,  $P =$  not significant (n.s.)]. The C allele was found in 27.6% of exposed-uninfected cases compared with 16.7% of healthy controls ( $\chi^2 = 6.3$ ,  $P = 0.01$ , OR = 1.9, 95% CI = 1.1–3.1) and 18.3% of patients with chronic HCV infection ( $\chi^2 = 4.8$ ,  $P = 0.03$ , OR = 1.7, 95% CI = 1.1–2.8). The AA and AC genotypes were not significantly different between exposed-uninfected cases and any other group.

## Discussion

This study sought to identify genetic factors that may confer protection from HCV infection. We have demonstrated that the high IL-12-producing C allele, and in particular the homozygous CC genotype, are associated with apparent

resistance to HCV infection, as demonstrated by the absence of HCV infection despite a long history of high-risk behaviour.

Defining exposure to HCV infection in our exposed-uninfected cases is crucial to interpreting the relevance of this observation. By identifying injection drug users with a long history of injecting behaviour and sharing of needles and/or other injection equipment, we have studied those cases at highest risk of exposure to HCV. Within our cohort there were some individuals with even higher risk exposure through sharing of needles with people known to be HCV-infected. It is known that the highest incidence of HCV infection occurs during the first year of injection drug use, with HCV prevalence rising with continuing drug use [20,21]. A recently reported cohort of long-term injectors had an HCV seroprevalence of over 90% [22]. While exposure to HCV infection cannot be defined with certainty, we feel that our cohort has exhibited a form of resistance to infection by the fact that no evidence of HCV infection exists despite these risk factors. A similar concept has been used to study both protective immune responses [23] and genetic factors [24] that can confer resistance in cases at high risk of HIV infection but who remain uninfected.

It is known that Th1-type cellular immune responses are important in the resolution of HCV infection, both treatment-related and spontaneously occurring. Similar HCV-specific T cell responses have been demonstrated in two injection drug users identified during screening for HCV infection at a time when they had detectable HCV RNA but no HCV antibody. These cases went on to resolve HCV viraemia without seroconverting to become HCV antibody-positive [25]. Resolution of infection in the presence of an effective cellular immune response, but without antibody production, has also been described following low-dose inoculation of schistosomiasis, *Toxoplasma gondii* and microbial infections [26]. In our cohort, injection drug use is likely to expose individuals to relatively low doses of virus, potentially allowing an effective interferon gamma-producing cellular immune response to eradicate virus before infection is fully established, and without production of HCV antibodies. It is possible that a genetic background of enhanced IL-12 production is able to promote early, effective anti-viral cellular immune responses that can confer this apparent protection from HCV infection. Virus-specific interferon gamma production demonstrable by enzyme-linked immunospot (ELISPOT) has been reported recently in seronegative injection drug users from Australia [27]. We have used a similar ELISPOT technique to investigate HCV-specific T cell responses in our exposed but uninfected individuals, as well as in healthy controls and patients with chronic viral infection. This work supports the genotyping results with evidence of HCV-specific interferon gamma production in a significant number of exposed-uninfected cases [28].

While almost half of our exposed-uninfected cohort carried the apparently protective C allele of the IL-12B gene,

most cases did not. This raises the possibility that other inherited factors may be able to confer resistance to HCV infection. Further study of this population of individuals at very high risk of HCV infection but who remain uninfected has considerable potential to provide new insights into mechanisms of resistance and protection.

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