2005-01-01

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

Metzner, M

Gut

Journal of Medical Virology 87:1207–1217 (2015)

Haplotype Analysis Finds Linkage Disequilibrium in the IL-12 Gene in Patients With HCV

Annwyne Houldsworth,[1]* Magdalena Metzner,[1,2] Andrea Hodgkinson,[1] Steve Shaw,[4] Edward Kaminski,[1,3] Andy G Demaine,[1] and Matthew E Cramp[1,2]

[1]*Hepatology and Molecular Medicine Research Groups, Peninsula Medical School, Plymouth, United Kingdom*
[2]*Gastroenterology and Liver Unit, Derriford Hospital, Plymouth, United Kingdom*
[3]*Immunology Department, Derriford Hospital, Plymouth, United Kingdom*
[4]*School of Maths and Statistics, Plymouth University, Plymouth, United Kingdom*

HCV is a major cause of liver disease worldwide. IL-12 plays an essential role in the balance of T helper 1 (Th1) differentiation versus a T helper 2 (Th2) driven response from its naïve precursor. Linkage disequilibrium measures the degree to which alleles at two loci are associated and the non-random associations between alleles at two loci. Haplotypes of the three IL-12B loci studied were determined in the patient cases and the normal healthy control subjects. The frequency of the 12 possible IL-12B haplotypes on the 3 loci was determined in subjects heterozygous at only one of the loci within the studied haplotype. Haplotype frequencies were compared between the patient groups and controls ($n = 49$) to determine if any preferential combination of markers occurred using chi-squared and applying the Bonferroni correction. 45 HCV RNA negative patients; 88 HCV RNA positive patients; and 15 uninfected cases at high risk of HCV infection (EU) were studied. The haplotype "C" SNP of the 3′ UTR with the "E" 4 bp deletion of the intron 4 region was in linkage disequilibrium ($\chi^2 = 45.15$, $P < 0.001$, 95% CL). The haplotype analysis of the insertion allele of the promoter with the deletion allele of the intron 4("E") IL-12B polymorphism showed linkage disequilibrium ($\chi^2 = 5.64$, $P = 0.02$). Linkage disequilibrium of polymorphisms is reported in the IL-12 gene in patients with HCV infection and contributes to the understanding of patient genotype and expected production of IL-12, responding to infection. ***J. Med. Virol. 87:1207–1217, 2015***.

KEY WORDS: IL-12B gene; Proinflammatory cytokine; gene polymorphism; hepatitis C; virus clearance

INTRODUCTION

Hepatitis C virus (HCV) is one of several viruses that results in hepatitis and is a major cause of liver disease worldwide [Gravitz, 2011]. Infection with the virus causes chronic hepatitis in 80% of those infected and it is unclear why some individuals are able to initiate and maintain effective antiviral responses to the virus and others do not. HCV infection continues to be one of the leading bloodborne infections in some regions, despite the efforts taken to eradicate the transmission of the virus by blood products [Cornberg et al., 2011]. Evidence suggests that HCV-specific T helper cell (Th) responses of T1 helper cell type are involved in resolution of HCV infection and long-term control of viremia. Cytokines are known to drive T1 helper cell responses in response to viral infection and, in acute infection, HCV-specific T1 helper cell responses occur more frequently and are stronger in individuals who clear viremia [Diepolder et al., 1995] and the loss of HCV-specific T1 helper cell reactivity following apparent resolution of acute HCV infection has been linked to the reappearance of HCV RNA [Gerlach et al., 1999]. Furthermore, T1 helper cell-type responses against many viral antigens (multi-specific), rare in patients with a chronic infection, are found in the majority of HCV antibody positive cases without viremia many years after exposure to infection [Cramp et al., 1999; Cramp et al., 2000], suggesting a role in the long-term control of viremia. In treatment-related resolution of viremia, induction of T helper cell responses has been shown to be important and the enhanced sustained treatment response

*Correspondence to: Dr Annwyne Houldsworth, Abbey Leat, Milton Combe, Yelverton, Devon, PL20 6HP
E-mail: neamati@umich.edu

Accepted 30 January 2015

DOI 10.1002/jmv.24179
Published online in Wiley Online Library (wileyonlinelibrary.com).

rates seen with ribavirin in combination with alpha interferon may be related to its ability to promote a T1 helper cell response by suppression of interleukin-10 (IL-10) production [D'Andrea et al., 1993; Cramp et al., 2000]. A cohort of intravenous drug users have been identified who, despite a long history of intravenous drug use and sharing of injecting equipment, remain seronegative and aviremic for hepatitis C virus (HCV) and it is suggested that an innate immune activation may be the key to prevention of infection in this cohort. These individuals have cytokine profiles that infer innate immune responses when exposed to the virus [Warshow et al., 2012]. They also appear to have different T-cell responses [Thurairajah et al., 2008].

The functional IL-12 heterodimer is known as IL-12p75 (but sometimes referred to as p70) and is a proinflammatory cytokine with immunoregulatory function [Gubler et al., 1991]. The dimers are p40 and p35 and the physiological role of p40 is not well characterized and its role may be underestimated. It is thought to form dimers that possibly influence the regulation of early immune responses [Gillessen et al., 1995].

IL-12 is a key signal of the innate immune system, acts as a bridge between innate and acquired immunity and thus plays a crucial role in the development of antiviral immune responses [Trinchieri, 1995, 1998, 2003]. Its production is important in the generation of cell-mediated immunity against many intracellular pathogens, including bacteria, fungi, protozoans, and viruses [Johnson and Sayles, 1997; Trinchieri, 1998, 2003]. The cytokine can, however, enhance or inhibit humoral immunity depending on the Ig isotype and the stimulus to antibody formation. IgG2a,b and IgG3 responses are associated with T1 helper cell responses [Germain, 1995] and T2 helper cell responses are associated with IgG1 antibodies which are suppressed by IL-12 [Buchanan et al., 1995].

Although IL-12 plays a key role in intracellular microorganism defense, excessive production has been associated with some organ-specific autoimmune diseases, including type1 diabetes mellitus, multiple sclerosis, rheumatoid arthritis, and Crohn's disease [Ronningen et al., 2004]. The cytokine interleukin-12 (IL-12) mediates a variety of these responses from resting lymphocytes, cytolytic T-cell maturation and cell stimulation [Trinchieri, 1995; Sene et al., 2010].

The precise role of IL-12 in HCV infection is poorly understood but its involvement in enhancing the cytotoxic response and the fact that Th1 driven responses are important for HCV clearance suggest that IL-12 is an important factor in the disease. Further to this, IL-12 and IFN-γ levels are lower in patients with viremic, chronic HCV than in patients who spontaneously cleared the virus [Sarih et al., 2000]. IL-12 enhanced the immune response of these patients when cell cultures were treated with the cytokine [Fan et al., 2000; Bes et al., 2004]. However, patients that are infected chronically with HCV, higher serum IL-12 levels were associated with greater necroinflammatory activity and lower serum levels of IL-12 showed fewer signs of piecemeal necrosis [Quiroga et al., 1998]. The differentiation of uncommitted T cells, during the induction of a cytotoxic antiviral host response, is characterised by the secretion of lymphokines associated with cell-mediated immunity rather than those secreted during a humoral response. [Gately et al., 1991; Diepolder et al., 1995]. A strong Th1 CD4+ response is also thought to be essential for spontaneous viral clearance and this is facilitated by the expression of IL-12 from macrophages and dendritic cells (Fig. 2). IL-12 mediates some physiological activities by acting as a potent inducer of IFN-γ secretion by Th1 and natural killer cells and is extremely important in the clearance of viremia [Ma et al., 1996]. Although the production of IL-12 is vital for HCV viral clearance initiating Th1 responding cells, unregulated and ongoing IL-12 production can result in infection-induced immunopathology and liver damage. Existing intrahepatic memory CD8+ T cells targeting dominant epitopes of HCV, in spontaneously resolved infection, failed to prevent persistence after reinfection. This indicates a need for a broader T cell epitope response to reduce the risk of a second persistent infection and the recently successful of IFN-free direct-acting antiviral therapy appears to restore the CD8+ antiviral response (Fig. 1). [Callendret et al., 2014; Martin et al., 2014].

Although APCs produce most of the cytokine, T cells also produce IL-12 [Trinchieri, 2003], they also have IL-12 receptors that respond the cytokine in an immune response [Grohmann et al., 2001]. An important property of IL-12 is to induce the production of large amounts of IFN-γ from resting and activated T and NK cells [Dorman and Holland, 2000]. The most distinctive role of IL-12 is to regulate the balance between Th1 and Th2 cells (Fig. 2). Allogeneic CTL responses are promoted by IL-12 in Th1-differentiated peripheral cells; IL-12 induced the synthesis of IFN-γ; IL-2 and TNFα expression [Chan et al., 1991; Bertagnolli et al., 1992]. However, IL-10 has been

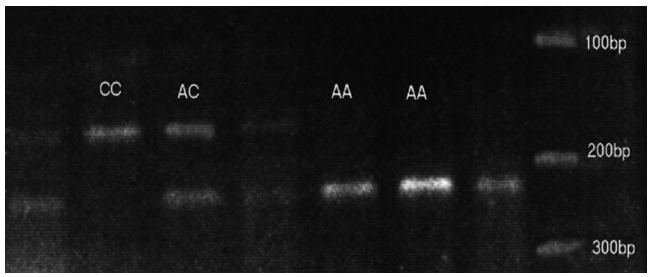

Fig. 1. shows the RFLP digest products of the Taq-1 enzyme after digestion of the amplified products from the IL-12B 3′ UTR gene, run out on an agarose gel using electrophoresis. The cut site contains a "C" allele and uncut products are seen as "A" alleles. The two different sized products can be clearly seen as white bands.

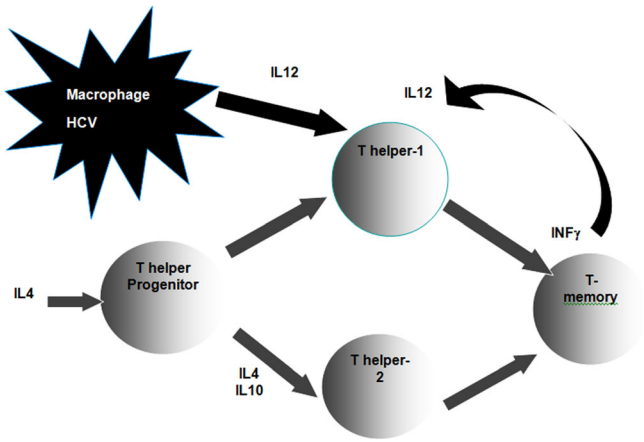

Fig. 2. Differentiation of Th1/Th2 driven responses according to cytokine environment.

shown to inhibit lymphocyte production of IFN-γ by suppressing IL-12 synthesis in accessory cells [D'Andrea et al., 1993]. It has also been reported that individuals with a defective production of IL-12 only respond to *staphylococcus aureus* when supplemented with IFN-γ but not with *staphylococcus aureus* alone [Frucht and Holland, 1996].

Several polymorphisms have been described on the IL-12B gene both in the translated and untranslated regions [Huang et al., 2000]. Some differences in expression in IL-12B associated with polymorphic differences have been reported and mutations in the IL-12 p40 gene or its receptor have been shown to lead to reduced IL-12 signalling [Seegers et al., 2002]. Polymorphisms have been described showing associations with several other diseases such as asthma, Crohn's disease, multiple scherosis and diabetes [Morahan, 2001; Morahan et al., 2002; Seegers et al., 2002; Muller-Berghaus et al., 2004; Randolph et al., 2004].

Strong linkage disequilibrium with the T1DM susceptibility locus in Australian and British diabetes families [Morahan, 2001]. However in a group of Danish, European and American patients, evidence for the presence of T1DM linkage disequilibrium of the (C1159A) SNP was not found in patients with T1DM. Typing of three microsatellites in the region of this SNP and construction of haplotypes did not reveal a T1DM association either. IL-12 expression levels described in this study contradict other group findings where the "A" allele rather than the "C" is found to be the higher p40 producer [Bergholdt et al., 2004].

Some polymorphisms in the IL-12B gene have been investigated in HCV cases. A difference in the distribution of the 3′UTR polymorphism A/C in exon 8 was found in HCV cases where the A allele was more prevalent in cases that spontaneously cleared HCV or remained uninfected after exposure to the virus [Houldsworth et al., 2003, 2005; Hegazy et al., 2008].

The gene for the 40-kD (p40) chain is known as IL-12B found on the distal region of the long arm of chromosome 5 (5q31–q33) [Warrington and Bengtsson, 1994] and p40 is comprised of at least 7 exons. An unusual feature of the IL-12B gene is that it has untranslated exons at both its 5′ and 3′ ends. Translation from mRNA begins at the first codon of exon 2 and terminates at the last codon of exon 7 [Huang et al., 2000]. Although several regions of the mRNA molecule are not translated into protein from the 3′ untranslated region and the 5′ untranslated region they can interact with specific RNA-binding proteins and often influence post-transcriptional gene expression from these regulatory regions as well as localization and export. Specific binding sites within the 3′UTR also have silencer regions that can bind to repressor proteins can also influence gene translation. The 3′UTR can stabilize the mRNA and also contain sequences that attract proteins to associate the mRNA with the cytoskeleton, transport it to or from the cell nucleus, or perform other types of localization. These untranslated regions can influence the protein folding of secondary structures [Pichon et al., 2012].

Another cytokine that appears to be associated with protection against HCV infection is IL28B where a polymorphism in this gene distinguishes exposed, uninfected individuals from spontaneous resolvers of HCV infection [Knapp et al., 2011; Bes, 2012].

Linkage disequilibrium sometimes occurs through a shared population history or through combinations of favored or unfavored alleles [Reich et al., 2001; Bes et al., 2004].

Undoubtedly genetic factors do influence, to a degree, the outcome of HCV infection. The viral immune response is not a simple mechanism but has multiple factors influencing its direction and there are unquestionably many more genetic factors to pursue. It is clear that environmental factors also have determining influences on the success of viral resolution [Gorham et al.,1996, 1997].

Patients with HCV infection investigated for linkage disequilibrium in other genes involved in the immune response to viral infection such as DQB1*0301 and the haplotype HLA-DRB1*1101-HLA-DQB1*0301 is also found more frequently in cases who spontaneously clear HCV [Alric et al., 1997; Cramp et al., 1998]. DRB1*04 and DQA1*03 alleles are protective in strong linkage disequilibrium with DQB1*0301 [Cramp et al., 1998]. Although MHC molecules that differ by one amino acid can have modified peptide binding ability for HCV epitopes [Peano et al., 1994], MHC class II associations with disease outcome may not be directly related to antigen presentation but through linkage disequilibrium with other genes or haplotypes as has been observed with the TNF-α promoter polymorphism and MHC genotypes [Wilson et al., 1993].

Studying patients and the clinical consequences resulting from defects in the IL-12 dependent IFN-γ pathway has shed much light on the Th1/Th2 paradigm during immune responses, which has largely been based on animal models [Lammas et al., 2000].

Identification of mutations of the IL-12 p40, IL-12 receptorβ1 and the IFN-γ receptor gives insight into the mechanisms involved in the human host defense against intracellular pathogens [Dorman and Holland, 2000].

Linkage disequilibrium was identified in the IL-12B gene in asthma patients [Muller-Berghaus et al., 2004; Randolph et al., 2004].

MATERIALS AND METHODS

Patients

133 European Caucasoid patients who are HCV antibody positive were included in this study, recruited from The Institute of Liver Studies at King's College Hospital. Three groups of patients were studied; HCV antibody positive but HCV RNA negative (n = 45) cases with spontaneous viral clearance, HCV antibody and HCV RNA positive patients with chronic infection (n = 88). The third group consisted of individuals who had proved exposure to the virus but were not found to be HCV antibody positive (n = 15). There was also a group of healthy controls that had not been exposed to HCV (n = 49). All patients in the study were recruited over some time and the study was a retrospective study.

Patient Testing for HCV

Serological testing for anti-HCV. In all cases, the presence or absence of HCV antibodies was determined by second or third generation assay (Abbott IMx, Abbott Diagnostics, Maidenhead, UK).

Some patients infected with HCV resolved the virus spontaneously and had undetectable HCV RNA. Anti-HCV specificity was confirmed using a third generation line immunoassay (INNO-LIA HCV AbIII, Innogenetics) as an antibody confirmatory test. ME Cramp et al. at King's College Hospital, London, performed the tests. No false positive HCV antibody results were identified.

Detection of HCV RNA. Less than 100 copies/ml of HCV RNA could be detected using a commercially available assay (Amplicor, Roche, Basel, Switzerland) [Gerken et al., 1996]. Results were confirmed by using nested PCR (Innogenetics, Gent, Belgium).

Extraction of high molecular weight DNA. The DNA isolation process consists of first eliminating the erythrocytes with red cell lysis buffer. Secondly, the membranes of the white cells are lysed along with the nuclei. The DNA from the nuclei is treated with Proteinase K, which is a recombinant enzyme identical to the protease originally isolated from *Tritrachium Album*, a mould that is one of the most active endopeptidases known. The activity of Proteinase K can be enhanced by the addition of SDS.

Finally DNA is precipitated out of solution by adding cations from 5M NaCl and ice-cold ethanol. Then washed with 70% ethanol to remove any salt contamination.

Reagents.

Red Cell Lysis Buffer (RCLB): 0.144 M Cl (38.5 g/5 L), 0.001 M $NaHCO_3$ (0.42 g/5 L)

Nuclei Lysis Buffer: 0.01 M Trisma base (1.21 g/L), 0.4 M NaCl (23.38 g/L), 0.002 M EDTA (0.744 g/L) (The solution was adjusted to pH 8 using concentrated HCL or NaOH)

SDS

Proteinase K (A stock solution was made up using 2 mg/ml in sterile water and stored at −20°C)

6 M NaCl (350.64 g/L)

Absolute ethanol (100% Stored at −20°C)

70% ethanol

SDS (10% solution 10 g made up to 100 μl of sterile water)

5–20 ml of frozen venous blood was defrosted at room temperature, allowing 1 hr per 10 ml of blood. The blood was then transferred into 50 ml Falcon tubes and 25 ml of red cell lysis buffer was added. The solution was mixed by inversion and then left at room temperature for 10 min.

The Falcon tubes were centrifuged at room temperature for 10 min at 600*g*. The supernatant of the red cell lysis mixture was then discarded and the same process of adding RCLB and centrifugation was repeated. The pellet was resuspended in 6 ml of RCLB and centrifuged again for 10 min at 600*g*.

The haemolycate was discarded and the pellet was resuspended in 3 ml of NLB, 200 μl of 10% SDS and 600 μl of Proteinase K. The mixture was incubated at 40°C in a water bath for 24 hr.

1 ml of 6 M NaCl was added and shaken vigorously for at least 15 sec. The remaining content of the falcon tubes was then centrifuged for 15 min at 2000*g*. The resulting supernatant was transferred into a 15 ml falcon tube with 8 ml of absolute ethanol at a temperature of −20°C and mixed by gentle inversion until DNA could be seen to precipitate from the solution.

The DNA was transferred to an Eppendorf tube containing 500 μl of sterile water using an inoculating loop.

When DNA is extracted from cell lysates, it is unusual to remove all the proteins by digestion with proteolytic enzymes such as Proteinase K, even though these enzymes are usually active against a broad spectrum of native proteins. DNA samples often require further purification.

Summary of Genetic Studies

Interleukin-12B gene. The three described polymorphisms of IL-12B studied in the populations infected with HCV were IL-12B (1188A/C) 3′UTR polymorphism, IL-12B 4 bp insertion/deletion promoter polymorphism and the IL-12B 4 bp insertion/deletion intron 4 polymorphism (Table I).

Stock solutions for PCR.

TBE Buffer (10%): 107.8 g Triz base, 55 g Boric acid, 7.44 g EDTA, 1 L distilled water

TABLE I. Interleukin-12B Polymorphisms Investigated

Region	Taq-1 3′ UTR	Promoter	Intron 4
Location	(1188A/C) chromosome 5q 31–33	3 kb upstream of the transcriptional start site chromosome 5q 31–33	Chromosome 5q 31–33
Polymorphism/ method	Single nucleotide polymorphism (SNP) "A"/"C" substitution RFLP*	4 bp insertion PAGE**	4 bp deletion PAGE**
Clinical significance	"C" allele associated with increased IL-12 expression; different IL-12 levels associated with autoimmune diseases	Heterozygote associated with IL-12 expression and more severe form of asthma	In linkage with Taq-1 polymorphism
Reason for investigation	Increased IL-12B expression may confer improved resolution of viremia	May preferentially drive a Th2 response thus associated with RNA positive patients	Shows similar distribution to the Taq-1 polymorphism
Results	HCV RNA negatives significantly lower "A" allele than HCV RNA positives; allele expresses lower levels of IL-12B	No significant differences between groups	Linkage disequilibrium seen with Taq-1 polymorphism

*RFLP restriction fragment length polymorphism using Taq-1 enzyme digestion.
**PAGE polyacrylamide gel electrophoresis using $P^{32}\gamma$ radio-labeled primer.

(All ingredients were mixed using an automatic stirrer in a conical flask).

0.5X TBE for agarose gels
0.5X TBE for gel tray buffer
1X TBE for polyacrylamide gel buffer

dNTPs: 100 μl of each nucleotide (C,T,G,A) were placed into 1.5 ml tube then put 40 μl in each tube and freeze (−20°C) (used for PCR)
Taq: 200 μl buffer (Mg−) was mixed with 100 μl Taq. Aliquot 40 μl into tubes and frozen (−20 °C) (used for PCR)
Ladder: 20 μl 100 bp ladder was mixed with 20 μl Orange G or Dye and 160 μl water and frozen (−20 °C) (used for agarose gel electrophoresis).

Restriction fragment length polymorphism (RFLP) analysis. RFLP refers to heritable differences in the length of DNA fragments from the specific region of DNA generated by restriction enzymes due to DNA sequence differences.

This process was used to investigate a 3′UTR single nucleotide polymorphism on the IL-12B gene and a promoter region of the mitochondrial antioxidant, manganese superoxide dismutase gene. The process involved designing genomic primers for amplification of the required DNA fragment.

IL-12B (1188A/C) 3′UTR polymorphism. A single-nucleotide polymorphism (SNP) at the 3′UTR end of the IL-12B gene at position 1188A/C was investigated. An "A" to "C" change in the variant allele results in a Taq-1 (*Thermus aquaticus* YT1) digestion site and a restriction digest protocol was followed (Fig. 1).

The polymorphic area of interest was amplified and Taq-1 (Promega) restriction enzyme digestion was performed on the amplified DNA in a water bath at 65°C for 90 min of the 1188A/C polymorphism of IL-12B gene produces two fragments of 156 and 71 bp when the "C" allele is present. When the "A" allele is present the Taq-1 enzyme cut site is abolished and produces a single product of 227 bp long.

Recognition sequence for Taq-1

5′T^CG A-3′
3′A GC^T-5′

Digestion products were separated and visualized under UV light using 2% agarose gel electrophoresis and staining with ethidium bromide (Table II).

IL-12B 4 bp insertion/deletion promoter polymorphism. The IL-12B insertion/deletion promoter gene has a 4 bp microinsertion polymorphism arising at the 5′ promoter end of the gene 3 kb upstream of the transcriptional start site. Two primers were used to amplify the DNA products the reverse primer was radiolabelled with ^{32p}ATP. This resulted in a 281 bp fragment and 285 bp fragment with the insertion.

Molecular genotyping was performed using the PAGE method to separate the 4 bp larger insertion allele (designated allele "2") from the smaller allele (designated allele "1") as described above. A 6% gel concentration was used (Table II).

IL-12B 4 bp insertion/deletion inton-4 polymorphism. The IL-12B gene insertion/deletion, intron 4 has a 4 bp microinsertion polymorphism. Two primers were used to amplify the DNA products the reverse primer was radiolabelled with ^{32p}ATP. This resulted in a 254 bp fragment and 258 bp fragment with the insertion.

Molecular genotyping was performed using the PAGE method described previously and a 6% gel concentration (Table II).

TABLE II. PCR Conditions for the IL-12 Polymorphisms Studied

Conditions for PCR	IL-12B promoter	IL-12B Intron 4	IL12B 3′UTR
Fragment size	281 bp	256–258 bp	227 bp
Optimised $MgCl_2$	12 μl	12 μl	3 μl
Anealing Temperature	55°C	53°C	55°C
Cycle 1	95°C for 3 min	95°C for 5 min	95°C for 3 min
2	95°C for 20 sec	95°C for 1 min	95°C for 20 sec
3	55°C for 20 sec	53°C for 1 min	55°C for 20 sec
4	72°C for 30 sec	72°C for 2 min	72°C for 30 sec
5	Cycles 2–4 × 35	Cycles 2–4 × 35	Cycles 2–4 × 35
6	72°C for 2 min	72°C for 10 min	72°C for 2 min
7	4°C for ever	4°C for ever	4°C for ever
References	Huang et al. [2000]	Morahan et al. [2002]	Huang et al. [2000]

Polymerase chain reaction specific conditions for genotyping of the IL-12 polymorphisms studied in patients with HCV.

Primers Designed for This Study

Areas in italic highlight the forward primer, bold areas highlight the reverse primer and green areas highlight the polymorphism.

IL-12 Promoter Region Insertion/Deletion Polymorphism

A*TCAGACACA TTAACCTTGC A*GCTCAGCAC GCCCTCTGTT TGTCAGCAGA
CCTTCCTCGC CCATAGGGTA AGCAATAGAA AGCTTATAGG TATCAGTTTA
TTTTGCCTGG GATCAGGGTC TGGATTGTGA AGTGGGACAT GTTGATAAAC
CTCTTCTCCA AAATTAGGTC AATGGGCATT TGGCTCATAT TACCAGAATG
CTGGCTGGCC ATGTACAGCC TGTCTCCGAG AGAGGCTCTA ATGTGGCCCC
CACATTAGAA CA**ACCTGCCA ATGACCACAT TA**GAACCTCC

Forward primer **5' TCA GAC ACA TTA ACC TTG CA**

Reverse primer **ACC TGC CAA TGA CCA CAT TA**
5'TAA TGT GGT CAT TGG CAG GT

IL-12B Intron 4 Insertion/Deletion Polymorphism

GGAT*GGTTAC ATAATCATAT GTA*TACAATT TGATCCTAGT TCATAAAAAT
AAAATCTATA TGTATAAGTA AAATATATAT AGTGGATATA TATAATGTAG
AGATGTATAT AACATGGATT ATATATATAA TGTGTGTATA CATATGTGTG
TGTGTGTGTG TGTGTATATA TATATATATA TA**TA**AAATGT GTATACAATT
ATCTTGAATA TTCATTGAAA AAGTTCTGGC CAGGCACAGT GG**CTCACACC**
TGAAATCCTA ACCCTTTGGG

Forward primer 5'GGT TAC ATA ATC ATA TGT A

Reverse primer CTC ACA CCT GAA ATC CTA AC
5'GTT AGG ATT TCA GGT GTG AG-3'
Insertion/deletion **TA**

IL-12B 3′ UTR Polymorphism

AGGTT*CTGAT CCAGGATGAA AATTTGG*AGG AAAAGTGGAA GATATTAAGC
AAAATGTTTA AAGACACAAC GGAATAGACC CAAAAAGATA ATTTCTATCT
GATTTGCTTT AAAACGTTTT TTTTAGGATC ACAATGATAT CTTTGCTGTA
TTTGTATAGT **TAGA**TGCTAA ATGCTCATTG AAACAATCAG CTAATTTATG
TATAGATTTT C**CAGCTCTCA AGTTGCCATG GG**CCTTCATG

Forward 5'CTG ATC CAG GAT GAA AAT TTG G-3'
Reverse CAG CTC TCA AGT TGC CAT GGG
5'CCC ATG GCA ACT TGA GAG CTG G-3'

Statistical Analysis

The frequency of the alleles and genotypes in both patient subgroups and controls were compared for significance with one sided testing, using contingency tables and chi-squared test with Yates correction where appropriate. P values of <0.05 were considered to be significant. We also included odds ratios with 95% confidence limits where appropriate. The results are described using percentage frequency, P values, chi-squared and odds ratio values. A Yates correction was applied to all P values.

A power calculation found that groups of size approximately 80 gives a power of 0.8 for detecting a difference in proportions of 0.16 based on a chi-square test with a 0.05 significance level. It was agreed that the sample size was adequate to give sufficient power to find significant statistical difference in this cohort.

In order to ensure that differences between the patient groups were not found by chance, a Bonferroni calculation was applied to the data [Benjamini and Hochberg, 1995].

Hardy–Weinberg Distribution

In a non-selected population, the relative frequencies of different alleles tend to be constant and are described by a single equation. Several factors may distort the gene frequencies in a population. The patients infected with HCV were recruited from the South East of England whereas the healthy control subjects. A high frequency may indicate the possibility that evolutionary forces are in operation and applying selective pressure in favor of heterozygotes or homozygotes for mutant genes that cause the more common inherited disorders. We tested for Hardy Weinberg distribution in the population of the genotypes that were studied using the calculation $(p^2+2pq+q^2=1)$. The frequency of $C=p$ and the frequency of $T=q$. This tested for genetic variation of a population in equilibrium [Emigh, 1980].

Testing for Linkage Disequilibrium With Haplotypes

The non-random association of haplotype frequencies of two genotypes, encoded at different loci was assessed for linkage disequilibrium. When a pair of alleles occurs together more frequently than predicted from the individual allelic frequencies, they are described as being in linkage disequilibrium. The genotype data collected was tested for linkage disequilibrium.

Percentage Frequency of Haplotypes

Haplotypes of the three IL-12B loci studied were determined in the patient cases and the normal healthy control subjects.

The frequency of the 12 possible IL-12B haplotypes on the 3 loci was determined in subjects heterozygous at only one of the loci within the studied haplotype. Haplotype frequencies were compared between the patient groups and controls to determine if any preferential combination of markers occurred using chi-squared and applying the Bonferroni correction, in order to ensure that differences between the patient groups were not found by chance [Benjamini and Hochberg, 1995].

RESULTS

IL-12B Promoter Polymorphism

One hundred fifty nine patients and 90 controls for the IL-12B 4 bp insertion/deletion promoter polymorphism were compared for genotype frequency using contingency tables and Yates correction.

The HCV RNA positive (n = 84) and HCV RNA negative patient (n = 58) groups were comparable in terms of age, sex, route and duration of infection. The EU cases were taken from the Kings and South West cohorts (n = 17).

No significant differences were found in the distribution of the IL-12B insertion/deletion promoter polymorphisms between the HCV RNA positive and HCV RNA negative cases.

Patient Data for the IL-12B Intron 4 Polymorphism

Two hundred fourteen patients and 94 ethnically matched healthy controls were genotyped for the IL-12B 4bp insertion/deletion intron 4 polymorphism.

No significant differences were found in the distribution of the IL-12B insertion/deletion intron 4 polymorphisms between the HCV RNA positive (n = 120) and HCV RNA negative (n = 66) cases or any of the EU cases (n = 28).

Patient Data for the IL-12B 3′UTR Polymorphism

Two hundred forty nine patients and 105 controls were typed for the IL-12B 3′UTR (1188A/C) polymorphism.

The HCV exposed uninfected (n = 54), HCV RNA positive (n = 123) and HCV RNA negative patient (n = 72) groups were comparable in terms of age, sex, route and duration of infection.

RNA positive cases had increased frequency of the AA genotype compared to the HCV RNA negative cases (66% vs 50%, $\chi^2 = 4.12$, $P = 0.04$, OR = 1.93 ($1.02 < OR < 3.65$)).The healthy control cases (n = 105) also had an increased frequency of "AA" genotype than the RNA negative cases (68% vs 50%, $\chi^2 = 4.83$, $P = 0.03$, OR = 2.09 ($1.08 < OR < 4.06$)). There was a significantly higher frequency of "A" alleles in the healthy control group (83.3%) when compared to the RNA negative group (72.9%) ($P = 0.03$, $\chi^2 = 4.99$, OR = 0.5 ($0.31 < OR < 0.93$)) [Houldsworth et al., 2005].

No other comparisons were significantly different.

Linkage Disequilibrium

Analyzing all cases together (i.e., all HCV cases and controls) we found that the 3′UTR "C" and intron 4 "E" alleles are in strong linkage disequilibrium ($\chi^2 = 45.15$, $P < 0.0001$). There are 4 kb between the IL-12B 3′UTR A/C Taq1 polymorphism and IL-12B intron 4 insertion/deletion polymorphism (Table III). As the distance in kb increases between polymorphisms towards the 5′ end of the gene linkage disequilibrium diminishes [Fig. 3].

There were no significant differences observed either between the 3′UTR and promoter or between the promoter and the intron 4 haplotype groups. However there was a non-significant trend before Bonferroni correction for 12 different groups (×11) between the 3′UTR and the intron 4 haplotype and the healthy control group, =0.034, $\chi^2 = 4.46$.

TABLE III. Percentage Frequency of Haplotypes of IL-12B (1188A/C) 3′ UTR Polymorphism (AC), IL-12B 4 bp Insertion/Deletion Intron 4 Polymorphism (EF) and IL-12B 4 bp Insertion/Deletion Promoter Polymorphism (1/2)

Haplotype	RNA positive	RNA negative	Controls
"A"-"1"	66.7% (45)	50.0% (22)	44.8% (30)
"A"-"2"	33.3% (29)	32.2% (19)	49.2% (33)
"C"-"1"	0.0% (0)	6.7% (6)	3.0% (2)
"C"-"2"	0.0% (0)	11.1% (8)	3.0% (2)
"A"-"F"	77.5% (138)	73.3% (66)	85.7% (84)
"A"-"E"	10.1% (18)	12.2% (11)	4.4% (4)
"C"-"F"	5.6% (10)	6.7% (6)	5.5% (5)
"C"-"E"	6.8% (12)	7.8% (7)	5.5% (5)
"1"-"F"	47.5% (38)	37.7% (20)	40.2% (37)
"1"-"E"	10% (8)	11.3% (6)	3.3% (3)
"2"-"F"	28.8% (23)	34.0% (18)	47.8% (44)
"2"-"E"	13.7% (11)	17.0% (9)	8.7% (8)

The number of subjects is given in parentheses. "A" = A allele in IL-12B gene (1188A/C) polymorphic site, "C" = C allele in IL-12B gene (1188A/C) polymorphic site, 1 = 4 bp deletion on IL-12B promoter region, 2 = 4 bp insertion on IL-12B promoter region, F = 4 bp insertion on IL-12B intron 4 region, E = 4 bp deletion on IL-12B intron 4 region.

After Bonferroni correction for 12 degrees of freedom (n−1 = 11) there were no significant P_c differences between any of the haplotypes.

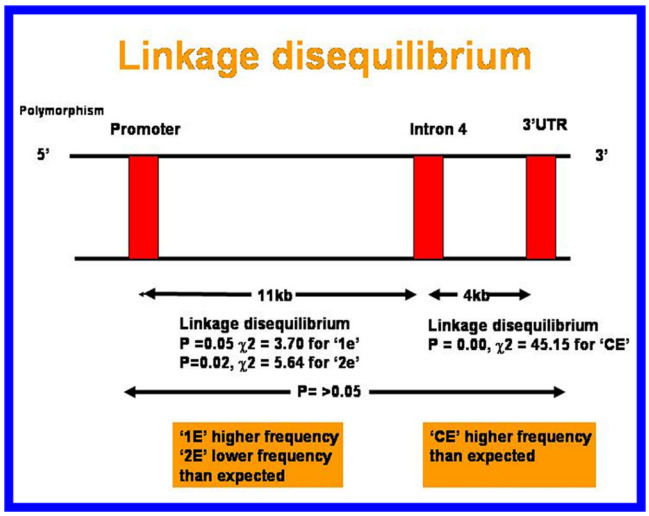

Fig. 3. Shows the kb distances between polymorphisms in linkage disequilibrium on the IL-12 gene. Linkage disequilibrium is more significant between polymorphisms in close proximity at the 3′ UTR end of the gene.

There was a non-significant trend before Bonferroni correction between the promoter and the intron 4 haplotype for patients versus the healthy control group for "2/F", $\chi^2 = 6.69$, $P = 0.01$, OR = 1.55. P_c was not significant between any of the groups.

When the expected frequency of alleles of all subjects studied was compared to those observed we found that the haplotype combining "C" SNP of the 3′UTR with the "E" 4 bp deletion of the intron 4 region was in linkage disequilibrium with the rest of the gene giving a χ^2 of 45.15 for the CE haplotype giving a $P \leq 0.05$ at the 95% confidences limit (Table III).

Haplotype Analysis of IL-12B Promoter and Intron 4 Polymorphisms

The haplotype analysis of the insertion allele of the promoter ("2") with the deletion allele of the intron 4 ("E") IL-12B polymorphism occurred significantly more frequently than expected ($\chi^2 = 5.64$, $P = 0.02$) showing linkage disequilibrium between these two polymorphisms (Table III).

DISCUSSION

In general, pathogenic mechanisms for diseases of immune disregulation often exhibit cytokine patterns dominated by either a Th1 or Th2 differentiation. Different genotypes of immune compounds such as KIR/HLA can affect the kinetics of human antiviral innate immunity [Ahlenstiel et al., 2004, 2008].

IL28B rs.1297860-CC polymorphism is also associated with a population of individuals that remain uninfected with HCV and are antibody negative, despite proved exposure to the virus and are a distinctly different population from patients that spontaneously resolve infection after testing HCV antibody positive [Knapp et al., 2011].

Although there is no effective vaccine for HCV in humans, antiviral treatment has recently greatly improved for patients infected with HCV. Recent advances, using a chimpanzee based HCV vaccine strategy, with durable, broad, sustained, and balanced T cell responses, characteristic of those associated with viral control has just been published offering an optimistic future for individuals exposed to HCV [Swadling et al., 2014]. The elucidation of innate immune responses to HCV infection holds great potential for the development of new therapies against the virus. Effective cellular immune responses are critical in the resolution of viral infections including HCV, and the possible role of immunogenetic factors such as cytokine gene polymorphisms in determining disease outcome via their influence on immune reactivity is of much interest [Hill, 1998]. IL-12 is a key cytokine and plays a central role in the promotion and maintenance of anti-viral immune responses, [Morahan, 2001; Piazzolla et al., 2001; Morahan et al., 2002; Tsunemi et al., 2002]. The IL-12 gene is a prime candidate gene for infectious as well as Th1 mediated autoimmune diseases and many chronic infections including HIV [Wolday et al., 2000] and HCV are associated with impaired IL-12 production [Schlaak et al., 1998; Piazzolla et al., 2001]. Whether this lower IL-12 secretion is a cause or consequence of chronic infection remains unclear, but an individual's genetically influenced ability to produce IL-12 in response to antigen exposure is likely to be important in influencing the outcome of infection. Part of this study was specifically designed to address the influence of IL-12B polymorphisms on the resolution of HCV infection by comparing the findings in a large group of patients without persistent viremia following HCV infection, with a group individuals that have a chronic infection.

Spontaneous resolution of hepatitis C viremia showed a non-significant trend of association with the presence of one or more "C" alleles at the IL-12B 3′UTR (1188A/C) polymorphism. The lower producing "A" allele was significantly associated with chronic HCV infection and lower IL-12 production indicating a role for the IL-12B polymorphism in IL-12 secretion and the outcome of HCV infection. No other IL-12B individual polymorphism studied had any association with the outcome of disease in the patients studied [Houldsworth et al., 2003, 2005].

The "A" allele of IL-12B, in our investigation, was of particular interest where an unfavourable clinical outcome has been shown to be associated with lower IL-12 production. It is worthy of mention that whilst not significant when corrected for multiple comparisons, overall just 34% of RNA positive cases carried one or more copy of the "C" allele that has been shown to enhance IL-12 production [Seegers et al., 2002] compared to 50% of patients that are HCV

RNA negative. In the study by Seegers et al. [Seegers et al., 2002], in vitro secretion of the biologically active IL-12 p70 heterodimer from stimulated monocytes was found to be significantly greater in heterozygous cases (A/C) than in those with the homozygous "AA" genotype, and higher still for those homozygous "CC". A plausible biological explanation for the improved clinical outcomes we have shown is that an enhanced ability to produce IL-12 following antigenic stimulation promotes and maintains the Th1 response needed to control and clear HCV infection [Diepolder et al., 1995; Takaki et al., 2000]. This explanation is in keeping with a previously reported in vitro T cell studies from King's College Hospital (involving some of the patients in this study) demonstrating more vigorous Th1 cell responses and greater IFN-gamma production in response to HCV antigens in those patients who resolved viremia [Cramp et al., 1999].

No polymorphisms in the coding region of the IL-12B gene have been identified, indicating a high level of conservation of genes in humans. However several intronic polymorphisms have been discovered and some are associated with disease. Although the polymorphisms studied are not in the coding region, a single alteration in a gene, including polymorphisms in intronic, non-coding sequences, may affect the protein transcription, function or activity. Gene polymorphisms can alter the tertiary structure or the binding ability of a protein either to enable intracellular transport, binding to substrates or co-ordination with cofactors. This may explain why the variant "C" allele in the 3′UTR (1188A/C) of the IL-12B gene produces more IL-12. As the 3′UTR region is known to be able to influence quantity of protein translation by effects on mRNA stability [Grzybowska et al., 2001], and transcriptional activity [Le Cam and Legraverend, 1995].

One research group carried out the 3′UTR polymorphism investigation in treated HCV cases but did not report the same association as us with self-limiting cases but did find that the SNP was associated with a reduced relapse rate in patients undergoing antiviral combination therapy [Mueller et al., 2004]. In contrast to our study, Mueller et al. studied mainly women in the patient group that had spontaneously resolved infection and they had treated all of the cases with antiviral therapy whereas the RNA negative group in this study resolved the viremia spontaneously without treatment. These differences in population may have influenced the genetic frequency of the Mueller group cases.

Modified peptide binding ability for HCV epitopes happens with at least one amino acid change [Peano et al., 1994] but this is not the only reason for MHC class II associations with disease outcome and may not be directly related to antigen presentation. Some HLA associations with disease are found in one population but not in another, possibly because of linkage disequilibrium with other genes or haplotypes. This phenomenon has been observed with the TNF-α promoter polymorphism and MHC genotypes [Wilson et al., 1993].

Linkage disequilibrium can be influenced by several factors, including selection, the rate of recombination, the rate of mutation, genetic drift, non-random mating, and population structure as well as genetic linkage [Ardlie et al., 2002]. Haplotypes can be informative provided associations within haplotypes are significant as in the Saami and African populations described earlier [Pritchard and Przeworski, 2001].

Linkage disequilibrium was found in the IL-12B gene at the 3′UTR end with intron 4 and exon 8. The two polymorphisms, "C" SNP of the 3′UTR (exon 8) and the 4 bp deletion of intron 4, both appeared together with a much higher frequency than expected. Although we did not find any haplotype associations associated with resolution of viremia in the IL-12B genes, other haplotype combinations may be interesting to investigate. The data was generated from a cohort from the London area, which has a high degree of ethnicity but we selected a European Caucasoid population from this area, however it is difficult to assess the purity of this gene pool in a community with such a broad ethnic mix.

There was a trend in the control group compared to the patient groups with an increased frequency of IL-12B promoter insertion/intron 4 insertion haplotype compared to the patient groups. However, no significant difference was observed in haplotype associations, after the p value was corrected using a Bonferroni correction ($\times 11$), There was also a trend of increased frequency of the 3′UTR "A"/intron 4 insertion in the control group. This could have been due to ethnic differences between the control population from the South West and the patient group from the South East. It may be necessary to take this into consideration with any further study by a further correction or by ensuring that the controls come from the same area as the patient cohort.

It would be interesting to investigate the IL-12B haplotypes in different ethnic groups to see if the same haplotype association occurs. These findings support the concept that an individual's genetically determined ability to produce IL-12 is another factor that may influence the outcome of HCV infection.

Associations of genes with immune responses to infection may also provide clues to the development of end-stage complications such as cirrhosis or hepatocellular carcinoma. Further analysis of the human genome may eventually allow us to determine the natural outcome of HCV infection and enable us to design appropriate antiviral therapy as well as predict an individual's response to therapy. Direct antiviral therapies have led to the most dramatic breakthroughs in treatment and understanding immune responses depending on patient genotype may help to improve treatments [Zuckerman, 2012].

More study is needed to investigate further haplotype combinations associated with immune responses to HCV in order to fully understand protective mechanisms against viremia. Understanding how haplotypes interact in the cytokine response to HCV infection may lead to enhanced understanding of immune responses in patients. Interleukin-12, as a genetic adjuvant, was found to enhance hepatitis C virus NS3 DNA vaccine immunogenicity [Naderi et al., 2013].

REFERENCES

Ahlenstiel G, Woitas RP, Rockstroh J, Spengler U. 2004. CC-chemokine receptor 5 (CCR5) in hepatitis C—At the crossroads of the antiviral immune response? J Antimicrob Chemother 53:895–898.

Ahlenstiel G, Martin MP, Gao X, Carrington M, Rehermann B. 2008. Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses. J Clin Invest 118:1017–1026.

Alric L, Fort M, Izopet J, Vinel JP, Charlet JP, Selves J, Puel J, Pascal JP, Duffaut M, Abbal M. 1997. Genes of the major histocompatibility complex class II influence the outcome of hepatitis C virus infection. Gastroenterology 113:1675–1681.

Ardlie KG1, Kruglyak L, Seielstad M. 2002. Patterns of linkage disequilibrium in the human genome. Nat Rev Genet 3:299–309.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate. A practical and powerful approach to multiple testing. J R Statist Soc B 57:289–300.

Bergholdt R, Ghandil P, Johannesen J, Kristiansen OP, Kockum I, Luthman H, Ronningen KS, Nerup J, Julier C, Pociot F. 2004. Genetic and functional evaluation of an interleukin-12 polymorphism (IDDM18) in families with type 1 diabetes. J Med Genet 41:e39.

Bertagnolli MM, Lin BY, Young D, Herrmann SH. 1992. IL-12 augments antigen-dependent proliferation of activated T lymphocytes. J Immunol 149:3778–3783.

Bes M, Sauleda S, Campos-Varela I, Rodriguez-Frias F, Casamitjana N, Homs M, Piron M, Quer J, Tabernero D, Guardia J, Puig L, Esteban JI. 2004. IL28B genetic variation and hepatitis C virus-specific CD4(+) T-cell responses in anti-HCV-positive blood donors. J Viral Hepat 19:867–871.

Buchanan JM, Vogel LA, Van Cleave VH, Metzger DW. 1995. Interleukin 12 alters the isotype-restricted antibody response of mice to hen egg white lysozyme. Int Immunol 7:1519–1528.

Callendret B, Eccleston HB, Hall S, Satterfield W, Capone S, Folgori A, Cortese R, Nicosia A, Walker CM. 2014. T cell immunity and HCV reinfection after cure of chronic hepatitis C with an interferon-free antiviral regimen. Hepatology 60:1531–1540.

Chan AC, Irving BA, Fraser JD, Weiss A. 1991. The zeta chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. Proc Natl Acad Sci USA 88:9166–9170.

Cornberg M, Razavi HA, Alberti A, Bernasconi E, Buti M, Cooper C, Dalgard O, Dillion JF, Flisiak R, Forns X, Frankova S, Goldis A, Goulis I, Halota W, Hunyady B, Lagging M, Largen A, Makara M, Manolakopoulos S, Marcellin P, Marinho RT, Pol S, Poynard T, Puoti M, Sagalova O, Sibbel S, Simon K, Wallace C, Young K, Yurdaydin C, Zuckerman E, Negro F, Zeuzem S. 2011. A systematic review of hepatitis C virus epidemiology in Europe, Canada and Israel. Liver Int 30–60.

Cramp ME, Carucci P, Underhill J, Naoumov NV, Williams R, Donaldson PT. 1998. Association between HLA class II genotype and spontaneous clearance of hepatitis C viraemia. J Hepatol 29:207–213.

Cramp ME, Carucci P, Rossol S, Chokshi S, Maertens G, Williams R, Naoumov N. 1999. Hepatitis C virus (HCV) specific immune responses in anti-HCV positive patients without hepatitis C viraemia. Gut 44:424–429.

Cramp ME, Rossol S, Chokshi S, Carucci P, Williams R, Naoumov NV. 2000. Hepatitis C virus-specific T-cell reactivity during interferon and ribavirin treatment in chronic hepatitis C. Gastroenterology 118:346–355.

D'Andrea A, Rengaraju M, Valiante NM, Chehimi J, Kubin M, Aste M, Chan SH, Kobayashi M, Young D, Nickbarg E. 1992. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. J Exp Med 176:1387–1398.

D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 178:1041–1048.

Diepolder HM, Zachoval R, Hoffmann RM, Wierenga EA, Santantonio T, Jung MC, Eichenlaub D, Pape GR. 1995. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 346:1006–1007.

Dorman SE, Holland SM. 2000. Interferon-gamma and interleukin-12 pathway defects and human disease. Cytokine Growth Factor Rev 11:321–333.

Emigh H. 1980. A comparison of test for Hardy Weinberg equilibrium. Biometrics 36:627–642.

Fan XG, Tang FQ, Yi H, Liu WE, Houghton M, Hu GL. 2000. Effect of IL-12 on T-cell immune responses in patients with chronic HCV infection. APMIS 108:531–538.

Frucht DM, Holland SM. 1996. Defective monocyte costimulation for IFN-gamma production in familial disseminated Mycobacterium avium complex infection: abnormal IL-12 regulation. J Immunol 157:411–416.

Gately MK, Desai BB, Wolitzky AG, Quinn PM, Dwyer CM, Podlaski FJ, Familletti PC, Sinigaglia F, Chizonnite R, U Gubler U. 1991. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). J Immunol 147:874–882.

Gerken G, Pontisso P, Roggendorf M, Grazia Rumi M, Simmonds P, Trepo C, Zeuzem S, Colucci G. 1996. Clinical evaluation of a single reaction, diagnostic polymerase chain reaction assay for the detection of hepatitis C virus RNA. J Hepatol 24:33–37.

Gerlach JT, Diepolder HM, Jung MC, Gruener NH, Schraut WW, Zachoval R, Hoffmann R, Schirren CA, Santantonio T, Pape GR. 1999. Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. Gastroenterology 117:933–941.

Germain RN. 1995. Binding domain regulation of MHC class II molecule assembly, trafficking, fate, and function. Semin Immunol 7:361–372.

Gillessen S, Carvajal D, Ling P, Podlaski FJ, Stremlo DL, Familletti PC, Gubler U, Presky DH, Stern AS, Gately MK. 1995. Mouse interleukin-12 (IL-12) p40 homodimer: A potent IL-12 antagonist. Eur J Immunol 25:200–206.

Gorham JD, Guler ML, Steen RG, Mackey AJ, Daly MJ, Frederick K, Dietrich WF, Murphy KM. 1996. Genetic mapping of a murine locus controlling development of T helper 1/T helper 2 type responses. Proc Natl Acad Sci U S A 93:12467–12472.

Gorham JD, Guler ML, Murphy KM. 1997. Genetic control of interleukin 12 responsiveness: Implications for disease pathogenesis. J Mol Med 75:502–511.

Gravitz L. 2011. "A smouldering public-health crisis." Nature 474: S2–S4.

Grohmann U, Belladonna ML, Vacca C, Bianchi R, Fallarino F, Orabona C, Fioretti MC, Puccetti P. 2001. Positive regulatory role of IL-12 in macrophages and modulation by IFN-γ. J Immunol 167:221–227.

Grzybowska EA, Wilczynska A, Siedlecki JA. 2001. Regulatory functions of 3'UTRs. Biochem Biophys Res Commun 288, 291–295.

Gubler U, Chua AO, Schoenhaut DS, Dwyer CM, McComas W, Motyka R, Nabavi N, Wolitzky AG, Quinn PM, Familletti PC. 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. Proc Natl Acad Sci U S A 88:4143–4147.

Hegazy D, Thurairajah P, Metzner M, Houldsworth A, Shaw S, Kaminski E, Demaine AG, Cramp ME. 2008. Interleukin 12B gene polymorphism and apparent resistance to hepatitis C virus infection. Clin Exp Immunol 152:538–541.

Hill AV. 1998. The immunogenetics of human infectious diseases. Annu Rev Immunol 16:593–617.

Houldsworth A, Metzner M, Rossol S, Kaminski E, Demaine AG, Cramp ME. 2003. IL-12B gene polymorphism and the outcome of HCV infection. Hepatology 38:314.

Houldsworth A, Metzner M, Rossol S, Shaw S, Kaminski E, Demaine AG, Cramp ME. 2005. Polymorphisms in the IL-12B gene and outcome of HCV infection. J Interferon Cytokine Res 25:271–276.

Huang D, Cancilla MR, Morahan G. 2000. Complete primary structure, chromosomal localisation, and definition of polymorphisms of the gene encoding the human interleukin-12 p40 subunit. Genes Immun 1:515–520.

Johnson LL, Sayles PC. 1997. Interleukin-12, dendritic cells, and the initiation of host-protective mechanisms against *Toxoplasma gondii*. J Exp Med 186:1799–1802.

Knapp S, Warshow U, Ho KM, Hegazy D, Little AM, Fowell A, Alexander G, Thursz M, Cramp M, Khakoo SI. 2011. A polymorphism in IL28B distinguishes exposed, uninfected individuals from spontaneous resolvers of HCV infection. Gastroenterology 141:320–325.

Lammas DA, Casanova JL, Kumararatne DS. 2000. Clinical consequences of defects in the IL-12-dependent interferon-gamma (IFN-gamma) pathway. Clin Exp Immunol 121:417–425.

Le Cam A, Legraverend C. 1995. Transcriptional repression, a novel function for 3′ untranslated regions. Eur J Biochem 231:620–627.

Ma X, Chow JM, Gri G, Carra G, Gerosa F, Wolf SF, Dzialo R, Trinchieri G. 1996. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. J Exp Med 183:147–157.

Martin B, Hennecke N, Lohmann V, Kayser A, Neumann-Haefelin C, Kukolj G, Böcher WO, Thimme R. 2014. Restoration of HCV-specific CD8+ T cell function by interferon-free therapy. J Hepatol 61:538–543.

Morahan G. 2001. Identification of a T1D susceptibility gene. Sci World J 1:198–199.

Morahan G, Huang D, Wu M, Holt BJ, White GP, Kendall GE, Sly PD, Holt PG. 2002. Association of IL12B promoter polymorphism with severity of atopic and non-atopic asthma in children. Lancet 360:455–459.

Mueller T, Mas-Marques A, Sarrazin C, Wiese M, Halangk J, Witt H, Ahlenstiel G, Spengler U, Goebel U, Wiedenmann B, Schreier E, Berg T. 2004. Influence of interleukin 12B (IL12B) polymorphisms on spontaneous and treatment-induced recovery from hepatitis C virus infection. J Hepatol 41:652–658.

Muller-Berghaus J, Kern K, Paschen A, Nguyen XD, Kluter H, Morahan G, Schadendorf D. 2004. Deficient IL-12p70 secretion by dendritic cells based on IL12B promoter genotype. Genes Immun 5:431–434.

Naderi MI, Saeedi A, Moradi A, Kleshadi M, Zolfaghari MR, Gorji A, Ghaemi A. 2013. Interleukin-12 as a genetic adjuvant enhances hepatitis C virus NS3 DNA vaccine immunogenicity. Virol Sin 28:167–173.

Peano GI, Menardi G, Ponzetto A, Fenoglio LM. 1994. HLA-DR5 antigen. A genetic factor influencing the outcome of hepatitis C virus infection? Arch Intern Med 154:2733–2736.

Piazzolla G, Tortorella C, Fiore G, Fanelli M, Pisconti A, Antonaci S. 2001. Interleukin-12 p40/p70 ratio and in vivo responsiveness to IFN-alpha treatment in chronic hepatitis C. J Interferon Cytokine Res 21:453–461.

Pichon X, Wilson LA, Stoneley M, Bastide King HA, Somers J, Willis AE. 2012. "RNA binding protein/RNA element interactions and the control of translation". Curr Protein Pept Sci 13:294–304.

Pritchard JK, Przeworski M. 2001. Linkage disequilibrium in humans: Models and data. Am J Hum Genet 69:1–14.

Quiroga JA, Martin J, Navas S, Carreno V. 1998. Induction of interleukin-12 production in chronic hepatitis C virus infection correlates with the hepatocellular damage. J Infect Dis 178:247–251.

Randolph AG, Lange C, Silverman EK, Lazarus R, Silverman ES, Raby B, Brown A, Ozonoff A, Richter B, Weiss ST. 2004. The IL12B gene is associated with asthma. Am J Hum Genet 75:709–715.

Reich DE, Cargill M, Bolk S, Ireland J, Sabeti PC, Richter DJ, Lavery T, Kouyoumjian R, Farhadian SF, Ward R, Lander ES. 2001. Linkage disequilibrium in the human genome. Nature 411:199–204.

Ronningen Nerup J, Julier C, Pociot F. 2004. Genetic and functional evaluation of an interleukin-12 polymorphism (IDDM18) in families with type-1 diabetes. J Med Genet 41:e39.

Sarih M, Bouchrit N, Benslimane A. 2000. Different cytokine profiles of peripheral blood mononuclear cells from patients with persistent and self-limited hepatitis C virus infection. Immunol Lett 74:117–120.

Schlaak JF, Pitz T, Lohr HF, Meyer zum, Buschenfelde KH, Gerken G. 1998. Interleukin 12 enhances deficient HCV-antigen-induced Th1-type immune response of peripheral blood mononuclear cells. J Med Virol 56:112–117.

Seegers D, Zwiers A, Strober W, Pena AS, Bouma G. 2002. A TaqI polymorphism in the 3′UTR of the IL-12 p40 gene correlates with increased IL-12 secretion. Genes Immun 3:419–423.

Sene D, Levasseur F, Abel M, Lambert M, Camous X, Hernandez C, Pene V, Rosenberg AR, Jouvin-Marche E, Marche PN, Cacoub P, Caillat-Zucman S. 2010. Hepatitis C virus (HCV) evades NKG2D-dependent NK cell responses through NS5A-mediated imbalance of inflammatory cytokines. PLoS Pathog 6:e1001184.

Swadling L, Capone S, Antrobus RD, Brown A, Richardson R, Newell EW, Halliday J, Kelly C, Bowen D, Fergusson J, Kurioka A, Ammendola V, Del Sorbo M, Grazioli F, Esposito ML, Siani L, Traboni C, Hill A, Colloca S, Davis M, Nicosia A, Cortese R, Folgori A, Klenerman P, Barnes E. 2014. A human vaccine strategy based on chimpanzee adenoviral and MVA vectors that primes, boosts, and sustains functional HCV-specific T cell memory. Sci Transl Med 6:261.

Takaki A, Wiese M, Maertens G, Depla E, Seifert U, Liebetrau A, Miller JL, Manns MP, Rehermann B. 2000. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. Nat Med 6:578–582.

Thurairajah PH, Hegazy D, Chokshi S, Shaw S, Demaine A, Kaminski ER, Naoumov NV, Cramp ME. 2008. Hepatitis C virus (HCV)—specific T cell responses in injection drug users with apparent resistance to HCV infection. J Infect Dis 198:1749–1755.

Trinchieri G. 1995. Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol 13:251–276.

Trinchieri G. 1998. Interleukin-12: A cytokine at the interface of inflammation and immunity. Adv Immunol 70:83–243.

Trinchieri G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 3:133–146.

Tsunemi Y, Saeki H, Nakamura K, Sekiya T, Hirai K, Fujita H, Asano N, Kishimoto M, Tanida Y, Kakinuma T, Mitsui H, Tada Y, Wakugawa M, Torii H, Komine M, Asahina A, Tamaki K. 2002. Interleukin-12 p40 gene (IL12B) 3′-untranslated region polymorphism is associated with susceptibility to atopic dermatitis and psoriasis vulgaris. J Dermatol Sci 30:161–166.

Warrington JA, Bengtsson U. 1994. High-resolution physical mapping of human 5q31-q33 using three methods: radiation hybrid mapping, interphase fluorescence in situ hybridization, and pulsed-field gel electrophoresis. Genomics 24:395–398.

Warshow UM, Riva A, Hegazy D, Thurairajah PH, Kaminski ER, Chokshi S, Cramp ME. 2012. Cytokine profiles in high risk injection drug users suggests innate as opposed to adaptive immunity in apparent resistance to hepatitis C virus infection. J Viral Hepat 19:501–508.

Wilson KM, Labeta MO, G Pawelec G, Fernandez N. 1993. Cell-surface expression of human histocompatibility leucocyte antigen (HLA) class II-associated invariant chain (CD74) does not always correlate with cell-surface expression of HLA class II molecules. Immunology 79:331–335.

Wolday D, Berhe N, Britton S, Akuffo H. 2000. HIV-1 alters T helper cytokines, interleukin-12 and interleukin-18 responses to the protozoan parasite *Leishmania donovani*. Aids 14:921–929.

Zuckerman E. 719 2012. [Treatment of hepatitis C virus-2012: A real breakthrough] Harefuah 151:699–704.