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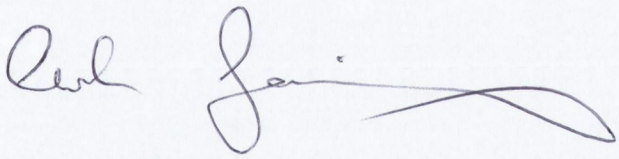
Title:

The Immune response to Hepatitis C; Host genetic factors influencing the out-come of Hepatitis C viral infection, in a cohort infected from a single source.

Submitted for M.D. thesis, Trinity College Dublin, 2007

Candidate: Carol Goulding



Declaration:

I, Carol Goulding, declare that this thesis entitled, 'The Immune response to Hepatitis C; Host genetic factors influencing the out-come of Hepatitis C viral infection, in a cohort infected from a single source', has not been submitted as an exercise for a degree at this or any other University. This thesis is entirely my own work. I agree that the library may lend or copy my thesis on request.

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Carol Goulding

Summary:

The Immune response to Hepatitis C; Host genetic factors influencing the out-come of Hepatitis C viral infection, in a cohort infected from a single source.

A coordinated response from both the innate and adaptive arms of the immune system is necessary for successful anti-viral immunity. This thesis looks at polymorphisms in genes influencing the innate and in turn the adaptive immune response and the impact that these polymorphisms may have on hepatitis C viral clearance and disease severity. The study group in this thesis provide a relatively unique opportunity to examine the host response to HCV exposure, including those who have cleared the virus spontaneously and those who remained infected. This group were all infected with HCV genotype 1B from a single donor and are all of Irish descent, with no other risk factors for liver disease. The fact that a significant number of the identified group had spontaneously cleared the virus meant that they were able to act as controls against those who remained infected.

All 233 women were genotyped for a total of 28 SNPs on 9 different genes; *CCR5, CCR2, RANTES, TLR7, TLR8, SKI2, BAT1, DDX3, RCK* and results analysed in relation to spontaneous HCV viral clearance and disease severity. An additional 50 women were genotyped for *CCR5, CCR2* and *RANTES* mutations only.

CCR5 and *CCR2* are chemokines and *RANTES* is a ligand for *CCR5*. The *CCR5* polymorphism examined in this thesis was the Δ32 deletion, using a PCR technique in which the wild type gives a band at 189bp, whilst the mutant gave rise to a band of 157bp (32bp deletion). Restriction fragment length polymorphism was used to perform genotyping on the *CCR264I* polymorphism, the PCR product being digested with the enzyme *Bsa BI* yielding fragments of 149 and 24bp if an isoleucine was present instead of valine at position 64. The initial PCR product remained uncut if valine was present, representing the wild type. Applied Biosystems Ltd. designed a 5' exonuclease assay using the 'Assay by_Design' service for TaqMan analysis to genotype the *RANTES 403* polymorphism. Following identification of a number of potentially important SNPs by using the NCBI dbSNP database and SNPTAGGER 9 polymorphisms in the toll like receptor genes; *TLR 7* and *8*, and 16 in the DEAD box genes;

SKI2, BAT1, DDX3, RCK were selected. These SNPs were then genotyped commercially by Kbiosciences (Hoddesdon, Herts, UK) using Amplifluor® technology.

All 283 women had been tested for the presence of HCV antibodies and HCV RT-PCR, only those who were RT-PCR positive underwent liver biopsy. These liver biopsies were all scored by a single histopathologist in each of the Hepatology centres, using the modified Hepatic Activity Index scoring system. Results from genotyping all of the above SNPs were then compared statistically, using both SPSS and Hitagene software, with HCV PCR status and HAI fibrotic and inflammatory scores.

This thesis in a homogenous patient group with few confounding factors showed significant associations with the polymorphisms examined and spontaneous viral clearance, but also with degree of hepatic inflammation and fibrosis. Those who were heterozygote for the CCR5Δ32 mutation were more likely to have had spontaneous viral clearance than the CCR5 wild type, $p = 0.044$, and in the DRB1*03011 negative subgroup this polymorphism was also associated with significantly less inflammation, $p = 0.043$. Conversely viral persistence was shown to be associated with a polymorphism in the BAT1 gene, those with the minor allele being significantly more likely to have remained RT-PCR positive, $p = 0.01$. This is the first time this association has ever been demonstrated. The wild type form of 2 of the TLR8 SNPs (TLR8C & D) were found to be associated with significantly more severe hepatic fibrosis, $p = 0.03$ & 0.018, respectively. The wild type of TLR8B was associated with more severe hepatic inflammation, $p = 0.008$, whilst the wild type of TL7B was associated with less severe inflammation, $p = 0.014$.

This study of polymorphisms in genes affecting the immune response in a cohort with very few confounding factors has shown some very clear gene associations and raised some very interesting questions for ongoing research. An association with the CCR5Δ32 mutation and spontaneous viral clearance was demonstrated as was an association with viral clearance and a BAT1 gene polymorphism, something which has never previously been demonstrated. It has also shown a link between polymorphisms in toll like receptor genes and disease severity.

Acknowledgements:

I would like to start by acknowledging the help and support of my supervisor, Professor Dermot Kelleher. His enthusiasm for research was infectious and his understanding and fore-sight remarkable.

Thanks also to Dr Ross Mc Manus and Dr Megan Dring and all others in the laboratory at the Dublin Molecular Medicine Centre, Trinity Centre for Health Sciences, for their patience and encouragement.

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I would like to sincerely thank the patients in this study and all patients who have such generosity of spirit to participate in this research and ongoing research projects.

I dedicate this thesis to my late parents, Eamonn and Sally, who taught me everything I know.

Index:

Figures:

Tables:

Chapter 1:

1.1. Hepatitis C Virus

Hepatitis C Virus (HCV) is the leading cause of chronic hepatitis, liver cirrhosis and indication for liver transplantation, and represents a major public health problem worldwide (Tong *et al* 1995, Fattovich *et al* 1997, Planas *et al* 2004, Verna *et al* 2006). Due to the tendency of HCV to progress to chronic disease and the relatively low treatment response rate, especially for genotype 1, liver disease secondary to HCV infection will remain a challenge for the foreseeable future. The host immune response in HCV is thought to be central to viral clearance but is incompletely understood (Alric *et al* 1997, Thursz *et al* 1999). Much progress has been made in delineating HCV immune escape strategies as well as viral and host genetic factors contributing to viral persistence, however, information on host genetic factors associated with HCV-related disease outcome is relatively sparse. This thesis explores the association between immune response encoding genes and clinical phenotype in relation to HCV infection and shows for the first time an association between Toll-like receptors (TLRs) and HCV-related disease progression and also between one of the DEAD box genes, BAT1, and HCV viral clearance. An understanding of the host genetic factors that influence disease outcome will aid in the design of more effective treatment regimes and in the development of novel vaccine strategies.

1.1.1. HCV Structure

Hepatitis C is a member of the flavivirus family. It is a blood-borne single stranded, positive sense RNA virus, which was first identified in 1989 (Kuo *et al* 1989). The HCV genome which is approximately 9.5 kb and comprises a single open reading frame (ORF), encoding a polyprotein of 3000 amino acids, is flanked by the 5' and 3' untranslated regions (UTR). The 5' UTR has an internal ribosome entry site for initiation of translation (Penin *et al* 2004). The 3'UTR plays an important role in RNA replication. The extreme 3' terminus is a highly conserved region of 98 nucleotides, known as the X-region; it has been shown to bind a polypyrimidine tract-binding protein (PTB), which is thought to be involved in cap-independent initiation of translation. Following translation the HCV polyprotein is cleaved

by both host and viral peptidases to form functional viral proteins. The HCV genome is divided into structural (Core and Envelope) proteins and non-structural proteins (NS2-NS5), *(figure 1)*.

1.1.2. The Core Protein

The core protein is highly conserved amongst HCV genotypes and contains a hydrophobic C terminus that functions as a signal peptide. It is the main structural component of the viral capsid. It is thought to play a role in regulation of cellular and viral promoters, inhibition and stimulation of apoptosis and activation of transcriptional factors (Ray *et al* 1995, Tellinghisen *et al* 2002).

1.1.3. Envelope Proteins

The HCV envelope proteins (E1 and E2) are membrane-associated glycoproteins, which form an integral part of the viral envelope. They are processed in the endoplasmic reticulum by host peptidases. The E2 protein possesses hypervariable regions (HVR), which vary not only amongst the major viral genotypes, but even within an infected individual indicating a high rate of mutation which presumably assists the virus to escape immune surveillance. Antibodies against the HVR-1 region of E2 have been shown to block HCV infection in tissue culture models (Shimizu *et al* 1996). HCV E2 has been shown to stimulate the secretion of RANTES via its interaction with CD81 which in turn attracts CCR5. However within the liver, interaction of HCV E2 with CD81 renders dendritic cells unresponsive to extra-hepatic chemokines and thereby inhibits their migration from the liver (Nattermann 2004, 2006).

1.1.4. Non-structural Proteins

There are 4 non-structural proteins, NS2-NS5, with NS4 and NS5 each being divided into 2 sub-units. NS3 possesses many enzyme activities (Gallinari *et al* 1998) including RNA helicase activity. The RNA helicase activity is found in the C-terminal of the NS3 and is structurally similar to other RNA helicases. The NS5A protein contains a region termed the 'interferon sensitivity determining region' (ISDR), variations in the amino acid sequence of which are thought to confer sensitivity to interferon (Enomoto *et al* 1996). HCV lacks

proofreading 3' to 5' exonucleause activity, therefore its replication results in the production of quasispecies within an individual, helping it to escape immune surveillance.

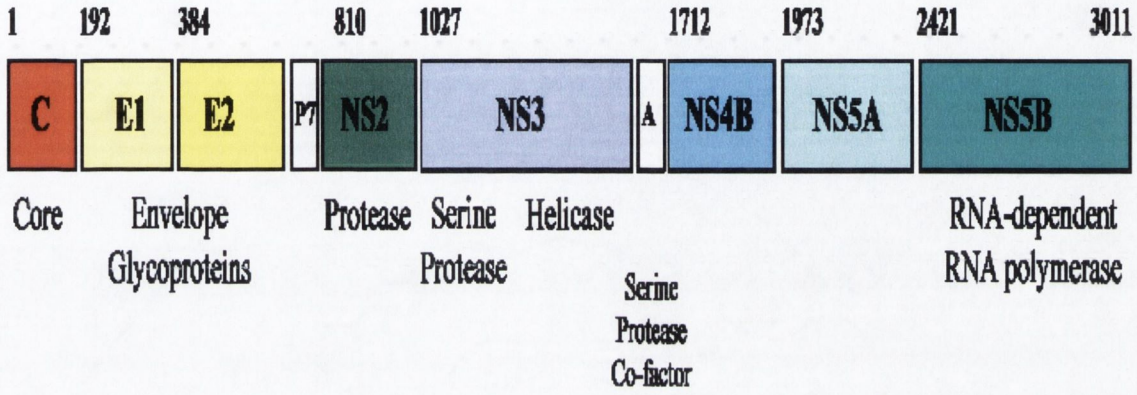

Figure 1: Hepatitis C Virus Proteins

The 9.6kb RNA genome is translated as a single polyprotein precursor, which is processed by cellular and viral proteases into mature structural and non-structural proteins. Amino acid positions are shown above each protein. (*Adapted from Moradpour and Blum, Liver International 2004; 24:519-525*).

1.1.5. HCV Replication

Studies on HCV have been limited by the lack of a tissue culture system or small animal model (Sheehy *et al* 2007). The exact mechanism for HCV replication is still unclear, but the following is postulated, based on knowledge of the HCV proteins and also on known mechanism of replication of other RNA viruses. The HCV virus binds to a host cell surface receptor via the envelope proteins, in order to gain entrance into the cell (Pileri *et al* 1998). Once the virus has entered the cell, it is uncoated in order to begin the replication cycle *(figure 2).* It is thought that non-structural proteins, along with the HCV RNA template and host cell factors combine to form a replication ribonucleoprotein complex (Egger *et al* 1996). It has been proposed that the mature HCV viron possesses a nucleocapsid and an outer envelope made up of a lipid membrane and envelope proteins.

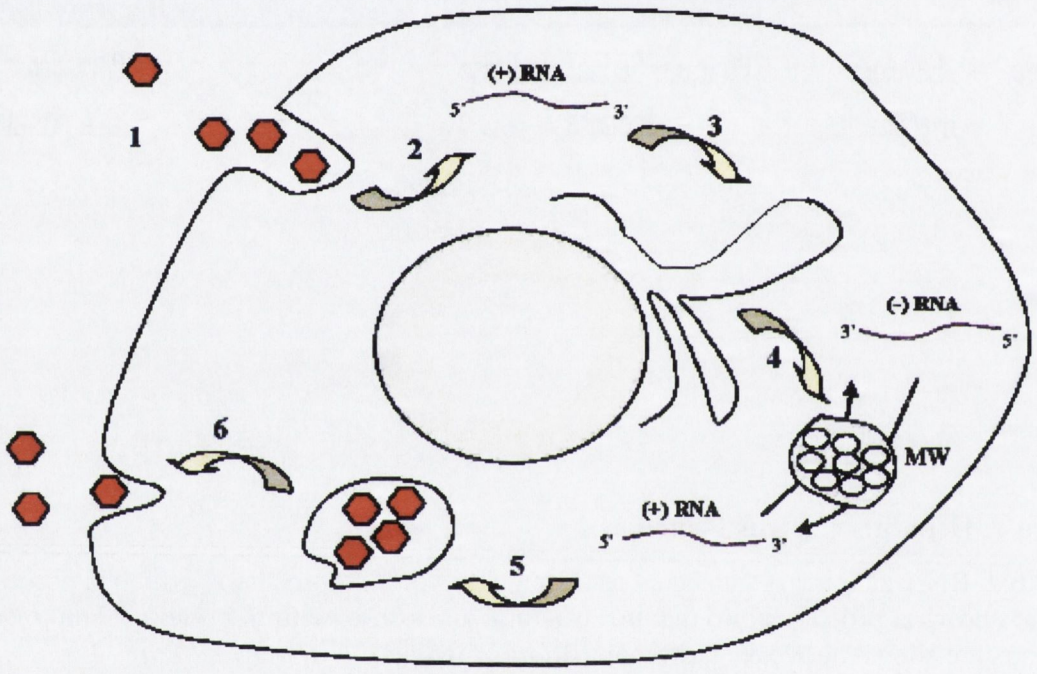

Figure 2: Life cycle of HCV

1. Virus binding and internalization. **2.** Cytoplasmic release and uncoating. **3.** IRES (internal ribosomal entry site)-mediated translation and polyprotein processing. **4.** RNA replication occurs in the membranous web (MW). **5.** Packaging and assembly. **6.** Virion maturation and release. (*Adapted from Moradpour and Blum, Liver International 2004; 24:519-525*).

1.1.6. Epidemiology of Hepatitis C

Current WHO figures suggest that up to 3% of the world's population is infected with HCV, leading to approximately 170 million carrier's world wide (WHO 2004). The prevalence of HCV varies greatly depending on geographical location and age group. The prevalence of HCV in Ireland is 0.1% with intra-venous drug abuse being the commonest risk factor. However it is considerably more common in other countries; for example, 18.1% of Egyptians are infected (WHO 1993). The high prevalence in Egypt has been traced back to mass parenteral therapy for schistosomiasis which was practised from the 1920's until 1988 (Frank *et al* 2000).

Six major genotypes (1-6) and approximately 50 subtypes have been described for HCV. The nucleotide sequence of the various genotypes can vary from each other by up to 30 % (Rosenberg 2001) and although evidence in relation to the affect of genotype on disease outcome is unclear, it is certain that genotype affects response to treatment. The evolution of genotypes has been influenced by numerous factors, including immune selection, infection patterns, replication efficiency, and population migration therefore there is distinct geographic variation in HCV genotypes (Dusheiko *et al* 1994, Lau *et al* 1996). Genotypes 1-3 are found world-wide whilst genotypes 4 and 5 are found primarily in Africa and genotype 6 in Asia.

HCV exhibits considerable genetic diversity and even within an infected individual closely related, but distinct quasi-species are found circulating (Martell *et al* 1992). Like that of other RNA viruses, the HCV polymerase enzyme has no proofreading ability, and is therefore unable to correct errors made during viral replication. The virus continues to mutate at a high but sustainable rate, as it proliferates, conferring it with a very significant survival advantage and making resistance to treatment a considerable problem. It has been shown that greater early quasispecies genetic complexity occurs in those with persistent viremia than in those with spontaneous clearance (Ray *et al* 1999).

1.1.7. Clinical Course of Hepatitis C

Chronicity after initial HCV infection occurs in approximately 85% of individuals infected (Villano *et al* 1999). It is a disease which often goes undiagnosed, as the acute and

indeed often the chronic infection are asymptomatic in a large number of infected individuals. If an individual has circulating antibodies to HCV (i.e. antibody positive) but HCV RT-PCR negative (i.e. copies of the HCV virus are not detectable in their serum using the reverse transcriptase-polymerase chain reaction) it means that they have been exposed to HCV but have cleared the virus and are no longer vireamic. Individuals who are HCV RT-PCR positive are chronically infected. Previously a liver biopsy was recommended in all those who were HCV RT-PCR positive in order to assess the degree of liver damage and guide treatment, however more recently, given the improvement in treatment out-come, particularly for genotypes 2 and 3, liver biopsies are no longer performed routinely pre-treatment. When liver biopsies are done they are scored according to the modified histological activity index (0-18 for inflammation, 0-6 for fibrosis) or by the metavir system (0-4 for inflammation, 0-4 for fibrosis). A study of 2235 HCV patients by Poynard et al suggested that the median time for progression to cirrhosis was 30 years of infection, with 31% never progressing. Rate of progression was not dependent on genotype, but was dependent on gender, age at infection and alcohol consumption (Poynard *et al* 1997). Other factors associated with more rapid progression include having a high BMI (Hourigan *et al* 1999, Hickman *et al* 2003), HIV (Benhamou *et al* 1999, Mohsen *et al* 2003) and frequent use of cannabis (Hezode *et al* 2005).

In those who do develop cirrhosis, but remain clinically compensated, the 5 year survival is > 90%, with a risk of developing hepatocelluar carcinoma of approximately 7% at 5 years (Fattovich *et al* 1997).The rate of progression in the Irish cohort examined here appears to be much slower with only 2% having cirrhosis after 17 years of infection (Kenny-Walsh 1999). Similar results were reported from the German cohort of 917 women infected with HCV from contaminated anti-D immunoglobulin; only 4% had overt cirrhosis 20 years after infection (Wiese *et al* 2000).

1.1.8. Treatment of HCV

The gold standard of treatment for HCV is pegylated-interferon alfa and ribavirin for 24 weeks for those with genotype 2 and 3 and 48 weeks for genotype 1 and 4. The aim of treatment is to achieve 'sustained viral response', i.e. the patient becomes RT-PCR negative (i.e. the virus is no longer detectable in the serum by RT-PCR) whilst on treatment, and remains RT-PCR negative six months after treatment has been completed. As mentioned previously genotype is known to play a role in response to treatment, with best results seen in

those who are infected with genotype 2 and 3, having sustained response rates of over 80%, whilst sustained response for genotypes 1 and 4 are approximately 45%. Prior to starting treatment patients need to be assessed carefully and educated about it, its potential side-effects and expected results. In most centres in Europe the patients are taught how to administer the pegylated-interferon as a sub-cutaneous injection themselves. Interferon can cause depression and any patient with previous psychiatric history or tendency to depression should be assessed by a psychiatrist prior to starting on therapy.

1.2. The Anti-D, HCV cohort

Routine screening of blood donors for HCV infection commenced in Ireland in 1991. Anti-HCV antibodies were identified in 15 women, 13 of whom were Rhesus negative, 12 of these 13 had received Anti-D immunoglobulin in 1977. Analysis of archived Anti-D immunoglobulin with RT-PCR revealed it to be contaminated with HCV. A National inquiry was set up which showed that the contaminated Anti-D was generated in 1977 from the plasma of a single donor who had herself received repeated exchange plasmaphoresis for the treatment of haemolytic disease of the newborn (Irish Gov. stationary office, 1997).

A national screening programme was set up, resulting in 62,667 women being tested, 704 of whom were HCV anti-body positive, over half (55%) of which were HCV RT-PCR positive. These women, all of whom were Irish, had been infected for 17 years by the time of diagnosis and they had a mean age of 45 years. They had no other risk factors for liver disease and showed a remarkably slow rate of progression, with only 2% being cirrhotic at the time of diagnosis (Kenny-Walsh 1999). This study by Kenny-Walsh was of all 363 patients who underwent biopsy, 43% had inflammatory grades < 3 and 83% had fibrotic stage < 1. The mild or slow disease progression seen in this group is similar to that in a German cohort of over 1000 women also infected during pregnancy (Wiese *et al* 2000). It is known that females and those infected below the age of 40 have less severe disease progression. In the rat model oesotrogen has been shown to be protective against fibrosis progression (Yasuda *et al* 1999), presumably due to its inhibition of production of pro-inflammatory cytokines and stimulation of anti-inflammatory cytokines such as IL-10. In a multivariate analysis by Di Martino it showed that the rate of fibrosis progression is higher in nulliparous and post-menopausal women, i.e. those with relatively low oestrogen states (Di Martino *et al* 2004). Not only is pregnancy associated with enormous changes in the immune response but so is the post partum period with significant increase in both CD4 and CD8 cells

upto 12 weeks post partum (0stenser *et al* 2005), which may have influenced disease outcome in this cohort of women all infected immediately post-partum.

HCV is often sub-clinical and therefore undiagnosed in those who have not become chronically infected. This is only one of the reasons why this cohort who were all exposed to HCV in whom some became chronically infected and others cleared it spontaneously are an extremely important and an extremely rare study population. The other great benefit of this group is that it has none of the confounding factors that most other studies face, i.e. different HCV genotypes, different racial origin, sex, age, other forms of liver disease and uncertainty about the duration of infection.

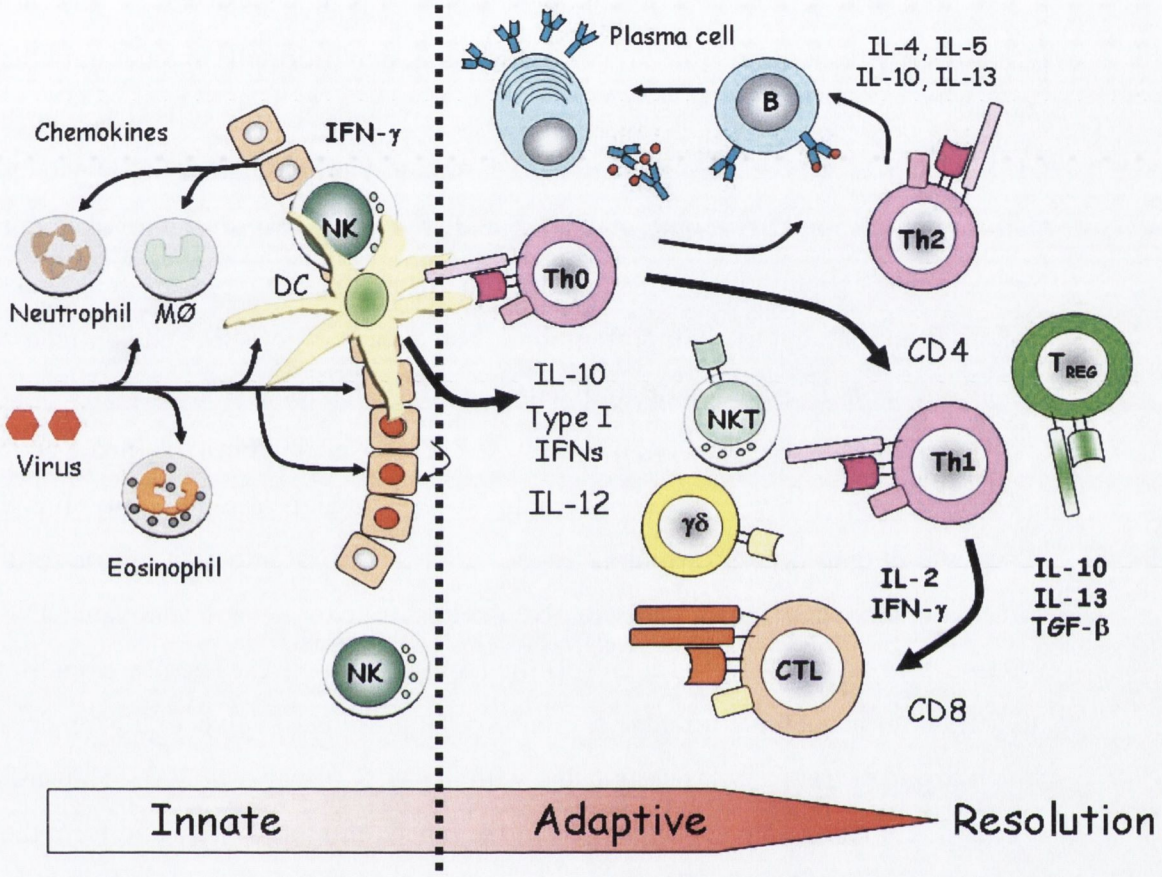

Figure 3: Interplay between the innate and adaptive immune system
A coordinated response from both the innate and adaptive arms of the host immune system is required for successful anti-viral immunity. This is a diagramatic representation of the interplay between the innate and adaptive immune system in response to virus infection. *(Adapted from Golden-Mason and Rosen, Liver Transplantation. 2006;12:363-372.)*

1.3. HCV and the Immune System

The innate immune system is evolutionary ancient and spans both the plant and animal kingdom. It provides the first line of defence against pathogens usually in the form of an inflammatory response mediated by macrophages, dendritic cells and natural killer cells (Janeway *et al* 2002). On exposure to a virus, activated dendritic cells (DCs) prime naïve $CD4^+$ T cells (Th0) thereby inducing a predominantly inflammatory (Th1) and cytotoxic $CD8^+$ T cell response. Not only may NKT and γδ-T cell responses be invoked but so may the production of antibody by B cells activated to differentiate into plasma cells. Innate responses are thought to be important in the acute stage of infection and antigen-specific or adaptive responses more important in chronic viral infection.

As represented by *figure 3* a coordinated response from both the innate and adaptive arms of the immune system is necessary for successful anti-viral immunity. It is the innate arm of the immune system which is important in early viral infection by controlling viral spread and replication sufficiently to give the host time to develop adequate CD4+ helper T, CD8+ cytotoxic T cell and antigen specific antibody responses. The key event in the induction of the adaptive immune response is the priming of naïve T cells by dendritic cells which express viral peptides complexed with the MHC (Sher *et al* 2003, Howard *et al* 2004).

Natural Killer (NK) cells are part of the innate immune system and form the earliest response to viral infection. It has been suggested that the liver contains approximately 1×10^{10} lymphocytes, 65% of which are Natural Killer (NK) cells (Norris *et al* 1998, Mehal *et al* 2001) and they produce both pro-inflammatory (Th-1) and anti-inflammatory (Th-2) cytokines (Chen *et al* 1997). In contrast to this T and B lymphocytes, i.e. part of the adaptive immune response, represent 85% of peripheral blood lymphocytes, suggesting a very important role for the innate immune response in the liver. However in chronic disease, HCV specific CD4+ and CD8+ T cells are present in much higher concentrations, (upto 30 times higher), within the liver than peripherally (He *et al* 1999, Grabowska *et al* 2001), presumably via a cytokine or chemokine driven gradient. Indeed it has been shown that intraheptic HCV specific CD8+T cells in chronic HCV have an impaired ability to secrete IFN γ (Spangenberg *et al* 2005), which would thereby lead to a gradient in favour of sequestration of CD8+T cells within the liver. Also the activity of HCV specific IFNγ is suppressed by CD4(+) CD25(+) regulatory T lymphocyte (T reg) levels, higher levels of

which have been found in those chronically infected with HCV. CD4(+) CD25(+) T reg cells which secrete transforming growth factor beta (TGF-beta) and IL-10 also suppress HCV-specific CD4(+) and CD8(+) T cell proliferation, higher levels are associated with higher viral loads (Cabrera *et al* 2004).

NK cells target any cell not expressing MHC (major histocompatibility complex) Class I antigens by binding to and releasing cytotoxic granules into the cell. They can also be stimulated to mediate antibody dependent cellular cytotoxicity and to produce cytokines IFNα, TNFα, MIP and RANTES. IL-12 along with virus induced INFα and $_{\beta}$ and TNFα are potent inducers of NK cells. NK cell function is inhibited by MHC class I molecules that stimulate inhibitory receptors on NK cell surfaces. MHC class I expression on hepatocytes is upregulated during HCV infection (Ballardini *et al* 1995). *In vitro* studies have shown that recombinant HCV E2 protein inhibits NK-cell functions, including cytotoxicity and IFNγ and TNFα secretion (Crotta *et al* 2002, Tseng *et al* 2002).

Invading pathogens are recognised by pattern recognition receptors (PRR), such as toll receptors which initiate activation and migration of immature dendritic cells (DC). DCs along with macrophages and B cells are professional antigen presenting cells (APC) which play a crucial role in stimulating multiple arms of the immune response. Immature DCs are excellent at antigen uptake, but not at antigen processing or presentation. Upon exposure to inflammatory mediators, such as cytokines and chemokines DCs undergo maturation, stop antigen uptake, and instead begin to process and present the antigen. IL-12 production by mature DCs promotes Th1 T cell differentiation, which in turn promotes the maturation of cytotoxic T cells, which play a major role in host defence against viruses (Koch *et al* 1996). Auffermann-Gretzinger *et al* showed that DCs from patients with chronic HCV failed to mature upon stimulation (Auffermann-Gretzinger *et al* 2001). The function of dendritic cells is also impaired by HCV infection, exhibiting impaired stimulation of T cells and decreased IFNγ production (Kanto *et al* 1999, Bain *et al* 2001). APCs form an antigen presenting complex composed of an MHC molecule and the foreign antigen, which they then 'present' to the antigen receptor on either CD4+ helper T cells or CD8+ cytotoxic T cells. This antigen specific, T cell mediated response forms the second line of defence, the 'adaptive' or 'antigen-specific' immune response. The MHC is located on the short arm of chromosome 6 and is one of the most polymorphic regions of the human genome. MHC class I antigens include HLA A, B and C and complex with APCs to present antigens to CD8+ cytotoxic/suppressor T cells. MHC class II antigens present to CD4+ helper T cells and include the DR and DQ antigens, variations in which have been shown to be associated with

HCV viral clearance (Thurz *et al* 1999, McKiernan *et al* 2004). Also of interest in the context of MHC encoded inter-individual differences to infection, several DEAD box genes are located on the MHC class III region, which encode helicases involved in RNA processing. Helicases have previously been shown to be important in the cells response to RNA virus infections (Kato *et al* 2005, 2006, Gitlin *et al* 2006).

The various steps of the immune responses are interdependent on each other, for e.g. in viral infection a strong cytotoxic T cell response is important as these are the cells which recognise and kill virus infected cells, CD4+T helper cells activate CD8+ cytotoxic T cells through the induction of Th1 cytokines, i.e. IL-2, IFNγ and TNFα (Zinkernagel *et al* 1993). Patients with HCV infection have much fewer cytotoxic T cells than is observed in other viral infections (Rehermann *et al* 1996). In a study in which mice were infected with vacina virus expressing HCV structural proteins, there was marked suppression of the cytotoxic T cell response and it was shown that it was the HCV core protein, which caused this suppression. This study also showed profound suppression of IFNγ production by mice infected with vacina virus expressing HCV core (Large *et al* 1999). Core protein has also been shown to protect primary hepatocytes from cytokine induced apoptosis thereby assisting in viral persistence (Lasarte *et al* 2003). In HCV, there seems to be predominantly a Th1 response in the liver (Napoli *et al* 1996, Dumoulin *et al* 1997). In a study of 28 patients with acute HCV, the 17 who cleared the virus all had a Th1 phenotype when stimulated with HCV antigens, as opposed to the 11 who remained chronically infected, all of whom displayed a Th2 phenotype (Tsai *et al* 1997). Not only is a strong Th1 response important for HCV viral clearance, but amongst those chronically infected, patients with little or no histological disease activity were shown to mount a stronger Th1 cytokine response to *in vitro* stimulation with HCV core antigen than those with severe disease (Woitas *et al* 1997). These results were supported by Kamal's findings that not only does an initially poor CD4+ response lead to chronicity it also leads to more severe hepatic fibrosis (Kamal *et al* 2001). Differentiation of naïve CD4+T cells in to Th1 or Th2 lineages is dependent on many factors including HLA haplotype and affinity between the T cell receptor and MHC complex, (Tite *et al* 1987, Murray *et al* 1989, Pfeiffer *et al* 1995, Pearson *et al* 1997) which may explain, in part, the influence of MHC Class II antigens on HCV viral clearance.

In patients with a high viral load, levels of circulating HCV have a half life of about 3 hours, with 10^{10} - 10^{12} virons produced daily (Neumann *et al* 1998). As mentioned previously studies on HCV are limited by the lack of a tissue culture system or small animal model. Also the fact that the majority of acute infections are asymptomatic, makes studying of the

initial immune response to HCV extremely difficult and potentially skewed. Attempts to circumvent this problem have lead to studies using chimpanzees as models of acute infection, all of which have shown that a strong intrahepatic CD4+ and CD8+ T cell response against a broad range of HCV specific antigens was essential for viral clearance (Thimme *et al* 2002). Thimme et al also looked at a small group of individuals, following accidental needlestick inoculation and very interestingly found that the first appearance of virus specific T cells in the peripheral blood occurred several weeks after a detectable vireamia (Thimme *et al* 2001), suggesting that HCV may initially be able to impair or 'stun' the normal immune response. In acute HCV, CD8+T cells have been shown to be unable to produce IFNγ during the peak of vireamia; recovery of their function coincides with the beginning of CD4+T cell response and a significant decrease in viral load (Lechner *et al* 2000, Thimme *et al* 2001). *In vitro* studies have shown that binding of the HCV core protein to the complement receptor on T cells inhibits proliferation and IL-2 and IFNγ production of antigen specific T cells (Yao *et al* 2001). Not only is the initial T cell response delayed but HCV has also been shown to significantly increase T cell apoptosis and decreases T cell viability, providing yet another method of survival and immunological evasion for HCV (Iken *et al* 2006). Further weight is given to this by a study which showed re-emergence of normal CD4+T cell responses in those who had cleared HCV infection following treatment (Cramp *et al* 2000).

1.3.1. HCV and Host Genetic Factors

As mentioned our study group were all exposed to HCV from a single source yet approximately 45% of those exposed managed to spontaneously clear the virus, the others remaining chronically infected thus individual host immune reactions clearly play an important role. The interaction between the hepatitis C virus and HLA alleles is important in relation to viral clearance and perhaps disease severity. Several class II HLA alleles (*DRB1*0301, DRB1*1101, DQB1*0301, DRB1*04, DQA1*03*) (Alric *et al* 1997, Cramp *et al* 1998, Minton *et al* 1998) have been found to significantly favour viral clearance, while the alleles *DRB1*0701* and *DRB4*0101* were found to be associated with viral persistence. In previous analysis of our study cohort *DRB1*03011* and *DQB1*0201* were associated with viral persistence, and with less severe hepatic inflammation (McKiernan *et al* 2000). Associations with class I HLA alleles and viral clearance and disease severity have also been found. Those from this cohort who had spontaneous viral clearance were previously found to

be more likely to have the following class I alleles; A*03, B07, B27 and CW01, compared with those who remained chronically infected carrying B*08 and B*12 (McKiernan *et al* 2004). Thio et al found the alleles A*1101 and B*57 associated with viral clearance (Thio *et al* 2002). Another study has suggested that not only viral clearance but also rate of progression of fibrosis may also be affected, with a minor increase in progression of fibrosis in those who were heterozygote at the B locus but homozygote at the A locus (Patel et al 2006).

Finally associations with class III polymorphisms and viral clearance or disease severity have also been shown. The TNFα-863A SNP is associated with viral clearance in African Americans (Yee et al 2000, Dai et al 2006), whilst the TNF-238 and TNF-308A SNPs were associated with more severe progression of fibrosis (Thio *et al* 2004). Heterogenicity in the IL-10 promoter region has also been shown to be influence viral clearance in a small Italian study (Mangia *et al* 2004).

1.4. HCV and Chemokines

Chemoattractant cytokines i.e. chemokines are small polypeptides, the main task of which is leukocyte recruitment and trafficking. They play a crucial role in activating receptors on lymphocytes that promote migration into tissues. Lymphocytes will only be recruited to tissues if they express receptors that allow them to respond to locally presented chemokines. Maintenance of leukocyte recruitment during inflammation requires intercellular communication between infiltrating leukocytes and the endothelium and parenchymal cells, mediated by early response chemokines *(figure 4).* The development of large numbers of chemokines and receptors in vertebrates coincides with the emergence of a more sophisticated immune system (Mackay 2001). The importance of chemokines in host anti-viral defence is emphasized by the several protective strategies viruses have developed to circumvent host chemokines such as, secreting chemokine homologs, chemokine receptor homologs and chemokine binding proteins (Kledal *et al* 1997, Boshoff *et al* 1997, Sozzani *et al* 1998).

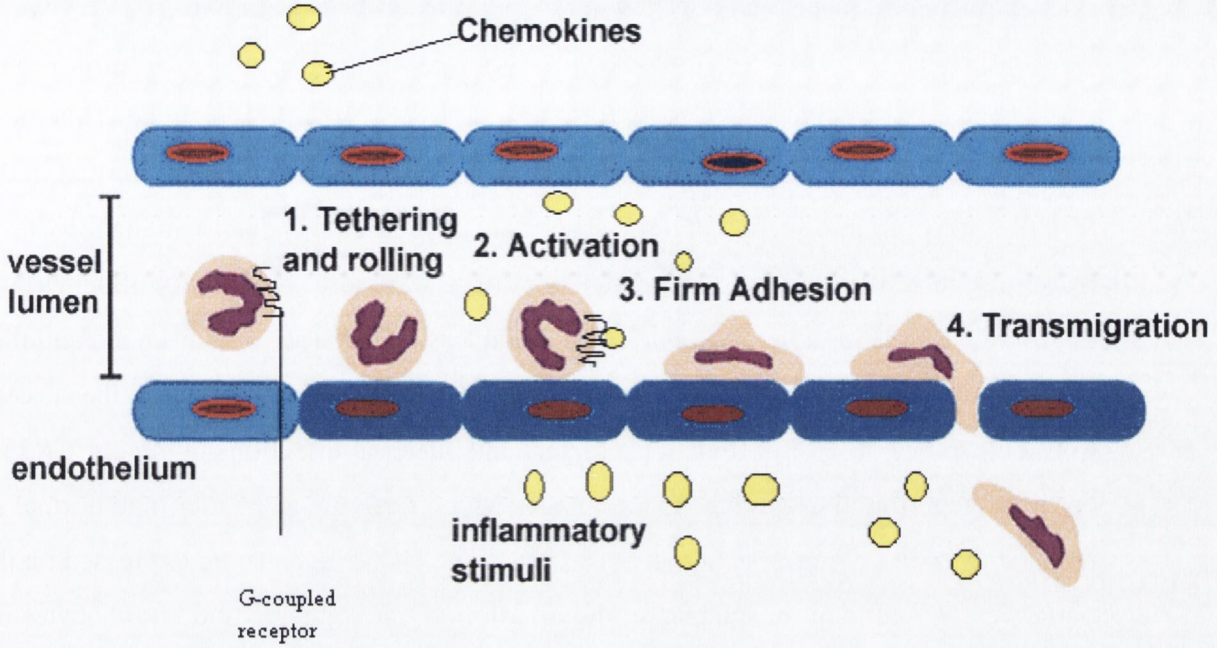

Figure 4: Chemotactic Cytokines – transendothelial migration of leukocytes. Chemokines bind to G-protein coupled receptors on the leukocyte, resulting in integrin mediated adhesion. Following adhesion the leukocyte migrates through the endothelium in response to a chemokine gradient within the tissue. (Adapted from www.mpi.muenster.mpg.de/nrg/wilde.shtml, Prof D Vestweber).

Chemokine receptor 5 (CCR5) facilitates the migration of T cells primed by antigen. CCR5 is expressed on Th (especially Th1 cells, which respond selectively to ligands for CCR5) and also on memory and activated T cells (Baggiolini 2000). As mentioned previously CD4+T cells can differentiate into Th1 (important for HCV viral clearance), or Th2, their differentiation is directed by several factors, including chemokines. Th1 cells are capable of activating macrophages by the release of IFN, which is a potent activator of macrophage phagocytic activity. CCR5 is pretty much ubiquitously expressed, therefore it may not be involved in determining the tissue specificity of lymphocyte homing, but may be more important in relation to retention of lymphocytes after tissue entry. Its ligands are

MIP1α (macrophage inflammatory protein 1α), MIP 1β and RANTES (regulated upon activation, normally T cell expressed and secreted). MIP1α and RANTES have key anti-viral roles in mice. The response of *MIP1α* knock out mice to Coxsackie virus (CVB) was assessed. None of the *MIP1α*-/- mice developed myocarditis (i.e. no T cell infiltration), but they managed to raise CVB antibody titres similar to *MIP1α* wild type mice. This implies that the antigen processing, T cell activation and B cell differentiation are normal in the *MIP1α* knock out mouse, and that the absence of myocarditis is due to an inability to recruit/retain cells in the infected heart. These mice were also exposed to influenza A and their lungs were subsequently found to be much less oedematous and inflamed and there was also a delay in viral clearance (Cook *et al* 1995). CCR5 is very important in the susceptibility to and clearance of several fungal, protozoan and bacterial infections in mice. *CCR5* knock out mice were found to produce macrophages which were much smaller than normal and had reduced cytokine production (Zhou *et al* 1998). The CCR5 ligands are expressed on the portal vessel endothelium and trigger the invasion of macrophages and lymphocytes into the liver (Shields *et al* 1999). It has been shown in HCV infected livers that the majority of liver infiltrating lymphocytes (LIL) showed expression of CCR5 (Shields *et al* 1999). This increased CCR5 expression was correlated with increased inflammation histologically (Boisvert *et al* 2003). It was suggested by Lichterfield *et al* that chemokines mediated trafficking and recruitment of T cells is specifically altered in chronic HCV (Lichterfeld *et al* 2002). Thus CCR5 expression may favour an intrahepatic accumulation of lymphocytes in the portal tracts of HCV infected livers. The progressive liver damage in HCV is associated with up-regulation of Th1 cytokines (IFN and IL-2), and indeed increased expression of these cytokines correlates with both inflammatory and fibrotic histological scores (Napoli *et al* 1996).

Dendritic cells (DC) are professional antigen presenting cells (APC) which play a crucial role in stimulating multiple arms of the immune response. These cells induce both tolerogenic and immunogenic responses and it has been proposed that the cytokines resulting in DC maturation may be the influencing factors as to which response dominates. Immature DCs migrate in response to the ligands of CCR5, (Sallusto *et al* 1998) but appear to lose their response to them upon maturation. As mentioned both the maturation and function of dendritic cells are impaired by HCV infection (Auffermann-Gretzinger *et al* 2001, Bain *et al* 2001, Fowler *et al* 2003). Given the pivotal role of dendritic cells in the immune response and their interaction with CCR5 they provide another enticing potential mechanism by which *CCR5* heterozygosity could result in increased HCV clearance.

CCR5 along with *CCR2* are two of a cluster of six chemokine receptor genes mapped to chromosome 3p21.The *CCR5* gene spans approximately 6kb and consists of 4 exons and 2 introns (OMIM). 17 naturally occurring *CCR5* mutations have been described; most work has been done on the 32bp deletion which has been found to be protective against HIV. This deletion results in a mutant protein which can not be detected on the cell surface (Liu *et al* 1996).

CCR2 codes for a minor HIV receptor designated *CCR264I*. The wild type version of *CCR2* is in complete linkage disequilibrium with *CCR5Δ32*, which is 10kb away. CCR2 is involved in Th1 development and lymphocyte recruitment to sites of infection (Traynor *et al* 2002), therefore we postulated that it may play a role in progression of fibrosis in HCV infection.

RANTES has been mapped to position 17q11.2-2.12. Mice deficient in RANTES infected with murine parainfluenza have been shown to have decreased viral clearance, increased airway inflammation and death (Tyner *et al* 2005). Its expression in HCV infected livers is significantly increased, especially in periportal areas (Apolinario *et al* 2002), again leading to the possibility of polymorphisms in this gene leading to increased hepatic fibrosis in HCV infection.

In chronic HCV chemokine receptors e.g. CCR5, that promote the accumulation of Th1 cells are increased. Studies on murine models have shown that this effect can be mitigated against by the use of anti-CCR5 and anti-MIP antibodies. This study was designed to assess whether the above mutations would impact on the immune response initiated by HCV infection.

1.5. HCV and Toll like Receptors (TLR)

The innate immune response plays an important role in inducing production of anti-viral factors (e.g. IFN, which may prevent replication and viral spread), as well as inflammatory cytokines e.g. IL-6 and TNFα. Involvement of the toll receptor in innate immunity was first described in Drosophilia (Belvin *et al* 1996). Toll like receptors (TLR) initiate the innate immune response by acting as pattern recognition receptors (PRR) that bind to components of the pathogen termed 'pathogen-associated molecular patterns' (PAMP). Recognition of microbial invasion by TLRs triggers activation of a signalling cascade that is evolutionally conserved from Drosophilia to mammals. These genes are type 1 transmembrane proteins with the following common structural features *(figure 5):*

- Multiple leucine rich repeats (LRR), this is the extra-cellular binding domain and comprises 19-25 tandem copies of LRRs.
- A short transmemebrane region
- A conserved cytoplasmic domain that is highly homologous among the individual TLRs and contains a TOLL/IR (TIR) domain similar to the cytoplasmic domain of the IL-1 receptor (cytoplasmic domain) (Takeda *et al* 2005).

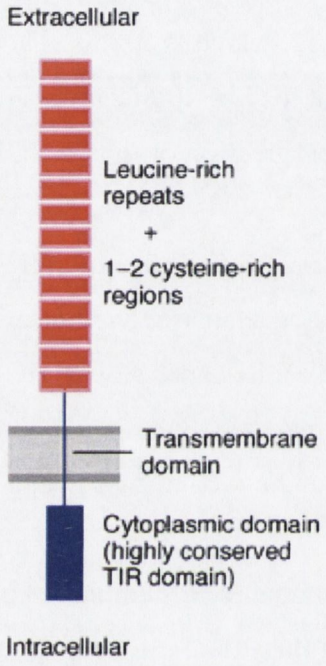

Figure 5: Toll like receptor structure

A diagrammatic representation of the basic structure which Toll like Receptors have in common, i.e. Leucine rich repeats, a transmemebrane domain and a cytoplasmic , highly conserved TIR domain. *(Adapted from Cario, Expert reviews in Molecular Medicine. 2003; 5: 1-15).*

Immature DCs express a variety of innate PRRs including members of the TLR family (Takeda *et al* 2003). Stimulation of TLR induces MyD88 dependent signalling via the Toll-IL1 receptor pathway, which activates DC and helps induce differentiation into professional APCs (Akira *et al* 2001). On maturation DCs produce IL-12 and TNFα, cause up-regulation

of costimulatory molecules (CD40, CD80 and CD86), alter expression of CCR5 and CCR2 and begin to function as APCs, thus initiating the antigen specific immune response (Akira *et al* 2001) (figure 6). TLRs therefore act as a link between the innate and the antigen specific immune response.

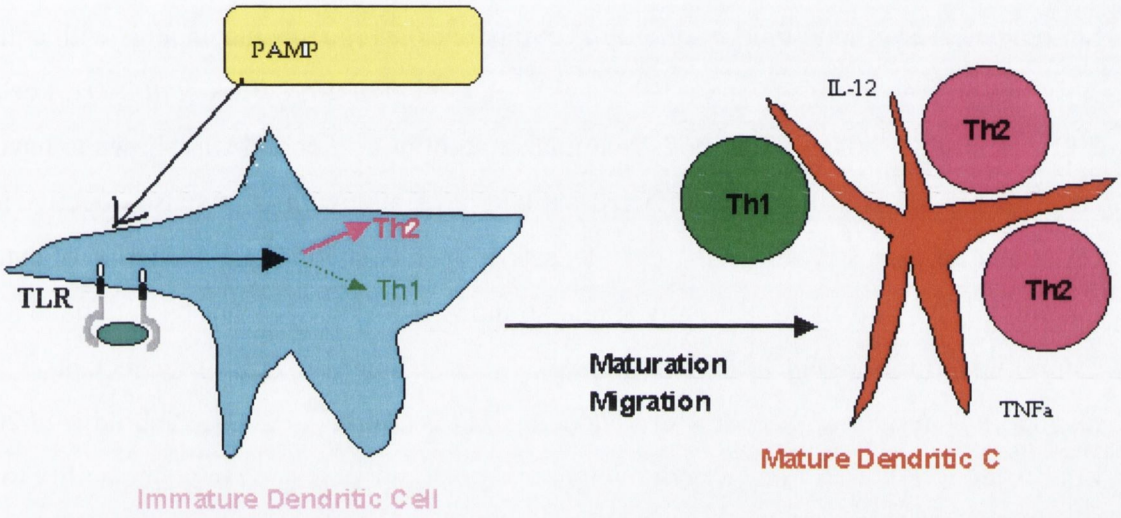

Figure 6: Dendritic cells and TLRs

A diagrammatic representation of the interaction between dendritic cells and TLRS on exposure to a pathogen (PAMP). This interaction affects the development and maturation of the dendritic cell. *(Adapted from David Segal, httphttp://rex.nci.nih.gov/researchbasic/eib/segal/htm)*

Activation of TLRs also results in downstream activation of other transcription factors such as NF-KB, along with other cytokines, chemokines and costimulatory markers (Medzhitov *et al* 1997). Macrophages and DCs express mRNA for most TLRs. Viral infections induce expression of TLR1, 2, 3 & 7 mRNAs in macrophages, this is suppressed by treatment with anti-IFNα/ß antibody, suggesting that IFNα/β may mediate virus induced

activation of innate immunity via modulation of TLR expression (Miettinen *et al* 2001). NK cells, which form part of the earliest response to viral infection strongly express TLR8 on exposure to viral particles, (Saikh *et al* 2003) but do not express it in the resting state (Muzio *et al* 2000).

TLR 7 and *8* are located in tandem on Xp22 and are encoded by 2 exons each. The genes for *TRL7* and *8* show 42.3% identity and 72.7% similarity in their amino acid sequences (Chuang *et al* 2000, Du *et al* 2000). By PCR analysis both TLR 7 and 8 have been shown to be expressed in liver and lung, and TLR8 to be expressed on peripheral lymphocytes. Synthetic imidazoquinoline compounds and guanosine analogs with antiviral activities are known ligands for TLR 7 and 8 (Hemmi *et al* 2002, Jurk *et al* 2002, Lee *et al* 2003, Heil *et al* 2003). Imiquimod, an imidazoquinoline compound was shown to have antiviral activity in guinea-pigs infected with herpes simplex virus; it's also effective against CMV and arbovirus (Chen *et al* 1998). Its activity is mediated via the induction of cytokines including IFNα and IL-12. Recently single-stranded GU-rich RNA has been defined as a natural ligand for human TLR8 and murine TLR7, with human TLR8 transfectants responding strongly to the ssRNA by inducing NFκB (Heil *et al* 2004, Diebold *et al* 2004, Lund *et al* 2004). TLR7 and 8 both recognize viral components and have the ability to induce type I IFN. These TLRs are exclusively localized to endosomal compartments (Takeda *et al* 2005) suggesting that they recognize nucleic acids released into endosomes after viruses are internalized and lysed.

Although *TLR7* and *8* are structurally similar, the cytokine profile they induce in response to agonists are different, with TLR7 selective agonists inducing IFNα from human peripheral blood monocytes (PBMC) and TLR8 selective agonists preferentially inducing TNFα, IL-12 and MIP1α (Gorden *et al* 2005).

TLR7 ligands enhance survival of DCs and increase their surface expression of the costimulatory molecules CD40, CD80 and CD86. TLR7 signalling also induces IFNα and IL-12 production from DCs, resulting in induction of a Th1 response, which is so important in HCV (Ito *et al* 2002). In studies on CMV infected T cells, DCs stimulated by TLR7 and 8 ligands have been shown to significantly increase CMV specific CD4+T and CD8+T cells. TLR7 ligands also showed up-regulation of MHC class II (HLA-DR) and costimulatory molecules (Lore *et al* 2003). IFNα, IL-12 and IL-6 production in response to the ssRNA vesicular stomatitis virus (VSV) is TLR7 dependent. Recognition of VSV by TLR7 appears to occur in the endosome (Lund *et al* 2004). They were also shown to have an increased expression of IFN response genes, ISG15 and OAS, suggesting it worked via stimulation of

the host immune response rather than direct viral suppression (Horsman *et al* 2005). In view of the important role that TLRs play in linking the innate and adaptive immune response, the anti-viral properties of some TLR7 and 8 ligands and the fact that TLR7 and 8 respond to ssRNA we feel they represent good candidate genes for a role in HCV immunity.

1.6. HCV and DEAD Boxes

DEAD box genes encode proteins that are required for a variety of metabolic processes involving nucleic acids and in particular ribonucleic acid (RNA). They are highly conserved amongst species (Linder 2000) and a number of them are located in the class III region of the major histocompatibility complex (MHC). Many putative RNA helicases are members of the DEAD box protein family. These are characterized by the 'DEAD' motif (Asp-Glu-Ala-Asp) as well as by 7 other conserved amino acid motifs including 2 adenosine triphosphate (ATP) binding domains (Gorbalenya *et al* 1988, Linder *et al* 1989, De la Cruz *et al* 1999). Helicases are essential to the normal metabolism of nucleic acids and are found in all cellular organisms. These enzymes use the energy of ATP hydrolysis to separate strains of deoxyribonucleic acid/ribonucleic acid (DNA/RNA). RNA helicases participate in many essential cellular processes such as transcription, translation, ribosome assembly, RNA processing and messenger RNA (mRNA) splicing (Schmid *et al* 1992, Luking *et al* 1998, Egelman *et al* 1998). ATPase activity has been demonstrated for all purified DEAD box and related proteins. The presence of the conserved motifs in DEAD box proteins provides the structural basis for the motor enzymes to be capable of translocation and unwinding long stretches of nucleic acid by using ATP (Henn *et al* 1998, Iost *et al* 1999).

SKI2 (Superkiller 12P) is a yeast DEAD box gene, encoding the protein Ski2p, which has been shown to have antiviral activity (Widner *et al* 1993, Dangel *et al* 1995, Johnson *et al* 1995, Lee *et al* 1995, Maison *et al* 1995, Qu *et al* 1998). The *SKI2* gene was originally characterized as a gene controlling the propagation of killer particles in yeast. These 'killer particles' are in fact yeast viruses with 2 double stranded RNA molecules in a viral capsid. Mutations in the *SKI2* gene result in a 'superkiller' phenotype due to an overproduction of viral RNA. Ski2p appears to be a major route for the destruction of mRNAs which are not (or are no longer) adenylated. Thus Ski2p has an important role in defence against infection by single and double stranded RNA viruses (Widner *et al* 1993, Dangel *et al* 1995).

Viruses are unable to multiply except inside living cells of a host; i.e. they are obligate parasites. Viral mRNA is translated into viral proteins by host cell ribosomes, transfer RNA

(tRNA) and translation factors. Once viral transcription begins, host cell synthesis is generally shut down. The human helicase gene *SKI2W* is located in the class III region of the MHC on chromosome 6 (Shen *et al* 1994). The protein product Ski2w shares striking amino acid sequence similarities to the yeast antiviral protein Ski2p that controls the translation of mRNA. Indirect immunofluorescence experiments show that human Ski2w is localized in the nucleoli and in the cytoplasm, i.e. at the sites of ribosome biogenesis and protein synthesis (Qu *et al* 1998). This raises the interesting possibility of Ski2w having an anti-viral role in humans; in particular in relation to HCV which is a positive sense, single strand RNA virus. A positive strand RNA virus means that the viral genome serves as mRNA in the host cell and serves as a template for a negative strand RNA intermediate during replication.

Once viral transcription begins, host cell mRNA synthesis is severely modified or attenuated by viral mechanisms. *BAT1* a gene encoding a helicase in the MHC, 40 kb telomeric to the *TNFα* gene (Spies *et al* 1989) was also considered as a candidate gene influencing the outcome of HCV infection. BAT1 has been implicated as a negative regulator of pro-inflammatory cytokine expression (Allcock *et al* 2001) and therefore we were particularly interested in its impact on histological staging in HCV. Another DEAD box gene, *DDX3*, located on chromosome X has been shown to interact with the HCV core protein (Owsianka *et al* 1999). *DDX6/RCK* is present on chromosome 11 and has been reported to be over-expressed in those with chronic HCV infection and to have subsequently decreased expression in response to treatment with Interferon therapy (Miyaji *et al* 2003).

1.7 AIM

The aim of this thesis is to look at three different aspects of the immune systems association with response to HCV. Firstly I look at the role of chemokines in HCV, secondly, the role of Toll receptors and finally I look at some DEAD box genes and their potential role in HCV clearance and disease activity. These are key players not only in the innate immune system but are also influential in the antigen specific immune response. In this thesis I assessed the impact that 28 different SNPs in 9 different genes had on the immune response to HCV infection in a cohort of 233 women, all infected by a single source. (An extra 50 patients were also assessed for the CCR5, CCR2 and RANTES polymorphisms.)

Chapter 2

2.1. Materials and Methods

2.1.1. Study Population

The study population of 283 women was recruited from the outpatient hepatology units in St. James, St. Vincent's and the Mater Misericordiae Hospitals in Dublin. All women attending these units who had been exposed to HCV via contaminated anti-D immunoglobulin in 1977 were invited to participate. The group includes those who are both chronically infected, persistently HCV RNA positive as determined by RT-PCR and those who have cleared the virus, i.e. remain HCV anti-body positive but RNA negative. None of the participants had any other risk factors for acquisition of viral hepatitis, e.g. blood transfusion or past history of intra-venous drug abuse. All had an alcohol consumption of less than 14U/wk and other forms of chronic liver disease were excluded in all cases. Of the 283 initially exposed to contaminated anti-D immunoglobulin, 196 remained chronically infected, i.e. RT- PCR (RNA) positive and 87 were anti-HCV antibody positive, but persistently RNA negative, i.e. they had cleared the HCV infection. The RNA negative individuals had an average of six RT-PCR reactions carried out at different time points to confirm spontaneous viral clearance. The majority of these subjects had already been genotyped for both class I and Class II HLA polymorphisms, and a significant association was found between viral chronicity and the presence of DRB1*03011 and DQB1*0201 (McKiernan *et al* 2004). All subjects gave informed consent prior to participating in the study, which received ethical approval from the Research and Ethics Committees at all three institutions. (Note: the patients from the Mater Misericordiae Hospital participated in the CCR5, RANTES and CCR2 study only, hence the difference in population size in the other studies).

2.1.2. Controls

To estimate the frequency of the CCR5Δ32 allele in the Irish population, a control group of 120 unselected, unrelated healthy volunteers were genotyped. These were health care workers and all of Irish descent.

2.2. Diagnosis of Hepatitis C Viral Infection

- HCV antibodies

A third generation enzyme immunoassay (ELISA) (Abbott Diagnostics, Germany) was used to test all subjects for HCV specific antibodies and a third generation recombinant immunoblot assay (RIBA 3) (Chiron Corp., Emeryville, CA,USA) was then used as a confirmatory test.

- RT-PCR testing (Reverse Transcriptase- Polymerase Chain Recation)

An RT-PCR assay (Amplicor; Roche Diagnostic Systems, NJ) was used to test for HCV RNA in all subjects.

2.3. Histological Evaluation

All 196 RT-PCR positive women had had a liver biopsy taken as part of their initial clinical evaluation, i.e. prior to the commencement of any treatment, and between 17-20 years post infection. The liver biopsies were all scored by a single histopathologist in each of the three centres. Biopsies were scored according to the modified histological activity index (0-18 for inflammation, 0-6 for fibrosis). The Hepatic Activety Index (HAI) scoring system used is illustrated below in *Table 1*. HCV RNA negative subjects did not have a liver biopsy performed.

Modified HAI Grading: Necroinflammatory Scores							
Periportal or Periseptal Interface Hepatitis (piecemeal necrosis) (A)	**Score**	**Confluent Necrosis (B)**	**Score**	**Focal (spotty) Lytic Necrosis, Apoptosis, and Focal Inflammation (C)**	**Score**	**Portal Inflammation (D)**	**Score**
Absent	**0**	Absent	**0**	Absent	**0**	None	**0**
Mild (focal, few portal areas)	1	Focal confluent necrosis	1	One focus or less per 10x objective	1	Mild, some or all portal areas	1
Mild/moderate (focal, most portal areas)	2	Zone 3 necrosis in some areas	2	Two to four foci per 10x objective	2	Moderate, some or all portal areas	2
Moderate (continuous around <50% of tracts or septa)	3	Zone 3 necrosis in most areas	3	Five to ten foci per 10x objective	3	Moderate/marked, all portal areas	3
Severe (continuous around >50% of tracts or septa)	4	Zone 3 necrosis + occasional portal-central (P-C) bridging	4	More than ten foci per 10x objective	4	Marked, all portal areas	4
		Zone 3 necrosis + multiple P-C bridging	5				
		Panacinar or multiacinar necrosis	6				
Total Modified HAI = __/18							

Modified Staging: architectural changes, fibrosis and cirrhosis	
Change	**Score**
No fibrosis	**0**
Fibrous expansion of some portal areas, with or without short fibrous septa	1
Fibrous expansion of most portal areas, with or without short fibrous septa	2
Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging	3
Fibrous expansion of portal areas with marked bridging [portal to portal (P-P) as well as portal to central (P-C)]	4
Marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis)	5
Cirrhosis, probable or definite	6

Table 1: Modified Hepatic Activity Index scoring system

2.4. DNA purification from blood

This was done using the QIAamp ® DNA Mini Kit. The reagents were prepared as follows;

Protease stock solution

5.5ml of protease solvent (nuclease-free water containing 0.04% sodium azide) was pipetted into the vial containing lyophilized protease. Dissolved protease is stable for 2 months when stored at -8°C, but storage at -20°C prolongs its life. In order to avoid repeated thawing and freezing an aliquot was stored at 4°C for frequent use.

Buffer AL (lysis buffer)

Stored at 15-25°C.

Buffer AE (elution buffer)

Stored at 15-25°C.

Buffer AW1 (wash buffer 1)

125ml of 96% ethanol was added to the 95ml concentrate to obtain a 220ml working solution of Buffer AW1. Stored at 15-25°C.

Buffer AW2 (wash buffer 2)

160ml of 96% ethanol was added to 66ml of concentrate to obtain a 226ml working solution of Buffer AW2. Stored at 15-25°C.

2.4.1. Method of DNA purification from blood

a) The samples were equilibrated to room temperature (15-25°C) and mixed on a roller.
b) 20ul of protease was pipeted into a 1.5ml microcentrifuge tube.
c) 200ul of the sample was then added to this microcentrifuge tube.
d) In order to inactive the hepatitis C virus in the samples 4ul of RNase A was added at this point.
e) 200ul of Buffer AL was then added and the solution mixed by pulse-vortexing for 15 seconds.

f) The tube was then incubated for 10minutes at 56°C in a pre-heated water bath.

g) The tube was centrifuged briefly to remove drops from the lid.

h) 200ul of 96% ethanol was added to the tube and mixed by pulse-vortexing for 15 seconds.

i) This was followed by centrifuging the tube briefly.

j) The mixture from step 9 was added to the QIAamp spin column (in a 2ml collecting tube), the cap was closed and the mixture centrifuged for 6000xg (8000rpm) for 1 minute.

k) The QIAamp spin column was placed in a clean 2ml collecting tube, and the tube containing the filtrate was discarded.

l) 500ul of Buffer AW1 was added to the QIAamp spin column. The cap was closed and this mixture was then centrifuged at 6000xg for 1 minute.

m) The QIAamp spin column was placed in a clean 2ml collecting tube, and the tube containing the filtrate was discarded.

n) 500ul of Buffer AW2 was added to the QIAamp spin column. The cap was closed and this mixture was then centrifuged at 14,000 rpm for 3 minutes.

o) In order to reduce the chance of Buffer AW2 carryover the QIAamp spin column was placed in a new 2ml collection tube, and the tube containing the filtrate was discarded. The mixture was centrifuged for 1 minute at 14,000rpm.

p) The QIAamp spin column was placed in a clean 1.5ml microcentrifuge tube and the tube containing the filtrate was discarded.

q) 200ul of Buffer AE was added to the QIAamp spin tube; this was incubated at room temperature (15-25°C) for 1 minute and then centrifuged at 6000xg for 1 minute.

r) The spin column was discarded and the DNA quantified.

s) The DNA was alloquoted out and stored at -20°C.

2.4.2. Quantification of Extracted DNA

2 ul of the extracted DNA was diluted in sterile dH_2O. The DNA was measured using a UV/Visible spectrophotometer at 260nm. The DNA concentration in the extracted DNA prep was calculated using the fact that a reading of 1 OD unit at 260nm is equal to 50ug/ml of double stranded DNA.

2.5. Polymerase Chain Reaction (PCR)

2.5.1. PCR reagents and their preparation for CCR5Δ32 Genotyping

Primers

Primers are ordered in lyophilised form and re-suspended in sterile H_2O and diluted to required stock concentrations (most commonly 10x of the working stock). The working stock of primer was aliquoted into small volumes and stored at -20°C. An aliquot for frequent use was defrosted and kept at 4°C to avoid repeated freeze-thawing which can damage primers. The primers for the *CCR5Δ32* mutation were as follows:

- Primer 1 (forward): 5'-CAAAAAGAAGGTCTTCATTACACC-3'
- Primer 2 (reverse): 5'-CCTGTGCCTCTTCTTCTCATTTCG-3'

dNTPs

These were ordered as stock solutions at a concentration of 100mM each. A working stock at the required concentration was prepared and this was aliquoted and kept frozen at -20°C. An aliquot for frequent use was kept at 4°c.

Taq Polymerase

As with most other reagents this was stored on ice and stored at -20°C.

Buffers and other components:

10X Buffer

The 10X buffer (50mM Tris-HCL, 100mM NaCl, 0.1mM EDTA, 1mM DTT, 50% glycerol and 1% Triton X-100) was aliqouted and stored at -20°C. An aliquot was kept at 4°C for frequent use.

$MgCl_2$ (2mM concentration) was stored at -20°C, once it had been vortexed and aliquoted. An aliquot was kept at 4°C for frequent use, and was vortexed before use each time.

This buffer has a high content of detergent, which can damage Taq polymerase, therefore this was always the last component added to the reaction.

Sterile H_2O

The water for PCR reactions must be DNase, RNase free and UV irradiated. The water for PCR was purified and auto-claved prior to being aliqouted in a laminar flow cabinet. The aliquots were stored at 4°C.

2.5.2. PCR Technique

A separate work station was used for the DNA preparation to that used for setting up PCR, to decrease the risk of cross contamination.

a) The 10X Buffer, dNTPs, primers and $MgCl_2$ were all thawed.
b) The Taq was taken out of the -20°C, just prior to use, and kept on ice.
c) A master mix was prepared using the following;

Component	Volume/reaction	Final concentration
10X Buffer	2.5ul	1x
$MgCl_2$	2ul	2mM
dNTP mix	2.5ul	200uM of each dNTP
Primer 1	1uM	
Primer 2	1uM	
Taq polymerase	0.2ul	
dH_2O	14.8ul	

Table 2: PCR Master Mix for *CCR5Δ32* genotyping

d) The 24ul of master mix was then placed in a sterile 0.5ml eppendorf tube. It was mixed gently by pipetting the master mix up and down a few times.
e) 1 ul of DNA was then added to the reaction.
f) The tubes were placed into the wells of a programmed thermocycler when the denaturation temperature was reached.

The thermocycling conditions were as follows:

Steps	Temperatures	Times	Comments
Step 1	95°C	10 minutes	Denaturation
Step 2	94°C	45 seconds	
Step 3	54°C	50 seconds	Annealing
Step 4	72°C	1 minute	Extension
Step 5	Back to step 2	x 34 times	
Step 6	72 °C	5 minutes	
Step 7	4°C	End	

Table 3: PCR conditions for *CCR5Δ32* genotyping

2.5.3. Agarose Gel Electrophoresis

Gelation of Agarose gels are results in a three-dimensional mesh of channels, the diameters of which range from 50nm to >200nm. Migration of the DNA through the gel depends on, the size of the molecules, the percentage of agarose in the gel, the presence of ethidium bromide and the voltage applied.

2.5.4. Preparation of 3% agarose gel

a) The open ends of a clean, dry gel electrophoresis tray were sealed with tape.
b) Powdered agarose was added to 100ml of TAE (Tris-acetate and EDTA, pH 8.0)
c) The lid was put on loosely and the solution heated in a microwave until the agarose powder had dissolved.
d) Once the gel had cooled sufficiently 0.5ug/ml of ethidium bromide was added and mixed by swirling.
e) A comb for forming wells in the gel was placed in the tray and then the gel was poured into the tray and allowed to settle (usually 30 minutes). It is important to remove any air bubbles from the gel.
f) Once the gel has set the comb and tape was removed and the gel placed in the electrophoresis tank and covered with 1x TAE buffer.

g) 5ul of a 25bp DNA ladder was mixed with loading dye and added to the outer well of the gel.

h) A control (i.e. H_2O) was placed in the second well and 10ul of each PCR sample, having been mixed with loading dye was added to the other wells.

i) The gel tank was closed and the electrical leads attached so that DNA migrated toward the positive anode.

j) A voltage of 120 volts was applied for 90 minutes.

k) The gel was then examined by UV light and photographed.

l) The *CCR5* gene wild type was visualised at 189bp, whilst the mutant gave rise to a band of 157bp (32bp deletion).

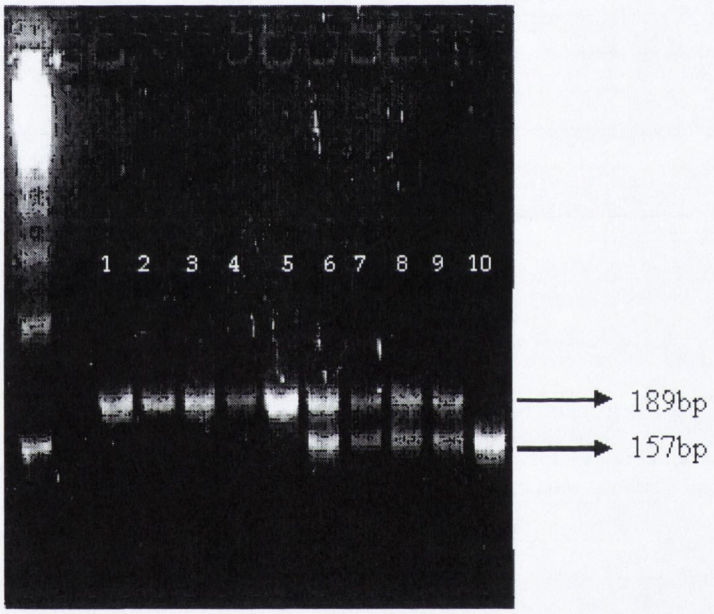

Figure 7: CCR5Δ32 PCR Gel electrophoresis

Gel electrophoresis showing wild type, heterozygous and homozygote mutant for the *CCR5 Δ 32* mutation. Lanes 1 to 5 show the CCR5Δ32 wild type with a single band visible at 189bp, lanes 6 to 9 show CCR5Δ32 heterozygotes with 2 bands visible, one at 189bp and one at 157bp, lane 10 shows a CCR5Δ32 homozygote mutant with a single band at 157bp (32bp deletion). A 25bp DNA step ladder was used.

2.7. Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism was used to perform genotyping on the *CCR264I* polymorphism. A PCR reaction was carried out in a similar fashion to that described above, using the following primers:

- Primer 1 (forward) 5'-TTG GTT TTG TGG GCA ACA TGA TGG-3'
- Primer 2 (reverse) 5'-CAT TGC ATT CCC AAA GAC CCA CTC-3'

The reagents for the PCR master mix are outlined in the table below:

Component	Volume/reaction	Final concentration
10X Buffer	2.5ul	1x
$MgCl_2$	1.5ul	1.5mM
dNTP mix	2.5mM	200uM of each dNTP
Primer 1	1uM	
Primer 2	1uM	
Taq polymerase	0.2ul	
dH_2O	15.3ul	

Table 4: PCR master mix for *CCR264I* genotyping

The conditions for the PCR reaction are outlined in the table below;

Steps	Temperatures	Times	Comments
Step 1	94°C	5 minutes	Denaturation
Step 2	94°C	30 seconds	
Step 3	65°C	1 minute	Annealing
Step 4	72°C	1 minute	Extension
Step 5	Back to step 2	x 39 times	
Step 6	72 °C	7 minutes	
Step 7	4°C	End	

Table 5: PCR conditions for *CCR264I* genotyping

This PCR reaction resulted in a 173bp amplicon, which was run on a gel, as described above under the 'gel electrophoresis' section. This PCR product was now digested with the enzyme Bsa BI (New England Biolabs), from Bacillus stearothermophilus, yielding fragments of 149 and 24bp if an isoleucine was present instead of valine at position 64. The 173bp remained uncut if valine was present and this represents the wild type.

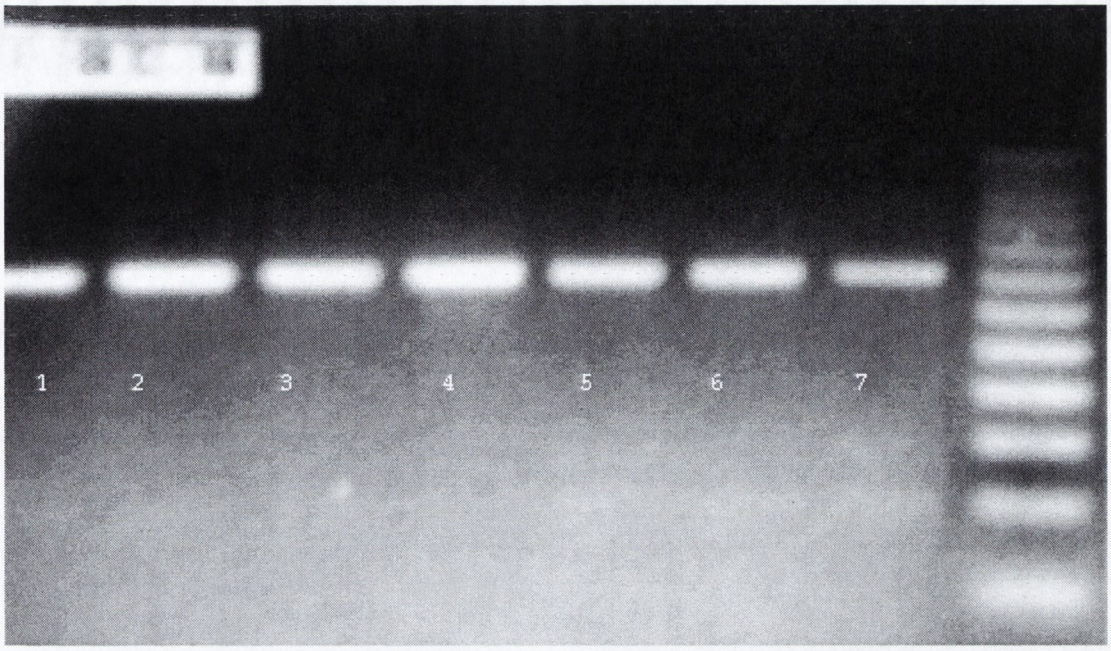

Figure 8: CCR2641 PCR Gel electrophoresis, step 1

Gel electrophoresis showing the first step in the *CCR2641* genotyping resulting in a 173bp amplicon, as seen in all 7 lanes. A 25bp DNA step ladder was used. (25bp DNA step ladder)

Bsa BI digestion of PCR product

Restriction digest of the PCR product was carried out with the following reagents;

Reagent	Volume
***Bsa BI* enzyme**	0.5ul (10,000 units/ ml)
Buffer	2.5ul
H_2O	12ul
DNA	10ul

Table 6: Reagents for *Bsa BI* restriction digest

a) This mixture was then incubated at 60°C for 1 hour, to allow enzymatic digestion to occur.

b) 10ul of the digest product was the run on an electrophoresis gel, as described above

c) The *Bsa BI* enzyme yields the restriction fragments of 149 and 24 bp where its site is present. The wild type remaining uncut and therefore continuing to give a single band at 173bp.

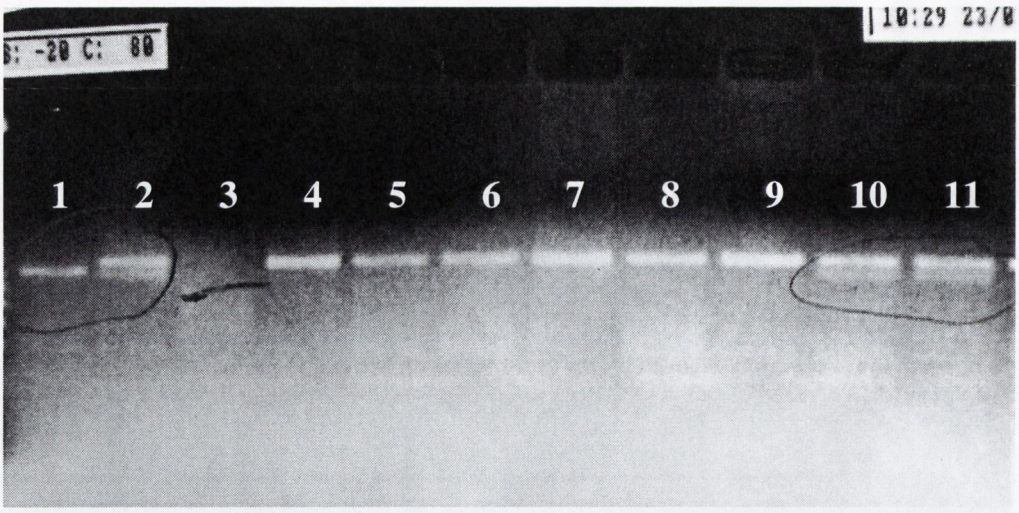

Figure 9: CCR2641 PCR Gel electrophoresis, step 2

Gel electrophoresis showing the second step in *CCR2641* genotyping following restriction digest with *BSA B1*. The wild type remains uncut and shows a single band of 173bp as seen in wells 4 to 9, wells 2, 10 and 11 represent heterozygotes showing a band at 173bp and at 149bp. Well 1 is a homozygote mutant, with a single band visible at 149bp. Well 3 is a control.

2.8. TaqMan ® Technique

TaqMan is based on the 5'-3' exonuclease activity of the Taq DNA polymerase, which results in cleavage of fluorescent dye-labelled probes during PCR.

Applied Biosystems Ltd. designed a 5' exonuclease assay using the 'Assay by Design' service for TaqMan analysis to genotype the *RANTES 403* polymorphism. The sequence of the SNP and surrounding DNA was sent to Applied Biosystems which then generated appropriate primers and probes for this sequence. The assay reagents for SNP genotyping by Assays-by-Design ™ consist of unlabeled PCR primers for amplifying the sequence of interest and TaqMan© MGB (minor binding groove) probes for distinguishing between the two alleles.

Each TaqMan MGB probe contains;

- A reporter dye at the end of each probe – one for each allele of the SNP. VIC ™ dye is linked to the 5' end of the Allele 1 probe, while a. 6-FAM ™ dye, is linked to the 5' end of the Allele 2 probe.
- A nonfluorescent quencher (NFQ) molecule at the 3' end of the probe

During the PCR reaction each TaqMan ® MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites. When the probe is intact, the proximity of the quencher dye to the reporter dye causes suppression of the

fluorescence of the latter by Forster-type energy transfer. Only probes that are hybridized to the target are cleaved by AmpliTaq DNA polymerase (the proprietary DNA polymerase supplied by ABI). Cleavage separates the reporter dye from the quencher dye, resulting in increased fluorescence by the reporter *(figure 10).* The increase in reporter fluorescence can be detected using a spectrometer or sequence detection system (SDS), e.g. ABI PRISM ™ 7000.

Allelic discrimination using this technique involves the use of two probes, each one specific for the target site and only differing by one base. These probes are labelled with different fluorescent dyes, as in this case, the probe for allele 1 is labelled with VIC and the probe for allele 2 with FAM. These dyes absorb at different wavelengths and are discriminated by the SDS.

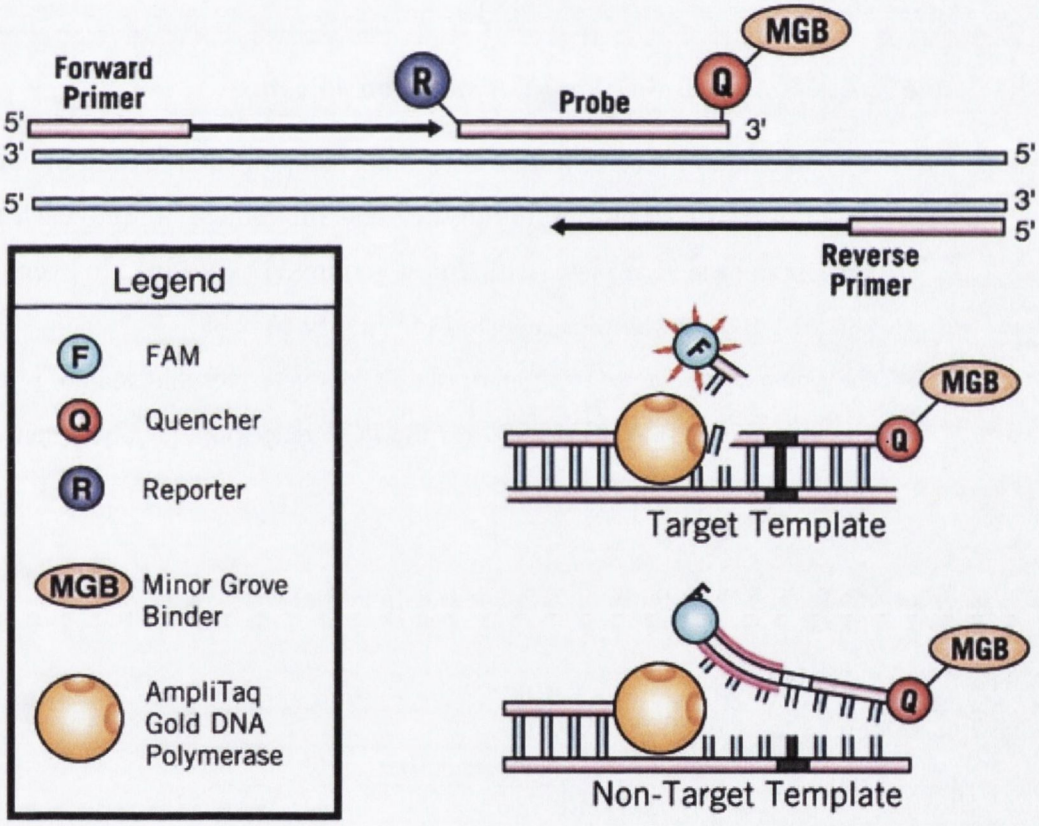

Figure 10: The steps in TaqMan ® PCR

This represents hybridization of the probes to the DNA, and subsequent cleavage by AmpliTaq DNA polymerase, showing separation of the fluorescent probe FAM from the quencher probe when the probe is hybridized to the target template, but no separation from the non-target template. *(Figure taken from Applied Biosystems Ltd.)*

The primer and probe sequences for the RANTES 403 polymorphism were as follows:

- Primer 1 (forward) 5'-GAG GAC CCT CCT CAA TAA AAC ACT TTA TAA AT-3'
- Primer 2 (reverse) 5'-ACT GAG TCT TCA AAG TTC CTG CTT-3'
- Probe 1 (VIC) CAT TAC AGA TCT TA**C** CTC CTT T
- Probe 2 (FAM) CAT TAC AGA TCT TA**T** CTC CTT T

The reagents for the TaqMan PCR reaction were as follows;

Reagents	**Volume/Well (12.5ul vol reaction)**	**Final Concentration**
TaqMan® Universal PCR Master Mix 2X	6.25 ul	1X
40X Assay Mix	0.3 ul	1X
Genomic DNA	2ul	
H_2O	3.9 ul	

Table 7: Allelic Discrimination PCR Reaction 40X mix

a) A master mix or stock solution was made using the above ratio of contents.

b) 10.5ul of the master mix solution was then alloqoutted out into each well of the 96 well optical reaction plate, with 2ul of genomic DNA added to every plat except the controls. 10% of samples repeated for quality control.

c) The plate was covered with an optical adhesive cover and sealed, prior to being placed in the ABI PRISM 7000 for the PCR reaction and subsequent measurement of fluorescent signalling.

Conditions for the PCR reaction were as out-lined below:

Times and Temperatures		
Initial Steps	Each of 40 cycles	
	Denature	Anneal/Extend
HOLD	CYCLE	
10 min 95°C	15 sec 92°C	1 min 60°C

Table 8: Thermal Cycler Conditions

Substantial increase in signalling	Allele
VIC dye fluorescence only	Homozygosity for Allele 1
6-FAM dye fluorescence only	Homozygosity for Allele 2
Both fluorescent signals	Heterozygotes

Table 9: Dye Signalling and genotype

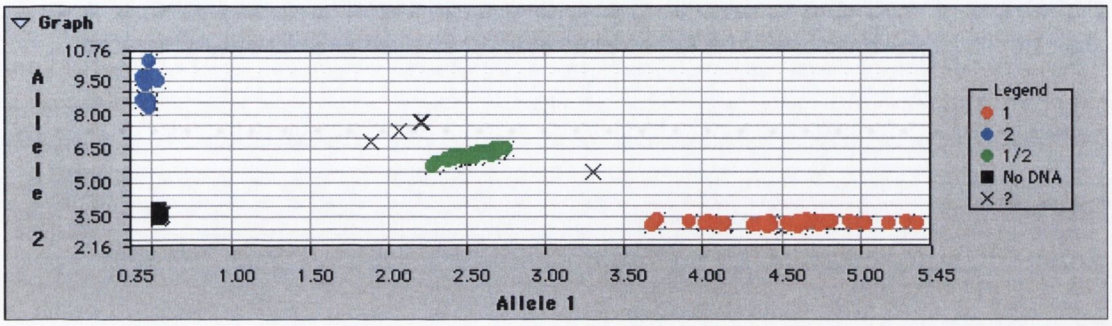

Figure 11: Results from TaqMan Allelic Discrimination Assay

There are typically 4 different clusters of points in the generated results. These points represent the H_2O controls (no amplification)(black), the wild type (red), the heterozygotes (green) and the homozygote mutants (blue), (x = unknown or unclear results). *(Figure taken from Applied Biosystems Ltd.)*

2.9. SNP Selection and Genotyping

2.9.1. TLR7 and 8

Single nucleotide polymorphisms were selected from the NCBI dbSNP database. Selecting all SNPs for which frequency data was available, we chose from these haplotype tagging SNPs (htSNPs) to tag all haplotypes present in caucasian samples at a frequency of 5% or greater as follows. Individual genotypes were downloaded from dbSNP and haplotye frequencies were estimated computationally using HITAGENE (www.hitagene.com) and /or Phase 2.1.1 software (Stephens *et al* 2001). Haplotype tagging SNPs (tSNPs) were identified using SNPTAGGER software (www.well.ox.ac.uk/~xiay/haplotype/) (Xiaji *et al* 2003). Haplotype tagging SNPs represent the minimum set of SNPs required to identify all common haplotypes in any given region of the genome, for a given population. In addition, SNPs with likely functional consequences were added to the minimum set required to tag haplotype variation. Thus we selected 4 SNPs in TLR7 and 5 SNPs in TLR8, *(table 10)* for genotyping using the Ampliflour technique (Myakiskev *et al* 2001). The sequence for the three primers required for each assay are outlined in *table 12.*

SNP Name	Rs number	Sequence
TLR7A	Rs179008	GGACACTGAAGAGAC[**A/T**]AATTCTTATCCTTTT
TLR7B	Rs5741880	GTATTGAGTCATATG[**G/T**]CAGATCCTTTGATCT
TLR7C	Rs5743780	CAGCCTCATCCAATT[**A/G**]GATCTGTCTTTCAAT
TLR7D	Rs5743779	GGGCCTCCCGCCTAG[**C/T**]TTACAGCTTCTCAGC
TLR8A	Rs3764880	TAGAACAACAGAAAC[**A/G**]TGGTAAGCCACTTCT
TLR8B	Rs3747414	TCTGCTAAAAACAAT[**A/C**]AACAAATCCGCA
TLR8C	Rs4830808	AATAATGTGGGTCTA[**C/T**]ACCTCACATTTT
TLR8D	Rs5744067	CAGTTCCTTGCAG[**C/T**]TGTTGTTCGACT
TLR8E	Rs5744077	CCTTCAGTCGTCA[**A/G**]TGCTGACCTGCA

Table 10: TLR SNP names, Rs numbers and sequences.

2.9.2. DEAD Box SNPs

Single nucleotide polymorphisms were selected from the NCBI dbSNP database. Selecting from SNPs for which frequency data was available, choosing those with a frequency for the rare allele above 20% and those which affect putative regulatory or coding regions. Individual genotypes were downloaded from dbSNP. Thus we selected 5 SNPs in SKIW2 and 5 in BAT1, 4 in DDX3 and 2 in RXK/DDX6, *(table 11)* for genotyping using the Ampliflour technique (Myakiskev *et al* 2001). The sequence for the three primers required for each assay is outlined in *tables 13 & 14.*

SNP Name	Rs number	Sequence
SKI1	Rs2280773	GCAGCTGGGA[**C/T**]GGCTTCCCCTGGAG
SKI2	Rs43899	CCAGAAGGGATAC[**C/T**]GAGTTGGATTT
SKI3	Rs437179	GAGGCTCCAACA[**T/G**]ACAGCTAAGGCTT
SKI4	Rs2734331	CACCGAAGGATCTC[**C/T**]GTGGTCATGAT
SKI5	Rs406936	GTTTCAACAT[**C/T**]TGTCCATGTTCC
BAT1A	Rs11796	TGGATGTCTTTTA[**A/T**]GATCAGAAT
BAT1B	Rs2071595	CTTCCTCCCCCC[**C/G**]CAACTTTTAGT
BAT1C	Rs2239709	CATTTTGTCCAGG[**A/G**]TTGTAGTAG
BAT1D	Rs2269476	CTTTGGGAGGC[**C/T**]GAGGCAGGAGGG
BAT1E	Rs2239527	CGAAGGAGGGAAAT[**C/G**]TGCCTTCACTT
RCK1	Rs581045	TCCTGTCAAGTA[**A/G**]TTACTATAGTA
RCK2	Rs524590	GAGAGCACATT[**A/T**]CAGAACAGAGAAGT
DDX31	Rs6610546	GAAAATGGGTC[**A/G**]AACATAGGAACAA
DDX32	Rs870208	CATGAAAGCACT[**C/T**]TTACTTTCTAAAAGT
DDX33	Rs10521420	CATTAGAATGTGA[**A/G**]ATGGGCTTC
DDX34	Rs953114	CTTCACTGGG[**A/C**]ACAAAACTTGGTGTTT

Table 11: DEAD box SNP names, Rs numbers and sequences

2.10. KBioscience (Amplifluor® assay development)

The *TLR7* & 8, *SKI2, BAT1, RcK* and *DDX3* SNPs were genotyped commercially by KBiosciences (Hoddesdon, Herts, UK) using Amplifluor® technology.

2.10.1. Introduction to Amplifluor® technology

The Amplifluor® (Chemicon, CA, U.S.A) is a one step genotyping system which analyses previously characterised SNPs, allowing the simultaneous amplification and detection of DNA. The universal Amplifluor® is targeted to the SNP of interest by unlabeled Z tailed oligonucleotides. It is the unique sequence of 21 bases at the Z tail at the 3'end of the Amplifluor® SNP primer which gives it is universality. The Z tail is one of four parts which make up the Amplifluor® molecule, the others being; a green and red ampliflour, a hairpin structure, and a quencher. The assay utilises two fluorescently labelled Amplifluor® SNP Primers and three unlabelled standard desalted oligonucleotides – two Z-tailed allele-specific primers (one on the 3' end of both the red and green ampliflour) and a common reverse primer (see Figure 2.7). The SNP primers have a hairpin structure which is specifically designed to give optimal thermodynamic stability. Unlabeled, allele-specific oligonucleotides which have the same green or red Z-tail, but on their 5' end, are synthesized. During competitive allele specific PCR it is the universal Ampliflour primers that generate the fluorescent signal whilst the distinction between the alleles is provided by the unlabeled primers. Incorporation of the Amplifluor® SNP Primer into an allele-specific amplicon melts its hairpin structure, thus separating the fluorophore from the quencher and generating a fluorescent signal.

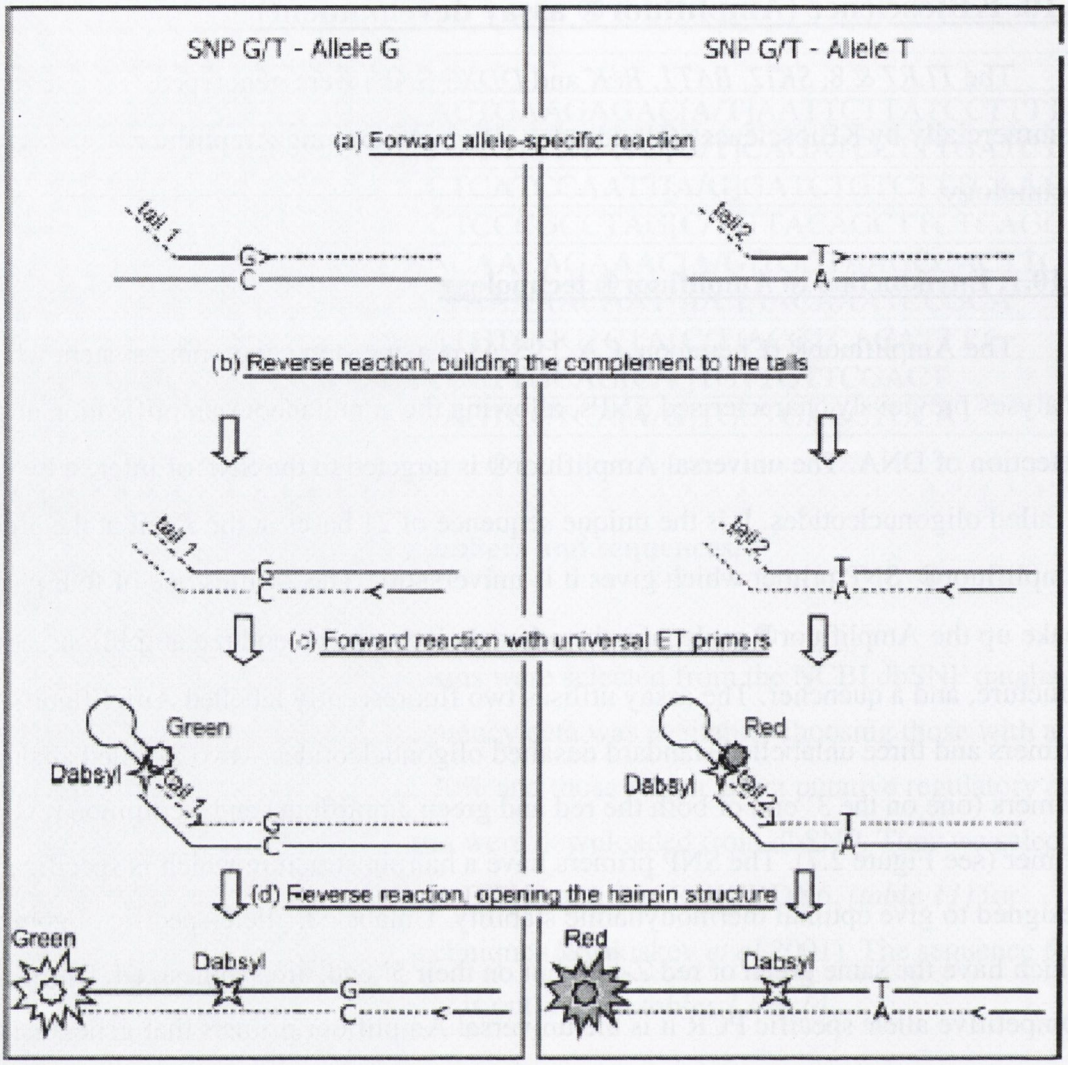

Figure 12: Procedure for Amplifluor SNP detection of a G/T genotype

a) Allele-specific primers anneal to the target and are elongated by Taq Polymerase. Each primer has a unique 5' tail sequence that is identical to the priming domain of one of the Amplifluor® SNPs Primers.
b) The common reverse primer anneals and is elongated by Taq Polymerase resulting in synthesis of the tail sequence complement.
c) Amplifluor® SNPs Primers anneal specifically to the products of the reverse reaction and are elongated by Taq Polymerase.
d) During PCR, the hairpin structures of the Amplifluor® SNPs Primers are unfolded and fluorescent signal are generated. *(Figure taken from www.chemicon.com).*

2.11. Statistical Analysis

The Mann-Whitney U test was used to compare the histological, inflammatory and fibrotic scores and the alanine aminotransferase (ALT) levels, between the different subgroups classified according to patient genotypes. The association between viral clearance and polymorphism was assessed by the Chi-square and Fisher exact test. A p-value of 0.05 was deemed as significant for all of the above tests. Initially multiple logistic regression analysis was run using all the SNPs that have been genotyped for this cohort, no hidden associations were found. A multiple logistic regression model was then made of all the SNPs which had shown significance on Chi square and cross tabs analysis, i.e; DRB1*03011, DQB1*0201, B*, CCR5Δ32, TNF308 and BAT1D in order to assess for the significance of association. A forward, stepwise conditional multiple logistical regression model was used. The odds ratio for each of the different polymorphisms and disease association was also calculated by Epi-Info (Dean *et al* 2002). All data was entered into the statistical package SPSS (SPSS Inc, version 12.0.1,Chicago, Illinois, USA) and Epi-Info.

SNP I.D.	Primer_Allele1	Primer_Allele2	Primer_Common	AlleleA1	AlleleA2
TLR7A	GAAGGTGACCAAGTTCATG CTTCCAATGTGGACACTGA AGAGACA	GAAGGTCGGAGTCAACGGA TTCCAATGTGGACACTGAA GAGACT	CCCAAGGAGTTTGGAAATT AGGATTATGTT	A	T
TLR7B	GAAGGTGACCAAGTTCATG CTGTAATCTCTAGATCAAAG GATCTGC	GAAGGTCGGAGTCAACGGA TTGTGTAATCTCTAGATCAA AGGATCTGA	CAGGGAAAATATTCAACTGT ATTGAGTCAT	G	T
TLR7C	GAAGGTGACCAAGTTCATG CTTTTCTCCCCAGCCTCATC CAATTA	GAAGGTCGGAGTCAACGGA TTCTCCCCAGCCTCATCCAA TTG	GCACGATAGACCTGAAGTT CAAAATTGAA	A	G
TLR7D	GAAGGTGACCAAGTTCATG CTCCTCAAGGCTGAGAAGC TGTAAG	GAAGGTCGGAGTCAACGGA TTCCTCAAGGCTGAGAAGC TGTAAA	CTGGATGGAAACCAGCTAC TAGAGAT	C	T
TLR8A	GAAGGTGACCAAGTTCATG CTAAAGAAATAGAAGTGGC TTACCAT	GAAGGTCGGAGTCAACGGA TTCTAAAGAAATAGAAGTGG CTTACCAC	CGCTGCTGCAAGTTACGGA ATGAAA	A	G
TLR8B	GAAGGTGACCAAGTTCATG CTGTCTTAGTTTCAAGTGCG GATTTGTTT	GAAGGTCGGAGTCAACGGA TTCTTAGTTTCAAGTGCGGA TTTGTTG	CACCTCGATTTAAGTTCCAA TCTGCTAAA	A	C
TLR8C	GAAGGTGACCAAGTTCATG CTGGAATTGTTTAGGTATAA AATGTGAGGTG	GAAGGTCGGAGTCAACGGA TTGGAATTGTTTAGGTATAA AATGTGAGGTA	GTTAGGGTAAATGGGTCTC CATTTAGAAA	C	T
TLR8D	GAAGGTGACCAAGTTCATG CTAGGTGATTTCAGTTCCTT GCAGC	GAAGGTCGGAGTCAACGGA TTCAGGTGATTTCAGTTCCT TGCAGT	GGTGGTGGGGTAGTGAGT CGAA	C	T
TLR8E	GAAGGTGACCAAGTTCATG CTTAGCAGGAAAATGCAGG TCAGCAT	GAAGGTCGGAGTCAACGGA TTGCAGGAAAATGCAGGTC AGCAC	GTGCTTCCACTTTTGATTTT CCTTAGGAA	A	G

Table 12: Primers for TLR SNPs

SNP I.D.	Primer_Allele1	Primer_Allele2	Primer_Common	AlleleA1	AlleleA2
Skiv1	GAAGGTGACCAAGTTCATG CTAACTGGGACTGCAGCTG GGAC	GAAGGTCGGAGTCAACGGA TTAACTGGGACTGCAGCTG GGAT	TTCTGTGACCTGACTCCAG GGGAA	C	T
Skiv2	GAAGGTGACCAAGTTCATG CTGTCCTTATGGGGAAATC CAACTCG	GAAGGTCGGAGTCAACGGA TTGTCCTTATGGGGAAATC CAACTCA	AAGAGTCACCTGGCCAGAA GGGATA	C	T
Skiv3	GAAGGTGACCAAGTTCATG CTCTGGACTACTAAGCCTTA GCTGTA	GAAGGTCGGAGTCAACGGA TTCTGGACTACTAAGCCTTA GCTGTC	CACCCAAATCCAGAGGCTC CAA	T	G
Skiv4	GAAGGTGACCAAGTTCATG CTTCCTGCCTCATCATGACC ACG	GAAGGTCGGAGTCAACGGA TTCTCCTGCCTCATCATGAC CACA	AGTGTCCATCTCTCACCGA AGGAT	C	T
Skiv5	GAAGGTGACCAAGTTCATG CTTTAGATGTGGCCACAGG AACATGGACAG	GAAGGTCGGAGTCAACGGA TTAGATGTGGCCACAGGAA CATGGACAA	CTCCACCTAGTGTTTCAACA T	C	T
Bat1A	GAAGGTGACCAAGTTCATG CTACTGTCCATTGCAGCATT CTGATCT	GAAGGTCGGAGTCAACGGA TTACTGTCCATTGCAGCATT CTGATCA	GTGTGCATTAGCAAAGTGG ATGTCTTTTA	A	T
Bat1B	GAAGGTGACCAAGTTCATG CTCACTCATGCTGGACTAAA AGTTGG	GAAGGTCGGAGTCAACGGA TTCACTCATGCTGGACTAAA AGTTGC	GTAGAGTTATCTGGAGACT GAAGTCTAAT	C	G
Bat1C	GAAGGTGACCAAGTTCATG CTTCGGGTCACGTAACTAC TACAAT	GAAGGTCGGAGTCAACGGA TTCGGGTCACGTAACTACTA CAAC	GGGTCAGGTACCAAGTCCT TCATTT	A	G
Bat1D	GAAGGTGACCAAGTTCATG CTCCCAGCACTTTGGGAGG CC	GAAGGTCGGAGTCAACGGA TTAATCCCAGCACTTTGGGA GGCT	GGCTTCTCTTGCACTTTTGA GCTCAA	C	T
Bat1E	GAAGGTGACCAAGTTCATG CTACAGCGACGAAGGAGGG AAATC	GAAGGTCGGAGTCAACGGA TTACAGCGACGAAGGAGGG AAATG	GGAAGCCTGCAACCGGAAG TGAA	C	G

Table 13: Primers for DEAD box SNPs (Ski and Bat gene)

SNP I.D.	Primer_Allele1	Primer_Allele2	Primer_Common	AlleleX	AlleleY
DDX31	GAAGGTGACCAAGTTCATG CTAATAATTTATTACCTAAAA TAGAAAATGGGTCA	GAAGGTCGGAGTCAACGGA TTAATAATTTATTACCTAAAA TAGAAAATGGGTCG	ATTGGAGAAGACTTTGGGT TGTTCCTAT	A	G
DDX32	GAAGGTGACCAAGTTCATG CTTGAAAGGATCTAACATGA AAGCACTC	GAAGGTCGGAGTCAACGGA TTCTTGAAAGGATCTAACAT GAAAGCACTT	CTTGCCCATGAATAGAGAG CATTTTACTT	C	T
DDX33	GAAGGTGACCAAGTTCATG CTGTTAAATGTTTTCATGTT ACATTAGAATGTGAA	GAAGGTCGGAGTCAACGGA TTAAATGTTTTCATGTTACA TTAGAATGTGAG	TGGTCTACATGACTTTATGA AGCCCAT	A	G
DDX34	GAAGGTGACCAAGTTCATG CTGGCAAGAACAGCTTCAC TGGGA	GAAGGTCGGAGTCAACGGA TTGCAAGAACAGCTTCACT GGGC	AAGGTGAAAGTTAAAGCAA ACACCAAGTTT	A	C
RcK1	GAAGGTGACCAAGTTCATG CTAGAAGTTGGATGTCTAC ATACTATAGTAAT	GAAGGTCGGAGTCAACGGA TTGAAGTTGGATGTCTACAT ACTATAGTAAC	AACCTATAAGGTAGGTATCC TGTCAAGTA	A	G
RcK2	GAAGGTGACCAAGTTCATG CTATTGAACCCTCAATAGAG AGCACATTA	GAAGGTCGGAGTCAACGGA TTGAACCCTCAATAGAGAG CACATTT	TGTCCCACAGCCTACTTCTC TGTT	A	T

Table 14: Primers for DEAD box genes (DDX3 and RcK)

Chapter 3

HCV and Chemokines

3.1. Introduction

Chemokines are small polypeptides with a significant role in leukocyte recruitment and trafficking. Leukocyte recruitment during inflammation requires intercellular communication between infiltrating leukocytes, endothelial cells and parenchymal cells, mediated by early response chemokines. The migration of T cells is modulated *in vitro* and *in vivo* by conditioning with chemokines (Baggiolini 2000). CCR5 is a receptor for the pro-inflammatory chemokines MIP1α, MIP1β and RANTES, which have key roles in host responses to viruses in both human and murine disease (Cook *et al* 1995). A 32 base pair (bp) deletion in CCR5 results in a protein that is not detectable at the cell surface (Samson *et al* 1996). Homozygosity for this deletion is found in 1% of Caucasians and has been shown to be protective against HIV infection, while the heterozygote state, which is found in 10% of Caucasians leads to a slower disease progression. Its role in other viral infections remains to be determined.

CCR5 and other chemokines play an important role in T cell differentiation. CD4+T cells can differentiate into Th1 or Th2 cells depending on their exposure to chemokines. The migration of antigen primed T cells is facilitated by CCR5 (Ansel *et al* 1999). When human T cell clones were analyzed, CCR5 appeared to be expressed at higher levels on Th1 cells, whereas many Th2 clones had no expression of CCR5. Sallusto *et al* demonstrated that CCR5 expression depends on the activation state of T cells and that its expression is up regulated by IL-2 (Sallusto *et al* 1998). It has been proposed that in HCV, there is predominantly a Th1 response in the liver (Napoli *et al* 1996, Dumoulin *et al* 1997). Indeed progressive liver damage in HCV is associated with up-regulation of Th1 cytokines (IFN and IL-2), as shown by Napoli *et al*, where increased expression of IFNγ and IL-2 correlated with both fibrotic and portal inflammatory histological scores (Napoli *et al* 1996).

Both CCR5 and CCR2 are mapped to 3p21. CCR2 codes for a minor HIV receptor, for which a G to A coding sequence polymorphism resulting in a valine to isoleucine substitution, designated CCR264I, has been described. It appears that CCR2WT is in complete linkage disequilibrium with CCR5Δ32, which is 10kb away. Thus, in a study of

3000 individuals, Smith et al (1997) demonstrated that the Δ32 mutation is never seen on the same haplotype as the CCR264I mutation. The distribution of CCR2 and CCR5 in cells and tissues is very similar. CCR2 signalling also promotes Th1 development in infection models and studies using a CCR2 knockout mouse have shown that these mice have a 46% reduction in lymphocyte recruitment to sites of infection and inflammation and an 80% reduction in CD4+T cells locally (Traynor *et al* 2002). RANTES, the CCR5 ligand, is also critical for lymphocyte recruitment, as it attracts memory and activated T cells. An A to G mutation of RANTES has been described at position -403 resulting in an additional GATA transcription factor binding site, with the mutant promoter having up to an 8 fold higher constitutive transcriptional activity than the wild type (Nickel *et al* 2000).

In normal liver, RANTES expression is restricted to a few scattered hepatocytes. However, in HCV infected livers its expression was significantly elevated, especially in periportal and lobular areas that had the most lymphocytic infiltration (Apolinario *et al* 2002). In view of this we postulated that genetic variation in either CCR5 receptors or in the chemokines binding to such receptors might have an impact on outcome of hepatitis C infection. The impact of such variation would be difficult to detect in populations in which there was heterogeneity in terms of ethnicity, viral genotype and source and dose of infection. Hence, we have undertaken a study of the association of the CCR5Δ32 mutation, the CCR2641 mutation and the RANTES position-403 mutation on the outcome of hepatitis C infection in a genetically homogenous population infected through a single source.

3.2. Patients and Methods

(See Study Population, Chapter 2, Materials and Methods)

Controls

To estimate the frequency of the CCR5Δ32 allele in the Irish population, a control group of 120 unselected, unrelated healthy volunteers were genotyped. These were health care workers and all of Irish descent.

Diagnosis of HCV Infection

(See Diagnosis of Hepatitis C Viral Infection, Chapter 2, Materials and Methods)

DNA extraction

(See DNA purification from blood, Chapter 2, Materials and Methods)

CCR5-Δ32 Genotyping

(See Chapter 2, Materials and Methods)

CCR264I Genotyping

(See Chapter 2, Materials and Methods)

RANTES Genotyping

(See Chapter 2, Materials and Methods)

Histological Evaluation

(See Chapter 2, Materials and Methods

Statistical Analysis

(See Chapter 2, Materials and Methods

3.3. Results

The heterozygous genotype was present in 17.6% of the HCV population and 17.9% of the control population, with the CCR5Δ32/32 genotype found in 0.32% and 0.69% respectively (p = n/s). As shown on the map below there is a North to South European gradient for CCR5Δ32 heterozygosity. North / South gradient.

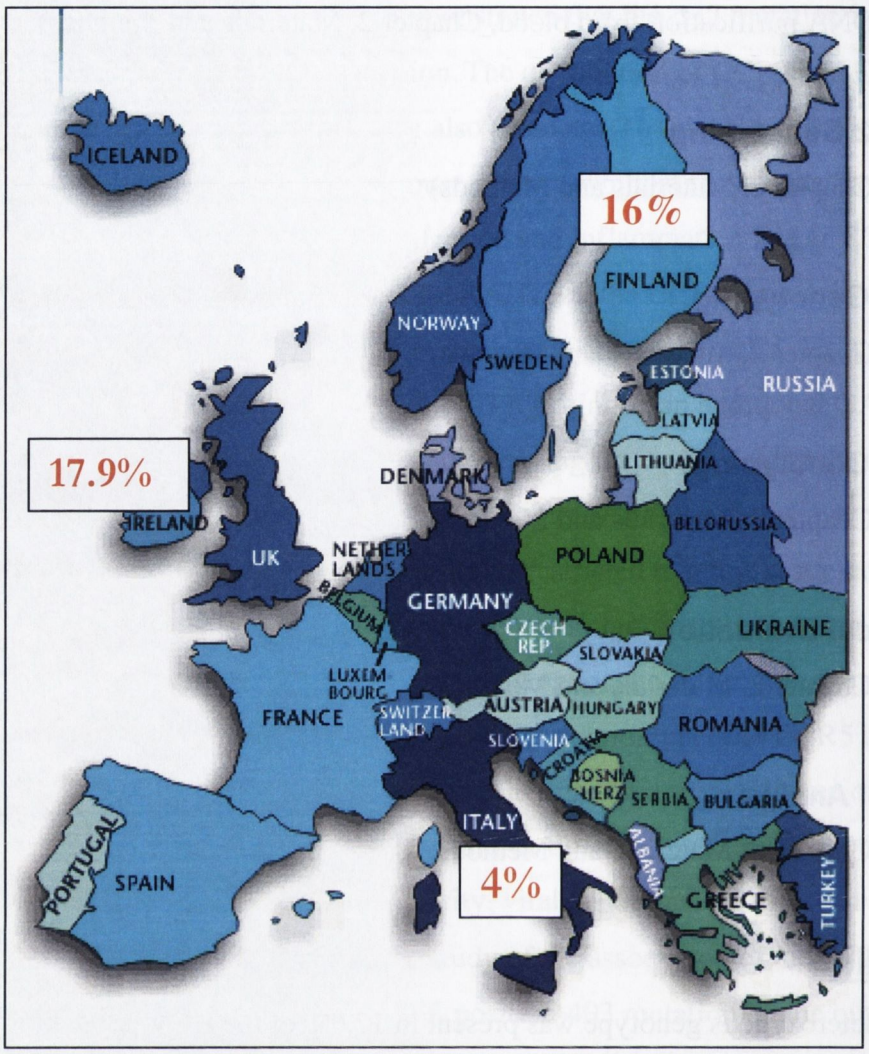

Figure 13: European distribution of CCR5Δ32 heterozygosity (www.euratlas.com)

This map of Europe shows the relatively high frequency of CCR5Δ32 heterozygosity in Ireland and Finland, versus the much lower figure in Sardinia.

3.3.1. Genotypes associated with viral clearance

The heterozygote frequency for the CCR5Δ32 mutation in the general population was similar to that of the HCV study group (17.9% and 17.6%, allele frequency 0.193 and 0.186 respectively).

There was only one CCR5Δ32/Δ32 individual in each group. The presence of the CCR5Δ32/WT (wild type) genotype was significantly associated with spontaneous viral clearance; 42.0% of those who were CCR5Δ32/WT were HCV PCR negative, versus only 28.3% of CCR5WT/WT (p= 0.044, one-sided Fisher's exact test, Odds Ratio (OR) = 1.9, 95%C/I = 1.1-3.6). This significance was lost on multiple logistic regression, performed as

described in section 2.11, with only DQB1*0201 and BT4 retaining significance in relation to viral clearance. Only one patient was homozygotic CCR5Δ32/Δ32 and she was HCV PCR negative. Allele frequency was in Hardy-Weinberg equilibrium for both cases and controls. When the association of CCR5 genotype and viral clearance was looked at in the DRB1*03011 and DQB1*0201 negative groups, none was found (p=0.563 and 0.68, respectively). Analysis of the CCR264I (p=0.327, OR =0.66, C/I =0.23-1.6) and RANTES (P=0.441, OR= 1.01, C/I=0.58-1.7) genotypes failed to reveal any relationship with HCV clearance.

3.3.2. Relationship between genotypes and histological severity

There was no significant difference in hepatic inflammatory scores between heterozygotes for the Δ32 mutation and those without a copy of this mutation (HAI; 3.82 vs. 4.53, p=0.098) in this cohort. Furthermore, in the DRB1*03011 positive group, previously found to be associated with less severe inflammation, CCR5Δ32 had no further additive impact on histological severity; with a mean inflammatory score of 4.16 for non-CCR5WT/WT and 3.80 for CCR5Δ32 heterozygotes, (p=0.78). In contrast, within the DRB1*03011 negative group, associated with more severe inflammation, CCR5Δ32 heterozygotes had significantly lower inflammatory scores than the CCR5WT/WT group (mean inflammatory score = 3.53 vs. 4.91, p= 0.043, *Table 16)*

3.3.3. Relationship between genotypes and ALT levels

The ALT levels were slightly higher in the CCR5 and CCR2 wild type group, compared with the heterozygotes, while the opposite was observed for the RANTES group. However none of these differences reached statistical significance (*table 17*).

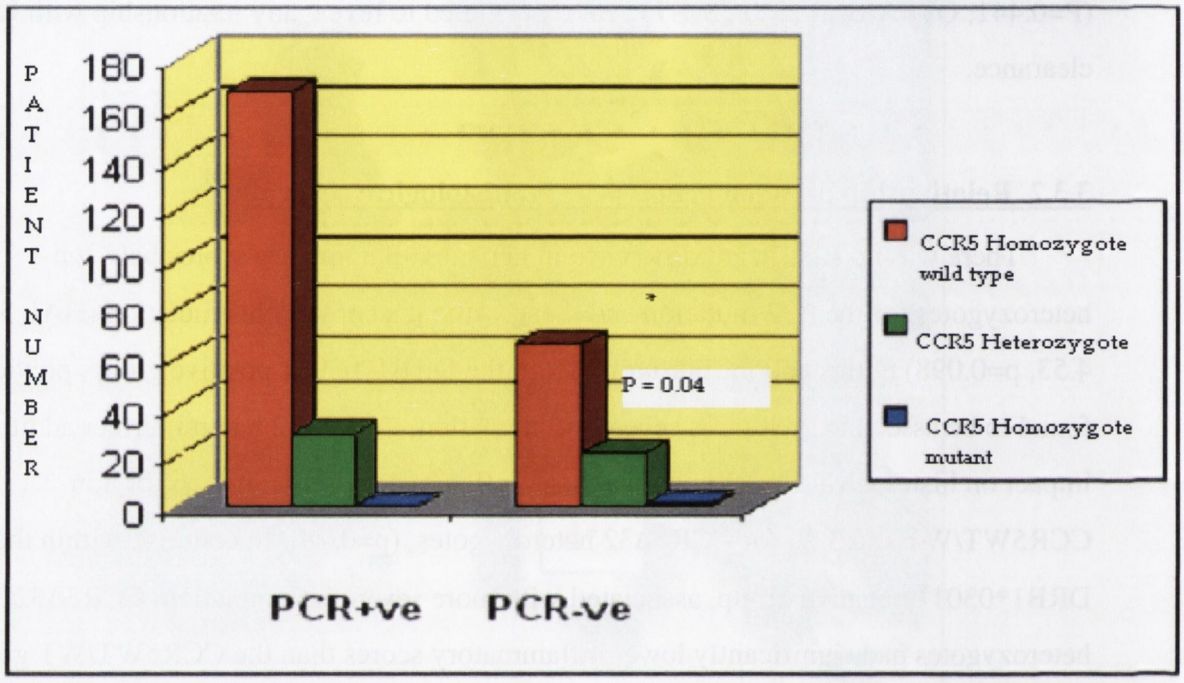

Figure 14: CCR5 genotyping and HCV PCR status

The CCR5Δ32 heterozygote genotype was significantly associated with spontaneous HCV viral clearance, (P = 0.044).

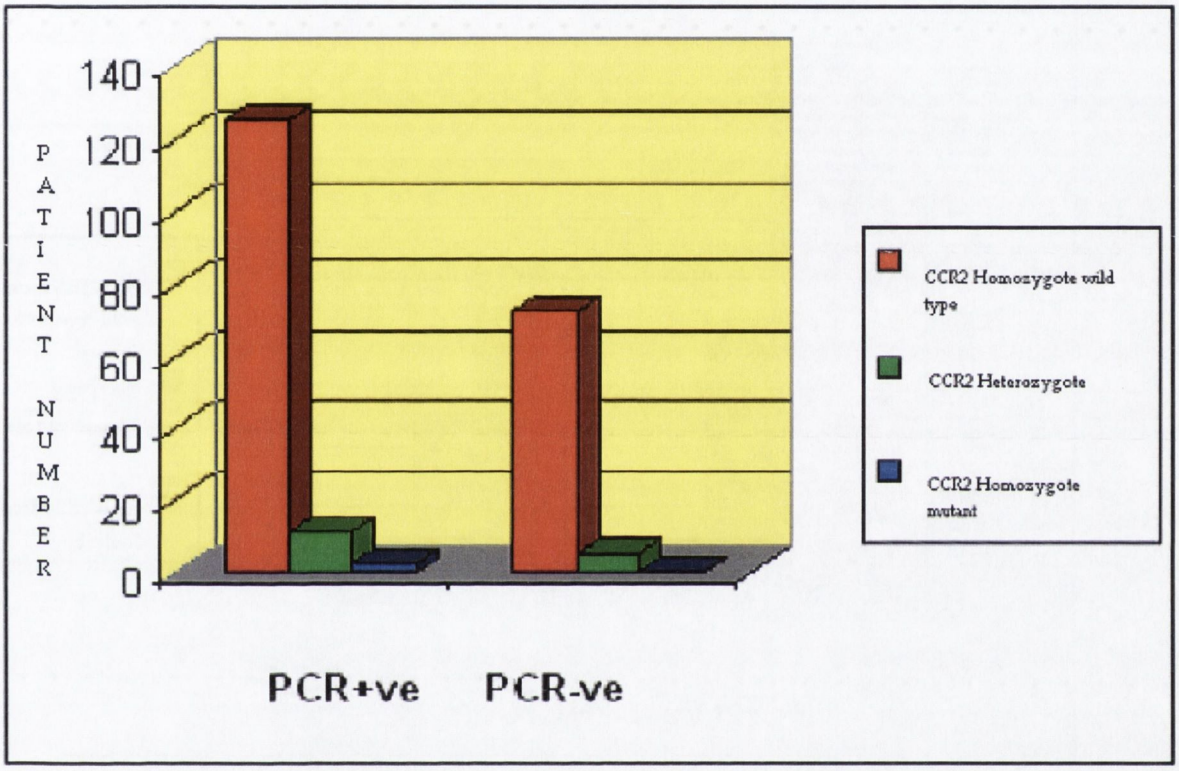

Figure 15: CCR2641 genotyping and PCR status

There was no significant difference between CCR261 genotypes and HCV PCR status.

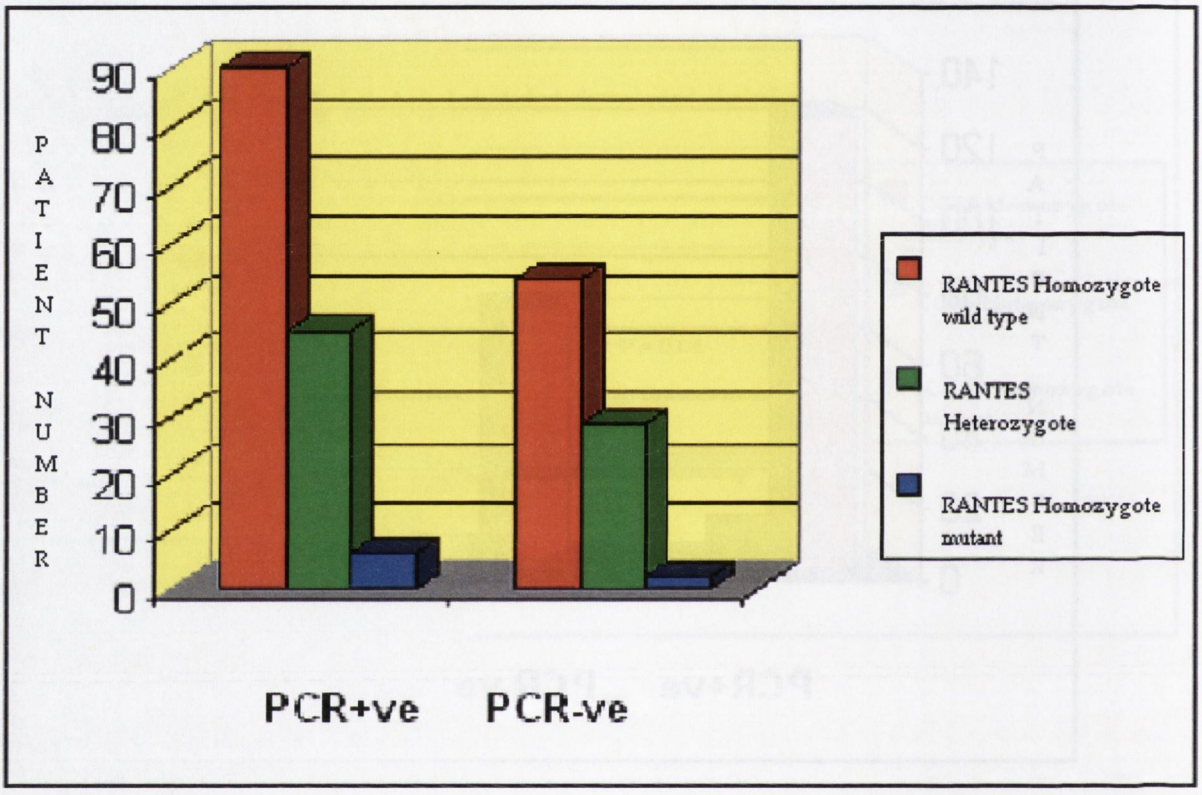

Figure 16: RANTES genotyping and PCR status

There was no significant difference between RANTES genotypes and HCV PCR status.

Genotyping	HCV PCR +ve (%)	HCV PCR –ve (%)	P-Value	OR (95% CI)
CCR5WT/WT	167 (85.2%)	66 (75.0%)	***p = 0.044***	1.9 (1.1-3.6)
CCR5Δ32/WT	29 (14.8%)	21 (23.9%)		
CCR5Δ32/Δ32	0 (0%)	1 (1.1%)		
CCR2WT/WT	125 (90.6%)	73 (93.6%)	p= 0.327	0.66 (0.23-1.6)
CCR264I/WT	11 (7.8%)	5 (6.4%)		
CCR264I/64I	2 (1.6%)	0 (0%)		
RANTESWT/WT	90 (63.8%)	54 (63.5%)	p = 0.441	1.01(0.58-1.7)
RANTES403/WT	45 (32.0%)	29 (34.1%)		
RANTES403/403	6 (4.2%)	2 (2.4%)		

Table 15: Shows the number of individuals with each genotype for the 3 different polymorphisms tested, divided according to their HCV PCR status. The figures in parenthesis are the percentage of those HCV PCR positive or negative, according to their genotype. P values were calculated using chi-square test, Odds ratio by Epi-Info.

	CCR5 WT/WT (%)	CCR5Δ32/WT (%)	CCR5Δ32 /Δ32	P- Value
HCV PCR +ve	167 (71.7%)	29 (58%)	**0**	***p= 0.044***
HCV PCR - ve	66 (28.3%)	21 (42%)	1	
Inflammation	4.53	3.82		p=0.098
Fibrosis	1.20	1.05		p=0.503
DRB1*03011-ve inflam, n=81	4.91	3.53		***p=0.043***
DRB1*03011+ve inflam, n=55	4.16	3.80		p=0.78

Table 16: details the impact of the CCR5Δ32 mutation on HCV PCR status, HAI scores for the entire group, and also for the sub-groups of DRB1*03011 positive and negative individuals. The p-value for the affect of PCR status was given by chi-square, and for HAI scores, as per Mann-Whitney U test.

	ALT levels	P-Values
CCR5WT/WT	52.76	0.419
CCR5Δ32/WT	44.69	
CCR2WT/WT	51.03	0.826
CCR264I/WT	40.67	
RANTESWT/WT	49.07	0.535
RANTES403/WT	54.46	

Table 17: Gives the mean alanine aminotransferase levels for all individuals with the above genotypes, and the associated p-values. P-value level of significance by Mann-Whitney U test.

3.4. Discussion

This study shows significantly higher spontaneous HCV viral clearance in the CCR5Δ32/WT over the CCR5WT/WT group (p=0.044). In 2002 Woitas *et al* reported that CCR5-Δ32 homozygosity occurred three times more frequently in anti HCV positive individuals and was also associated with increased HCV viral load (Woitas *et al* 2002). The major risk factor for HCV infection in this group was haemophilia. However, the Multicentre Haemophilia Cohort Study (MHCS) CCR5Δ32 genotyped 1419 haemophiliacs, 96% of whom were infected with HCV and found the CCR5Δ32 allele distribution to be very similar to that in the general population, i.e. as expected under Hardy-Weinberg equilibrium (Dean *et al* 1996). Similarly Mangia et al found the allele frequency in HCV to be the same as that in the general Italian population (Mangia *et al* 1999). Woitas' findings appear to relate only to a sub-group of HCV infected haemophilias who remain uninfected with HIV. It seems most likely that the increased CCR5Δ32 homozygosity seen by Woitas et al reflects resistance to HIV, rather than increased risk of HCV infection.

This highlights the need to have a study population with as few confounding variables as possible. In this regard our study population is relatively homogenous. All subjects are female, Caucasian and of Irish descent; all were infected by a single inoculum of HCV genotype 1b through anti-D immunoglobulin in1977. They have no other risk factors for liver disease or no other significant co-morbid illnesses. The heterozygous genotype was present in 17.6% of the HCV population and 17.9% of the control population, with the CCR5Δ32/32 genotype found in 0.32% and 0.69% respectively. This is higher than the generally reported figure of 10% for heterozygosity, but ties in with the results of Libert et al who investigated the gene frequency in 18 European countries where he found a North / South gradient, the highest frequencies being in Finland (16%) and the lowest in Sardinia (4%) (Libert *et al* 2002).It is thought that the large variation in CCR5Δ32 allele frequency is unlikely to be solely due to genetic drift and may represent some form of selection advantage.

The association of HCV viral clearance and CCR5Δ32 heterozygosity was not found in Hellier's (2003) or Promrat's (2003) studies, both of which had several confounding issues in relation to HCV genotype, gender, and ethnicity. Specifically, Hellier's study comprised individuals from multiple European populations and contained a lower percentage of viral negative patients. HCV genotype was not specified in this study. In addition, the infection came from multiple sources suggesting a high degree of HCV genetic heterogeneity. Hence a number of confounding variables are inherent to these studies that may have hindered the

ability to detect changes in viral clearance. Our study population has a number of relatively unique features, principally that (i) all subjects are female and Caucasians of Irish descent; and (ii) all were infected by a single inoculum of HCV genotype 1b through anti-D immunoglobulin in 1977 (Kenny-Walsh 1999). They have no other risk factors for liver disease and no other significant co-morbid illnesses. One possible weakness of our study is that the numbers in this homogenous group are relatively small and fixed and the p value is not particularly high. If it were possible to expand the numbers in this study it would be an important next step, as would looking at the impact of this genetic mutation on other HCV genotypes and in males as well as females. However it must also be noted that in this study the significance of the CCR5Δ32 mutation and viral clearance was lost when multiple logistical regression was performed, with only DQB1*0201 and BAT1D retaining significance. This suggests that the importance of the CCR5 mutation in relation to HCV viral clearance may purely be related to linkage with other genes.

The CCR5Δ32 mutation arises from a 32bp deletion causing a frame shift mutation and premature termination of the protein. The resultant CCR5 mutant protein is likely to be functionally inert since it not only lacks the last three of seven putative transmembrane regions, but also lacks the domains involved in G protein coupling and signal transduction (Samson *et al* 1996). Indeed, Liu et al showed that the resultant protein was not detectable on the surface of cells that would normally express it, and therefore cannot act as a receptor (Liu *et al* 1996). However the role of the CCR5 mutation in HCV is not the same as for HIV since the method by which HCV gains entry into the cell is unknown, but unlike HIV, it is generally not believed to be related to the CCR5 receptor.

In explaining how the CCR5Δ32 polymorphism could alter HCV clearance, we must consider that the effect of CCR5 heterozygosity in acute HCV may not be representative of what happens in chronic HCV infection. In acute HCV infection, clearance is associated with a strong T cell response to a wide range of HCV specific antigens (Lechner *et al* 2000). Counter-intuitively, lack of CCR5 may actually lead to increased T cell expansion. This was demonstrated using an acute lymphocytic choriomeningitis (LCM) infection model in CCR5 knock-out mice, where clonal expansion of antigen specific T cells was increased, not decreased, among CD8+ and CD4+ T cells (Nansen *et al* 1999). Likewise, lack of CCR5 has been associated with increased T cell production of IFNγ leading to the suggestion that CCR5 might be part of a negative regulatory feedback loop on acute T cell activation (Zhou *et al* 1998). In CCR5 deficient mice infected with mouse hepatic virus (MHV) there was

reduced T cell infiltration at day7, but by day 12, T cell infiltration was similar to wild type and this study also suggested that IFN production may be increased in the CCR5-/- group (Glass *et al* 2001). Infection with Leishmania donovani showed a shift from an initial low to an exaggerated antigen specific IFN response at 8 weeks post infection in CCR5-/- mice, suggesting that perhaps the impact of CCR5 alters during the course of an infection (Sato *et al* 1999). In contrast to the above, a study by Belnoue et al showed that CCR5-/- mice infected with cerebral malaria had significantly reduced T cell cerebral infiltration (Belnoue *et al* 2003). These contrasting results may reflect CCR5 interaction with parasitic rather than viral infection.

This study also shows a trend towards less severe hepatic inflammatory scores in CCR5WT/Δ32 vs. CCR5WT/WT individuals. In a previous study we identified HLA DRB1*03011 positivity as being associated with reduced hepatic inflammation in this cohort. We did not observe an additive effect of CCR5Δ32 in DRB1*03011 positive individuals suggesting a dominant role for this HLA allele. However we observed significantly lower hepatic inflammatory scores for the CCR5Δ32/WT groups who were DRB1*03011 negative ($p= 0.043$). In a recent publication by Hellier *et al*, a significant decrease in portal inflammation, but not in overall necro-inflammatory score was found amongst CCR5Δ32 heterozygotes (Hellier *et al* 2003). CCR5Δ32 is known to be associated with reduced migration of circulating lymphocytes in response to ligands such as MIP-1 alpha (Liu *et al* 1996).

In HCV there is predominantly a Th1 response in the liver. CCR5 is expressed on Th1 cells and facilitates the migration of T cells primed by antigen. Although the number of HCV specific cytotoxic lymphocytes (CTL's) in the liver is low during infection, there are many activated / memory T cells present, most of which express CCR5. It has been reported that in HCV patients, liver-infiltrating lymphocytes (LIL) showed increased expression of CCR5, (Shields *et al* 1999) which correlated with histological severity (Boisvert *et al* 2003). Similarly, animal studies have shown a key role for CCR5 in hepatic lymphocyte migration (Murai *et al* 1999). Hence reduced expression of CCR5, associated with heterozygosity for CCR5Δ32, could be mechanistically associated with less hepatic inflammation, due to reduced migration of CCR5 expressing cells.

Both Hellier and Promrat found an association with the RANTES -403 promoter polymorphism and reduced hepatic inflammation in a subgroup of patients, which was not found in this study. It is possible that ethnic variation in the RANTES polymorphism and the

patient numbers may partly explain differences between these studies. It should also be noted that the allele frequency for the mutant allele was higher in the Irish population (24%), compared with 15% in Helliers study (multi-centre European wide study population) and 17.5% in Promrats study (American based study population). As with the CCR5 mutation, there seems to be an ethnic variation in allele frequency of the RANTES-403 promoter polymorphism.

While there is a detectable effect on HCV clearance seen in this study, further studies are required to determine whether such data are generalisable to the broader HCV infected population. Such studies will require large numbers of patients and will also require either genetic homogeneity regarding ethnic origin or stratification given the wide diversity in allele frequency for this polymorphism even in Caucasian European populations. The effect that this mutation may have on HCV clearance and severity may be not only important in relation to those solely infected with HCV, but also of vital importance to the vast numbers who are coinfected with HIV, particularly as anti-CCR5 directed medications are already being investigated for the treatment of HIV (de Clercq *et al* 2001, Strizki *et al* 2001).

Chapter 4

HCV and Toll like receptors

4.1. Introduction

Toll like receptors (TLRs) are expressed by human antigen presenting cells (APCs) including monocytes/ macrophages, B cells and dendritic cells (DCs), all of which have distinct patterns of TLR expression. In turn, the identity of the TLRs expressed by individual APCs dictates their patterns of differentiation and cytokine production in response to different TLR ligands. Activation of innate immunity is characterized by the production of cytokines and chemokines, and provides the stimulus to engage the antigen specific immune system. The innate immune response also plays an important role in inducing the production of anti-viral factors, particularly IFNγ.

Low molecular weight compounds of the imidazoquinoline family, imiquimod and resiquimod (R-848) have anti-viral and anti-tumour properties and are known to be ligands for TLR7 and 8. Imiquimod has activity against herpes simplex virus and CMV and is also used for the treatment of genital warts caused by the human papilloma virus (Beutner *et al* 1998, Stanley *et al* 2002). Several studies have recently shown that TLR7 responds to ssRNA, using the influenza virus and vesicular stomatitis in murine models (Diebold 2004, Lund *et al* 2004). TLR8 is not active in mice, but in a study by Heil *et al* using transfected human embryonic kidney (HEK) cells, human TLR8 was shown to strongly induce NF-kB activation on stimulation with ssRNA (Heil *et al* 2004). Another study looking at the affect of different TLR ligands on human lymphocytes infected with CMV or HIV showed that when TLR7 expressing dendritic cells were stimulated with TLR7 ligands the result was a rapid up-regulation of co-stimulatory molecules as well as MHC class II (HLA-DR) antigens and increased production of IFNα (Lore *et al* 2003). We therefore examined the association

of TLR7 and 8 polymorphisms on outcome of infection with hepatitis C, a single stranded RNA virus, specifically in relation to potential association on viral clearance and disease severity in a highly defined patient population.

4.2. Patients and Methods

Study Population

(See Study Population, Chapter 2, Materials and Methods)

Diagnosis of HCV Infection

(See Diagnosis of Hepatitis C Viral Infection, Chapter 2, Materials and Methods)

DNA extraction

(See DNA purification from blood, Chapter 2, Materials and Methods)

TLR7 & 8 SNP Selection and Genotyping

(See, Chapter 2 Methods and Materials)

Statistical Analysis

(See, Chapter 2 Methods and Materials)

Histological Evaluation

(See, Chapter 2 Methods and Materials)

4.3. Results

The genotypes of the five TLR8 SNPs and the four TLR7 SNPs were compared with HCV PCR status, histological scores and alanine aminotransferase (ALT) levels. Although SNPs TLR7C and D both had reported frequencies of the rarer allele of 4% (www.ncbi.nlm.nih.gov), none of the 200 cases genotyped in this study carried the rare allele. All loci were in Hardy-Weinberg equilibrium ($p<0.05$).

4.3.1. Genotypes and viral clearance

Polymorphisms in TLR7 and 8 were not associated with PCR status in this study and thus do not appear to influence viral clearance. *(Table 18 and 19).* Multiple logistic regression was also performed and none of the TLR7 or 8 SNPs added any significance to the model.

SNP	HCV status	Genotype n (frequency)					Allele n (frequency)				
		A_1A_1	A_1A_2	A_2A_2	OR	P	A_1	A_2	P	OR	95%CI
TLR7A	PCR +ve	76 (.71)	27 (.25)	4 (.04)		0.51	179 (.84)	35 (.16)	0.58	1.18	.69-2.0
Rs179008 A/T	PCR -ve	55 (.65)	28 (.33)	2 (.02)	0.75		138 (.81)	32 (.19)			
TLR7B	PCR +ve	85 (.83)	17 (.16)	1 (.01)		0.24	187 (.91)	19 (.09)	0.1	1.72	.91-3.25
Rs5741880 G/T	PCR -ve	61 (.72)	21 (.25)	2 (.03)	0.56		143 (.85)	25 (.15)			
TLR8A	PCR +ve	58 (.54)	43 (.40)	7 (.06)		0.66	159 (.74)	57 (.26)	0.9	0.95	.59-1.5
Rs3764880 A/G	PCR -ve	48 (.58)	28 (.34)	7 (.08)	1.6		124 (.74)	42 (.25)			
TLR8B	PCR +ve	53 (.48)	45 (.40)	12 (.10)		0.34	151 (.69)	69 (.31)	0.32	1.27	.82-1.95
Rs3747414 A/C	PCR -ve	30 (.38)	40 (.50)	9 (.11)	0.66		100 (.63)	58 (.37)			
TLR8C	PCR +ve	69 (.63)	35 (.32)	5 (.05)		0.79	173 (.79)	45 (.21)	0.89	0.96	.58-1.58
Rs4830808 C/T	PCR -ve	56 (.66)	24 (.28)	5 (.06)	1.1		136 (.80)	34 (.20)			
TLR8D	PCR +ve	68 (.63)	35 (.32)	5 (.05)		0.83	171 (.79)	45 (.21)	1.0	0.98	.59-1.6
Rs5744067 C/T	PCR -ve	55 (.65)	25 (.29)	5 (.06)	1.1		135 (.79)	35 (.21)			
TLR8E	PCR +ve	104 (1)	0	0		0.44	208 (1)	0	0.44	n/a	n/a
Rs5744077 A/G	PCR -ve	83 (.99)	1 (.01)	0	n/a		167 (.99)	1 (.01)			

Table 18: TLR SNPs allele and genotype frequency in relation to HCV PCR status

SNP	HCV status	Allele A_1 carrier status					Allele A_2 carrier status				
		A_1A_1/A_1A_2	A_2A_2	P	OR	95%CI	A_1A_1	A_1A_2/A_2A_2	P	OR	95%CI
TLR7A Rs179008 A/T	PCR +ve	103 (.96)	4 (.04)	0.69	0.62	.11-3.47	76 (.71)	31 (.29)	0.35	1.3	.72-2.46
	PCR -ve	83 (.97)	2 (.03)				55 (.65)	30 (.35)			
TLR7B Rs5741880 G/T	PCR +ve	102 (.99)	1 (.01)	0.58	2.48	.22-27.9	85 (.83)	18 (.17)	0.11	1.78	.88-3.6
	PCR -ve	82 (.97)	2 (.03)				61 (.72)	23 (.28)			
TLR8A Rs3764880 A/G	PCR +ve	101 (.94)	7 (.06)	0.78	1.3	.45-3.95	58 (.54)	50 (.46)	0.66	0.85	.47-1.5
	PCR -ve	76 (.92)	7 (.08)				48 (.58)	35 (.42)			
TLR8B Rs3747414 A/C	PCR +ve	98 (.89)	12 (.11)	1	1	.4-2.6	53 (.54)	45 (.46)	0.16	1.6	.85-2.9
	PCR -ve	70 (.88)	9 (.11)				30 (.43)	40 (.57)			
TLR8C Rs4830808 C/T	PCR +ve	104 (.95)	5 (.05)	0.75	1.3	.36-4.6	69 (.63)	40 (.37)	0.76	0.89	.49-1.6
	PCR -ve	80 (.94)	5 (.06)				56 (.66)	29 (.34)			
TLR8D Rs5744067 C/T	PCR +ve	103 (.95)	5 (.05)	0.75	1.3	.36-4.6	68 (.63)	40 (.37)	0.88	0.93	.51-1.67
	PCR -ve	80 (.94)	5 (.06)				55 (.65)	30 (.35)			
TLR8E Rs5744077 A/G	PCR +ve	104 (1)	0	1	n/a	n/a	104 (1)	0	0.44	n/a	na/
	PCR -ve	84 (1)	0				83 (.99)	1 (.01)			

Table 19: TLR SNPs allele frequency in relation to HCV PCR status

4.3.2. Relationship between genotypes and hepatic fibrosis

Possession of the minor allele was associated with less severe hepatic fibrosis in two of the five SNPs assessed in TLR8. Specifically, the hepatic fibrosis scores of patients with the minor allele of **TLR8C** were significantly lower than the major allele 1.38+/- 0.5 vs. 0.78+/- 0.3, p = 0.03. Furthermore possession of the minor allele of **TLR8D** was similarly associated with a lower degree of fibrosis (1.39+/- 0.5 vs. 0.70+/- 0.4, p = 0.018). None of the polymorphisms examined in TLR7 showed any association with degree of hepatic fibrosis in this study. (*table 20*).

SNP name	Allele	ALT	(P value)	Inflammation	(P value)	Fibrosis	(P valu
TLR7A	A1A1	55	0.4	4.6	0.86	1.2	0.73
	A1A2, A2A2	48		4.7		1.1	
TLR7B	A1A1	47.8	**0.014**	4.4	**0.015**	1.1	0.7
	A1A2, A2A2	73.4		5.7		1.3	
TLR8A	A1A1	51	0.3	4.4	0.4	1.4	0.08
	A1A2, A2A2	55		4.8		0.9	
TLR8B	A1A1	66	**0.018**	5.1	**0.008**	1.4	0.06
	A1A2, A2A2	41		4.2		0.9	
TLR8C	A1A1	58	0.1	4.7	0.42	1.38	**0.03**
	A1A2, A2A2	44		4.4		0.78	
TLR8D	A1A1	58	0.1	4.7	0.45	1.39	**0.018**
	A1A2, A2A2	44		4.5		0.70	

Table 20: Summary of the genotyped SNPs and their affect on the liver histology (HAI) – inflammation and fibrotic scores and on serum ALT levels

4.3.3. Relationship between genotypes and hepatic inflammatory scores and ALT levels

The major allele of **TLR8B** was associated with significantly more severe inflammation in this patient cohort (major vs. minor; 5.1+/-1.5 vs. 4.2+/-1, p < 0.008). This allele was also associated with significantly higher mean ALT levels (66+/-10. vs. 41+/-9. p < 0.02) and fibrotic stage (1.44 vs. 0.89), however the latter result did not reach statistical significance p = 0.057. Conversely patients who had the major allele of TLR7B SNP, had lower hepatic inflammatory scores and ALT levels (4.4+/-1.2 vs. 5.7+/-1.9, p = 0.015, ALT levels; 47.8+/-8 vs. 73.44+/-14, p = 0.014). The fibrotic score for **TLR7B** major allele was lower than that for the minor allele (1.14 vs. 1.31), but again did not reach clear statistical significance.

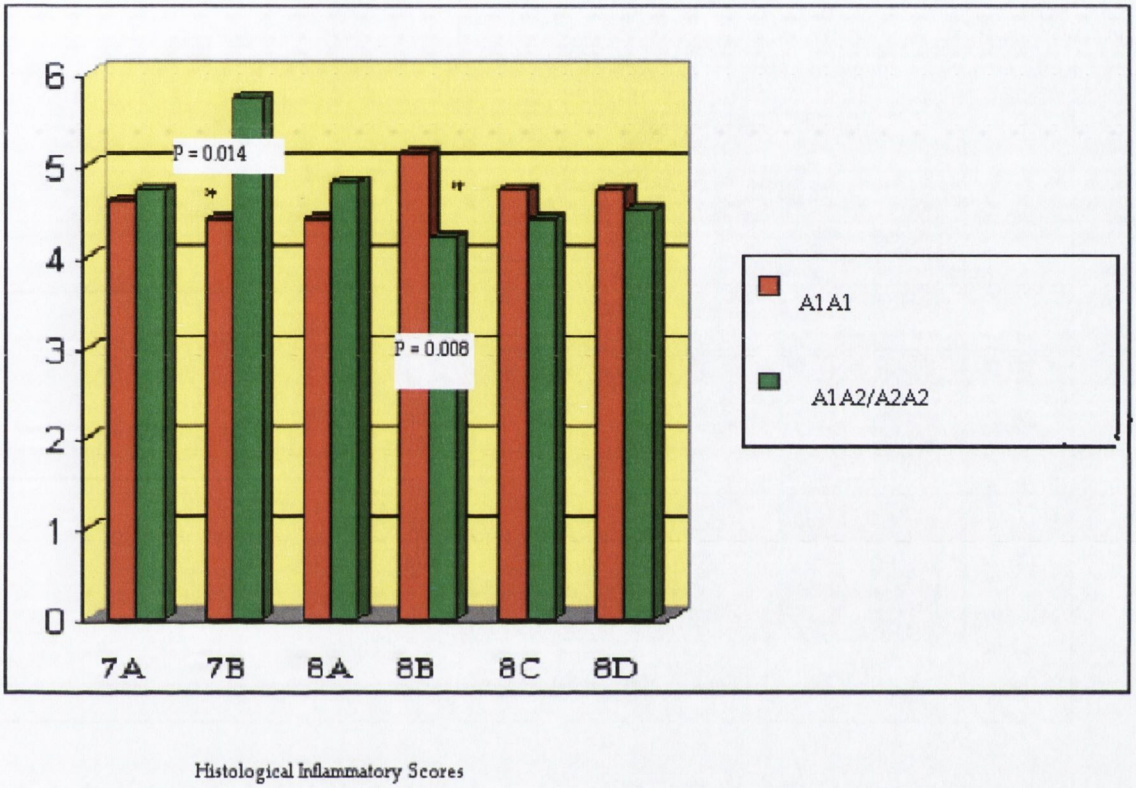

Figure 17: TLR7/8 SNPs and Inflammatory Scores

Individuals homozygous for the wild type, or major allele for TLR8B (A1A1) were associated with significantly more hepatic inflammation, P = 0.008, whilst the wild type genotype was associated with significantly less severe inflammation for the SNP TL7B, P = 0.014.

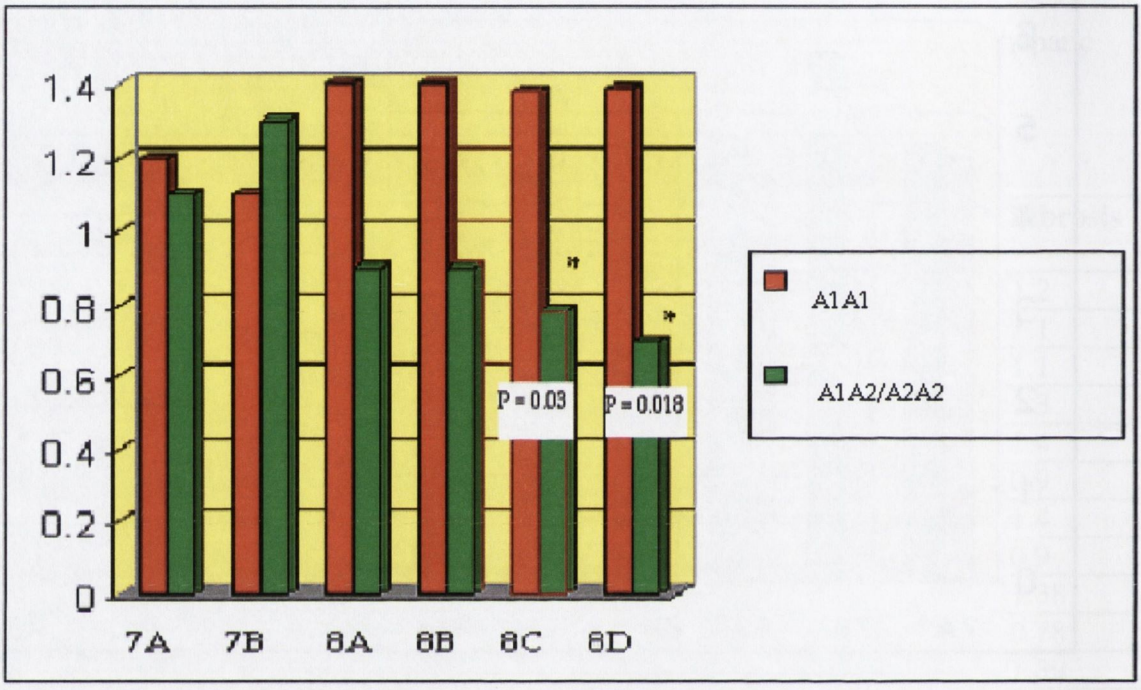

Figure 18: TLR 7/8 and Fibrosis Scores

Individuals homozygous for the wild type, or major allele of both TLR8C and TLR8D SNPs (A1A1) were associated with significantly more severe hepatic fibrosis, (P = 0.03, and 0.018, respectively).

4.3.3. Haplotype analysis

There was no association between haplotype distribution and persistent infection or clearance. We also assessed the association of haplotypes with fibrotic and inflammatory scores. To do this, we divided the patients into two groups, those with hepatic fibrosis, as indicated by a fibrotic grade of greater than zero, and those without. For inflammatory scores, we divided the group into those with an inflammatory score above the median, which was 4, and those with a score below this. Due to small numbers, haplotype differences between groups did not reach statistical significance overall. However permutation tests for differences in individual haplotype frequencies support the associations seen at individual loci: thus the haplotype containing all major alleles at the TLR8SNP loci is nominally associated with fibrosis with an estimated frequency of 0.664 compared with 0.512 in those without fibrosis (estimated $p=0.02$). Haplotype 2 containing minor alleles at all loci (TLR8E is practically monomorphic in this sample) is associated with lack of fibrosis (0.129 vs. 0.218 in those with fibrosis; $p=0.01$). No other differences were seen (data not shown).

4.3.4. Linkage disequilibrium

Patterns of linkage disequilibrium between SNPs were examined to establish the relationship between individual SNPs and each other. LD was examined in the entire group and for comparative purposes to those who had persistent infections only; patterns did not differ substantially between these groups and data pertaining to the entire group is presented. This shows that TLR7A and TLR7B ($D'=1$, $p=0.007$) are linked to each other but not to the TLR8 SNPs ($D'<0.5$). Similarly the TLR8 SNPS are all related to each other with D' values of >0.8 ($p<0.0001$ in each case), with the exception of TLR8A and TLR8B which do not show substantial LD ($D'=0.456$, $p<0.0001$). This indicates that associations seen between TLR7 and TLR8 and different clinical outcomes are most likely to be independent of each other, despite being coded contiguously, and in close physical proximity, on the X chromosome.

4.4. Discussion

This study assessed the association of SNPs in TLR7 and 8 with viral clearance and disease severity in 223 Irish Caucasians infected with HCV from a single source. As outlined in the results section, the TLR7B minor allele was associated with more severe inflammation, as was the TLR8B major allele, whilst the minor alleles of both of the TLR8 C and D were associated with significantly less severe hepatic fibrosis. There was no association between any of the SNPs genotyped and viral clearance.

Indeed in previous studies, we have clearly demonstrated a dominant role for the MHC in viral outcome in this cohort. Thus we have shown that the HLA A3, DR1 and 4, and HLA B27 alleles are strongly associated with clearance of the HCV virus. A study within this cohort has demonstrated that there is a selective drive for mutation in HLA B27 epitopes in B27+ patients in whom infection persists (McKiernan *et al* 2004). Hence, it is likely, at least in this cohort, that much of the process of viral clearance is dictated by host MHC types and related viral immune evasion processes. In other cohorts, it has been shown that NK cells may also play a major role in viral outcome. The innate immune system regulates the inflammatory process through a series of signalling events downstream of the TLR receptors. Such signalling events include the activation of NFkB and interferon gamma responses. As both of these processes are germane to the inflammatory response to HCV, we examined the role of TLR polymorphisms in influencing outcome in relationship to inflammation and fibrosis in Hepatitis C.

TLR7, and probably TLR8, has been demonstrated to recognize viral ssRNA rather than viral proteins and hence represented interesting candidate genes for examination. Exposure of viral ssRNA is likely to occur within an infected cell after endosomal degradation and uncoating of the virus (Finberg *et al* 2004). Lund *et al* demonstrated that recognition of the vesicular stomatitis virus (VSV) by TLR7 was dependent on lysosomal acidification, suggesting that the interaction occurred in the endosome (Lund *et al* 2004). Thus it may be that TLR7 and 8 do not recognize the HCV virus until it has already entered the cell, potentially limiting the ability of these molecules to affect elimination.

There are a number of potential mechanisms whereby TLR7 and 8 could influence inflammation and fibrosis in HCV infection. TLR7 ligands have been shown to enhance survival of dendritic cells (DCs) (Ito *et al* 2002). Progressive liver damage in HCV infection is associated with upregulation of Th1 cytokines (IFN and IL-2), as shown by Napoli *et al*, where increased expression of IFN and IL-2 correlated with more severe fibrotic and portal

inflammatory histological scores. In a study looking at cytokine induction by TLRs, TLR 7 and 8 were shown to induce TNFα, IL-12 and IFNα production from human peripheral blood monocytes (PBMCs), with TLR7 agonists preferentially inducing IFNα and TLR8 preferentially inducing TNF and IL-12. The high levels of TLR8-induced TNFα and IL-12 were also associated with high levels of IL-1, -6 and -8, and other pro-inflammatory cytokines (Gorden *et al* 2005). Given the role that these two TLRs play in induction of inflammatory cytokines, it is easy to see how polymorphisms of these genes may alter the degree of hepatic inflammation and/or fibrosis caused by the hepatitis C virus. There is no evidence however that any of the SNPs characterised in this study have a direct functional role in protein function or expression and hence, outcome of infection: as mentioned elsewhere, these SNPs were chosen as haplotype tagging SNPs, and at the time of study design there were no coding SNPs described of significant frequency in Caucasians.

Three of the four SNPs found to have significance for either fibrosis or inflammation are located in introns, the fourth being a synonymous substitution, thus none have an obvious functional role in influencing either the regulation or the function of either TLR. It is possible therefore that they are in linkage disequilibrium and thus acting as markers for other polymorphisms in the region which may exert functional effects. While we have identified effects of haplotypes on fibrosis and on inflammation, these should be interpreted with some degree of caution given the difficulties in estimating fibrosis as a continuous variable in this relatively small study. Nonetheless, given these constraints, we have identified associations with disease outcome in this homogenous group of recipients of Hepatitis C infected anti-D immunoglobulin.

This study suggests that TLR7 and 8 may play a role in degree of hepatic inflammation and fibrosis resulting from HCV infection. This is an exciting prospect, particularly given that several synthetic TLR7 and 8 ligands are already being used medically, such as Imiquimod for the treatment of genital warts, secondary to the human papilloma virus and Bropirimine, an orally active immunomodulator that induces production of cytokines, including IFN and is used in the treatment of renal cell carcinoma. To date we have examined haplotype tagging SNPs alone and these data suggest an association of these genes (which share an extended haplotype, being physically contiguous on chromosome 19) with outcome to HCV infection. Given the positive outcome of this analysis, it will be instructive to investigate the association of potentially functional SNPs in these genes with patient outcome. There is much work yet to be done on this; in particular it would be important to

look at more SNPs in these 2 genes, ideally in a larger population and also to perform functional studies specifically focussed on secreted mediators impacting on fibrotic activity.

Chapter 5

HCV and DEAD box genes

5.1. Introduction

DEAD box genes are highly conserved amongst species (Linder 2000) and encode proteins that are required for a variety of metabolic processes which involve ribonucleic acid (RNA). A number of these genes are located in the class III region of the major histocompatibility complex (MHC). *SKI2* (Superkiller I2P) is a DEAD box gene, encoding the protein Ski2p, which has been shown to have antiviral activity in yeast (Widner *et al* 1993, Dangel *et al* 1995, Johnson *et al* 1995, Lee *et al* 1995, Maison *et al* 1995, Qu *et al* 1998) and in general these genes are known to be important in the metabolism of nucleic acids. A number of observations as discussed here indicate that these genes may be important in viral biology. Thus they were considered candidate host response genes in viral infection.

As mentioned the human homolog of *SKI2*, the helicase gene *SKI2W,* is located centrally in the class III region of the MHC on chromosome 6 (Shen *et al* 1994). The similarity of the human protein product SKI2W with that of the yeast antiviral protein and also its location within the nucleus and the cytoplasm make it a strong candidate gene for having an antiviral role in humans. Another DEAD box gene encoded in the MHC at which we looked, was *BAT1* (which stands for HLA-B-associated transcripts; also called UAP56). This gene spans 10kb, encodes a 428 amino acid protein and has 10 exons. Its protein is localised to the nucleus and it has been shown to be highly conserved with 98% identity to the rat homologue and 99% to the pig (Peelman *et al* 1995). Allcock et al have claimed the *BAT1* gene to be a negative regulator of the pro-inflammatory cytokines; TNFα, IL-1 and IL-6 (Allcock *et al* 2001).

Other candidate genes investigated in this study include*: DDX3*, a DEAD box gene on the X chromosome which has been shown to interact with the HCV core protein (Owsianka *et al* 1999) and has been suggested to be involved in RNA splicing, RNA transport and translation initiation (Mamiya *et al* 1999, Zhou *et al* 2002, Yedavalhi *et al* 2004). The DEAD box gene, *DDX6/RCK* is reported to be over-expressed in those with chronic HCV infection and to have decreased expression in response to treatment with Interferon therapy (Miyaji *et*

al 2003) and thus was also investigated. DDX6/RCK is not located in the MHC, but rather is encoded on chromosome 11.We proposed that these DEAD box genes would be good candidate genes to examine for association with HCV viral clearance and disease severity.

5.2. Patients and Methods

Study Population

(See Study Population, Chapter 2, Materials and Methods)

Diagnosis of HCV Infection

(See Diagnosis of Hepatitis C Viral Infection, Chapter 2, Materials and Methods)

DNA extraction

(See DNA purification from blood,_Chapter 2, Materials and Methods)

SNP Selection and Genotyping

(See chapter 2, Methods and Materials)

Statistical Analysis

(See chapter 2, Methods and Materials)

Histological Evaluation

(See chapter 2, Methods and Materials)

5.3. Results

The results from the genotyping of the SNPs in the genes *SKI*, *RCK*, *DDX3* and *BAT1* were compared with PCR status of the 223 subjects and with the degree of histological inflammation and fibrosis on the liver biopsies of those who were PCR positive. There was a significant difference in PCR status and therefore viral clearance between those carrying the minor allele for the BAT1D SNP and the wild type. The allele frequency of the minor allele for those who remained PCR positive was .18 vs. only .08 in those who became PCR negative, (P = 0.01). When genotype was assessed for the BAT1D SNP this difference

remained significant, P = 0.005 *(table 21 & 22, figure 19, 20, 21).* Multiple logistic regression was also performed and the most significant model for viral clearance was that containing DQB1*0201 and BAT1D (*table 23*). When only DQB1*0201 negative individuals were looked at the polymorphism at BAT1D remained significant in relation to viral clearance (p = 0.005), this significance was not found when only DQB1*0201 positive individuals were looked at (p = 0.237). Likewise when we looked at only those who were BAT1D negative, the DQB1*0201 polymorphism retained its significance in relation to viral clearance (p = 0.000), but didn't amongst the BAT1D positive group (p= 0.124). Fourteen (19.7%) of those who were DQB1*0201positive were also BAT1D positive, whilst 66% of those who were DQB1*0201 negative were also BAT1D negative. This was the only SNP that showed an association with viral clearance and none of the SNPs showed a correlation between degree of histological damage, i.e. fibrosis or inflammation. *(table 24).*

SNP	HCV status	Genotype n (frequency)				Allele n (frequency)				
		A_1A_1	A_1A_2	A_2A_2	P	A_1	A_2	P	OR	95%CI
SKIV1 Rs2280773 C/T	PCR +ve	23 (.16)	66 (.47)	52 (.37)	0.5	112 (.40)	170 (.60)	0.54	1.15	.77-1.7
	PCR -ve	9 (.10)	44 (.52)	32 (.38)		62 (.36)	108 (.64)			
SKIV2 Rs4389 C/T	PCR +ve	109 (.82)	22 (.16)	2 (.02)	0.8	240 (.90)	26 (.10)	0.6	0.77	.37-1.57
	PCR -ve	67 (.86)	10 (.13)	1 (.01)		144 (.90)	12 (.08)			
SKIV3 Rs437179 T/G	PCR +ve	45 (.36)	57 (.45)	24 (.19)	0.54	147 (.58)	105 (.42)	0.41	0.83	.55-1.25
	PCR -ve	30 (.38)	38 (.49)	10 (.13)		98 (.63)	58 (.37)			
SKIV4 Rs2734331 C/T	PCR +ve	130 (.95)	5 (.04)	1 (.01)	0.67	265 (.97)	7 (.03)	0.4	1.69	.58-4.9
	PCR -ve	77 (.94)	3 (.04)	2 (.02)		157 (.96)	7 (.04)			
SKIV5 Rs406939 C/T	PCR +ve	101 (.76)	31 (.23)	1 (.01)	0.07	233 (.87)	33 (.12)	0.1	0.56	.28-1.11
	PCR -ve	71 (.87)	10 (.12)	1 (.01)		152 (.93)	12 (.07)			
BAT1A Rs11796 A/T	PCR +ve	52 (.38)	61 (.45)	23 (.17)	0.84	165 (.60)	107 (.40)	0.76	0.94	.63-1.4
	PCR -ve	33 (.38)	41 (.48)	12 (.14)		107 (.62)	65 (.38)			
BAT1B Rs2071595 C/G	PCR +ve	128 (.93)	9 (.06)	0	1	265 (.97)	9 (.03)	1	0.89	.29-2.7
	PCR -ve	80 (.94)	5 (.06)	0		165 (.97)	5 (.03)			
BAT1C Rs2239709 A/G	PCR +ve	129 (.93)	9 (.06)	0	1	267 (.97)	9 (.03)	1	1.05	.38-3.1
	PCR -ve	79 (.93)	6 (.07)	0		164 (.97)	6 (.03)			
BAT1D Rs2269476 C/T	PCR +ve	96 (.69)	35 (.25)	7 (.05)	**0.03**	227 (.82)	49 (.18)	**0.005**	0.41	.22-.77
	PCR -ve	72 (.85)	12 (.14)	1 (.01)		156 (.92)	14 (.08)			
BAT1E Rs2239527 C/G	PCR +ve	52 (.37)	62 (.45)	25 (.18)	0.74	166 (.59)	112 (.40)	0.55	0.88	.59-1.3
	PCR -ve	34 (.39)	41 (.47)	12 (.14)		109 (.62)	65 (.38)			

Table 21: Dead box SNPs allele and genotype frequency in relation to HCV PCR status

SNP	HCV status	Allele A_1 carrier status					Allele A_2 carrier status				
		A1A1/A1A2	A2A2	P	OR	95%CI	A1A1	A1A2/A2A2	P	OR	95%CI
SKIV1 Rs2280773 C/T	PCR +ve	89 (.63)	52 (.37)	1	1	.59-1.8	23 (.16)	118 (.84)	0.32	1.6	.7-3.7
	PCR -ve	53 (.62)	32 (.38)				9 (.11)	76 (.89)			
SKIV2 Rs43899 C/T	PCR +ve	131 (.99)	2 (.02)	1	0.85	.06-9.5	109 (.82)	24 (.18)	0.56	0.74	.34-1.6
	PCR -ve	77 (.98)	1 (.02)				67 (.85)	11 (.14)			
SKIV3 Rs437179 T/G	PCR +ve	102 (.81)	24 (.19)	0.3	0.63	.28-1.39	45 (.36)	81 (.64)	0.76	0.9	.49-1.6
	PCR -ve	68 (.87)	10 (.13)				30 (.38)	48 (.62)			
SKIV4 Rs2734331 C/T	PCR +ve	135 (.99)	1 (.01)	0.56	3.3	.3-37.8	130 (.95)	6 (.04)	0.75	1.4	.4-4.76
	PCR -ve	80 (.97)	2 (.03)				77 (.94)	5 (.06)			
SKIV5 Rs406939 C/T	PCR +ve	132 (.99)	1 (.01)	1	1.6	.1-26.4	101 (.76)	31 (.24)	0.08	0.49	.23-1.0
	PCR -ve	81 (.99)	1 (.01)				71 (.87)	11 (.13)			
BAT1A Rs11796 A/T	PCR +ve	113 (.83)	23 (.17)	0.7	0.79	.37-1.7	52 (.38)	84 (.62)	1	0.99	.57-1.7
	PCR -ve	74 (.86)	12 (.14)				53 (.38)	53 (.62)			
BAT1B Rs2071595 C/G	PCR +ve	137 (1)	0	1	n/a	n/a	128 (.93)	9 (.07)	1	0.88	.28-2.7
	PCR -ve	85 (1)	0				80 (.94)	5 (.06)			
BAT1C Rs2239709 A/G	PCR +ve	138 (1)	0	1	n/a	n/a	129 (.93)	9 (.07)	1	1.1	.37-3.2
	PCR -ve	85 (1)	0				79 (.93)	6 (.07)			
BAT1D Rs2269476 C/T	PCR +ve	131 (.95)	7 (.05)	0.16	0.2	.03-1.8	96 (.69)	42 (.31)	**0.01**	0.41	.2-.82
	PCR -ve	84 (.99)	1 (.01)				72 (.85)	13 (.15)			
BAT1E Rs2239527 C/G	PCR +ve	114 (.82)	25 (.19)	0.46	0.72	.34-1.54	52 (.37)	87 (.63)	0.89	0.93	.54-1.6
	PCR -ve	75 (.86)	12 (.14)				34 (.39)	53 (.61)			

Table 22: Dead box SNPs allele frequency in relation to HCV PCR status

	SNP	S.E.	P	O.R.
Step 1	DQB1*0201	0	**0.000**	3.4
	Constant/ Intercept	0.45	0.22	0.35
Step 2	BAT1D	0.34	**0.001**	3
	DQB1*0201	0.36	**0.000**	4
	Constant/ Intercept	0.66	0.18	0.45

Table 23; Multiple logistic regression model for PCR status. Multiple logistic regression was performed on all SNPs which had given significant values in relation to HCV viral clearance when tested individually. The DQB1*0201 and BAT1D SNPs were the only two to remain significant on logistic regression analysis.

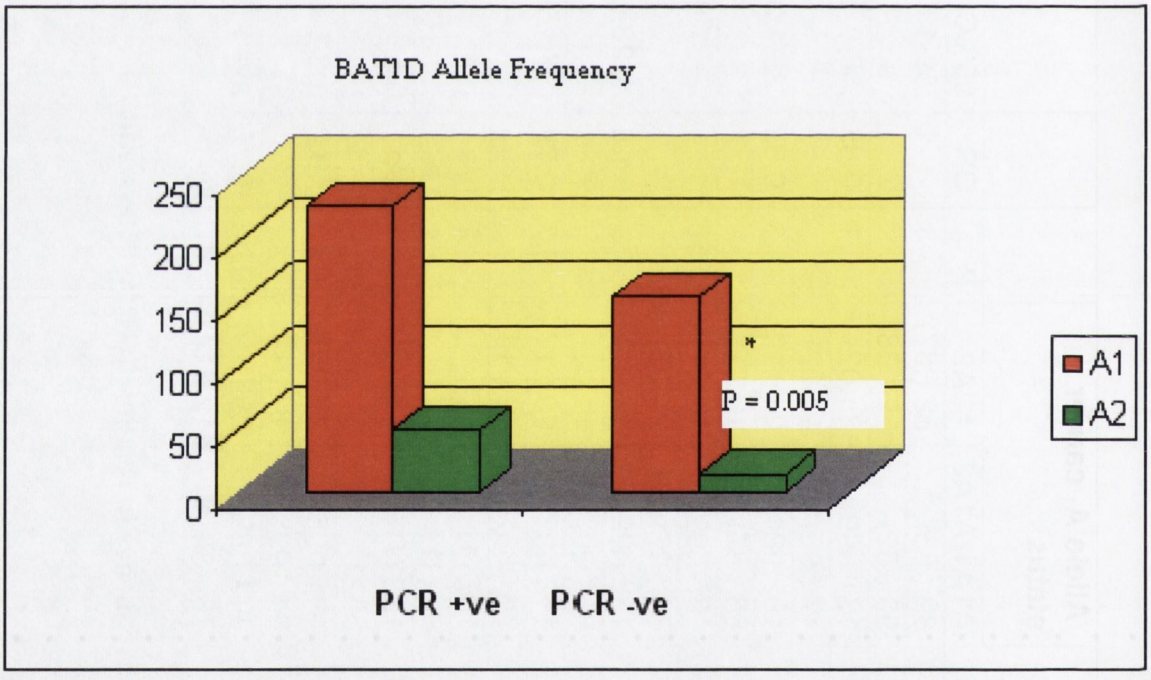

Figure 19: BAT1D allele frequency and HCV PCR status
The A2 allele was significantly less common in patients who cleared the HCV virus, i.e. became HCV PCR – ve, compared with those who remained HCV PCR +ve, (P = 0.005).

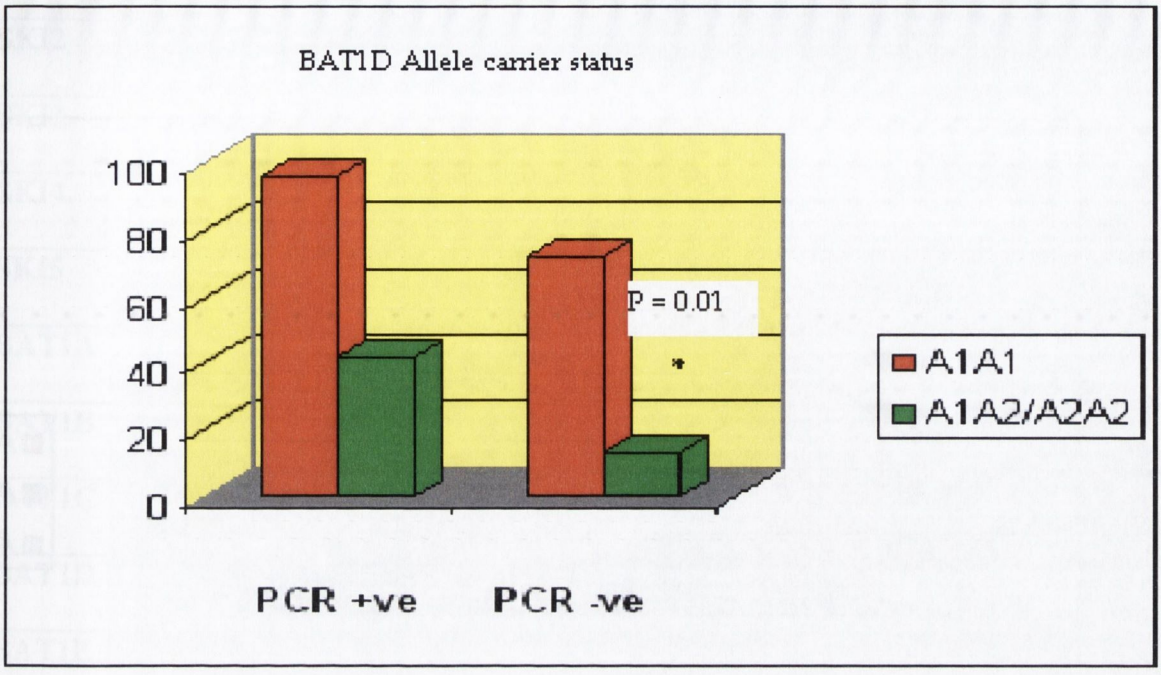

Figure 20: BAT1D Allele carrier status and HCV PCR status

Those carrying the A2 allele were significantly less likely to have cleared the HCV virus, i.e. be HCV PCR – ve, (P = 0.01)

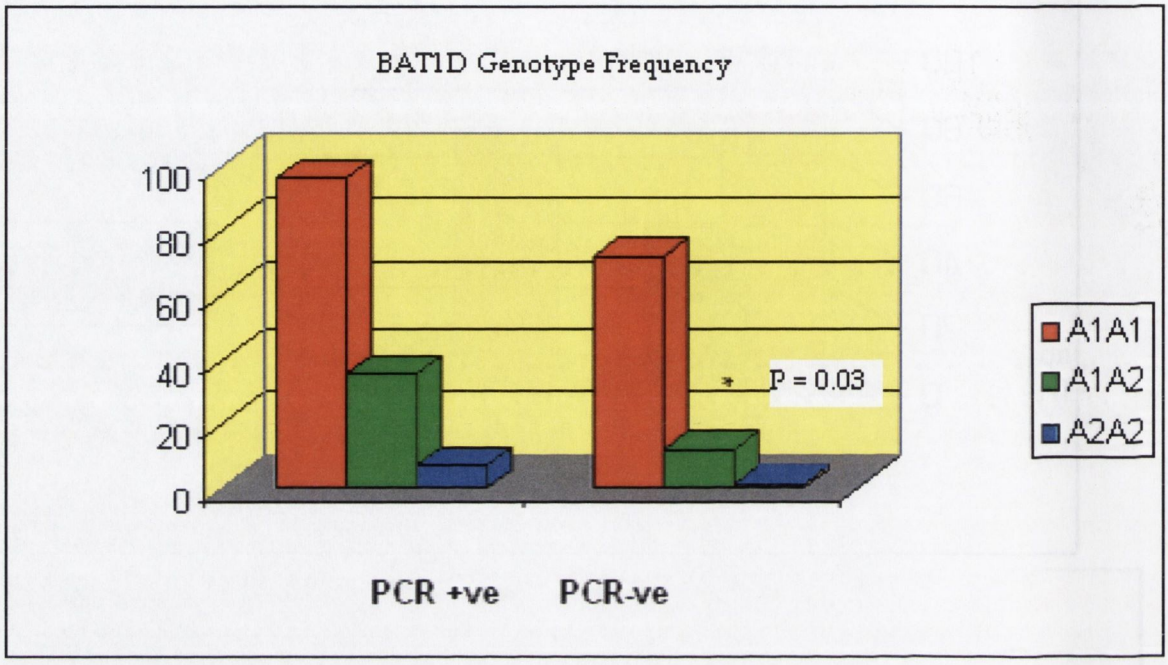

Figure 21: BAT1D genotype frequency and HCV PCR status

There was a significant difference between the above genotypes and HCV PCR status, (P = 0.03).

SNP	Genotype	Inf	S.E	P-value	Fib	S.E.	P-value
SKI1	A1A1	4.9	0.48	0.38	1.30	0.30	0.17
	A1A2/A2A2	4.4	0.19		0.97	0.13	
SKI2	A1A1	4.4	0.22	0.41	0.92	0.13	0.18
	A1A2/A2A2	4.7	0.45		1.57	0.39	
SKI3	A1A1	4.6	0.34	0.54	0.93	0.20	0.95
	A1A2/A2A2	4.4	0.25		1.04	0.17	
SKI4	A1A1	4.5	0.20	0.68	1.03	0.13	0.88
	A1A2/A2A2	4.8	0.91		0.83	0.50	
SKI5	A1A1	4.5	0.2	0.66	0.91	0.13	0.37
	A1A2/A2A2	4.7	0.45		1.35	0.32	
BAT1A	A1A1	4.9	0.36	0.22	1.00	0.21	0.84
	A1A2/A2A2	4.2	0.21		1.02	0.15	
BAT1B	A1A1	4.4	0.19	0.41	1.01	0.13	0.93
	A1A2/A2A2	5.1	0.99		1.14	0.60	
BAT1C	A1A1	4.4	0.19	0.42	1.00	0.13	0.92
	A1A2/A2A2	5.1	0.98		1.14	0.59	
BAT1D	A1A1	4.4	0.22	0.21	1.00	0.14	0.57
	A1A2/A2A2	4.9	0.38		1.23	0.27	
BAT1E	A1A1	4.9	0.36	0.20	1	0.21	0.93
	A1A2/A2A2	4.2	0.21		1	0.15	
RCK1	A1A1	4.7	0.30	0.27	0.98	0.16	0.30
	A1A2/A2A2	4.3	0.24		0.95	0.17	
RCK2	A1A1	4.4	0.25	0.79	0.97	0.16	0.76
	A1A2/A2A2	4.5	0.30		1.06	0.20	
DDX31	A1A1	4.2	0.20	0.07	0.93	0.14	0.47
	A1A2/A2A2	5.2	0.40		1.25	0.25	
DDX32	A1A1	4.2	0.20	0.24	0.97	0.14	0.83
	A1A2/A2A2	4.9	0.36		1.12	0.23	
DDX33	A1A1	4.5	0.19	0.32	0.98	0.13	0.18
	A1A2/A2A2	4.5	0.75		1.53	0.41	
DDX34	A1A1	4.3	0.25	0.98	0.90	0.17	0.83
	A1A2/A2A2	4.6	0.27		1.11	0.17	

Table 24: DEAD Box SNPs and hepatic inflammatory (inf) / fibrotic (fib) scores

5.4. Discussion

This study of polymorphisms in 4 DEAD box genes in a homogenous HCV infected population with minimal confounding factors shows a significant association with polymorphisms of the BAT1 gene and viral clearance. It does not show any impact on severity of hepatic fibrosis or of hepatic inflammation for any of the DEAD box genes tested.

BAT1 is an essential splicing factor; Fleckner *et al* showed that a HeLa nuclear extract depleted of BAT1 was unable to support a crucial step in pre mRNA splicing (Fleckner *et al* 1997). BAT1 has been shown to play an important role in RNA export from the nucleus to the cytoplasm in yeast, Drosophila and C. elegans (Gatfield *et al* 2001, Jensen *et al* 2001, MacMorris *et al* 2003, Strasser and Hurt 2001). In a recent study by Kapadia *et al* (2006) this role of BAT1 was also demonstrated in HeLa cells. siRNA to BAT1 and to URH49, which is 90% identical to BAT1, were used and resulted in the accumulation of poly(A)+ RNA within the nucleus, partial inhibition of gene expression and cell death. BAT1 has been demonstrated to have very low ATPase hydrolysis activity, but this is increased up to 20 fold with the addition of ssRNA. BAT1 interacts with and plays a crucial role in the life cycle of several viruses, e.g. it has been shown to be required for the replication and translation of the influenza A virus, (Momose *et al* 2001) which, like HCV, is also a ssRNA virus. Human CMV has a gene product UL69, which participates in the nuclear export of RNA into the cytoplasm. Liscka *et al* demonstrated that not only is pUL69 activity linked to BAT1 and URH49 but that pUL69 mediated mRNA export is in fact dependent on BAT1 interaction and nucleocytoplasmic shuttling (Lischka *et al* 2006). As mentioned above, BAT1 ATPase hydrolysis is increased in the presence of ssRNA, however in certain BAT1 mutants the ATPase hydrolysis activity is completely abolished, leading also to the loss of helicase activity. This may provide a possible clue as to why those with the presence of the minor allele of BAT1D in this study were less likely to have managed to clear the HCV virus. Of course BAT1 is located in the MHC which is the most gene dense region of the human genome and is highly polymorphic, it may be that the BAT1D polymorphism is linked to or forms part of a 'HCV disease' haplotype in that area, as has been suggested for its potential role in diabetes and autoimmune disease susceptibility (Wong *et al* 2003). Wong *et al* showed that there was lower BAT1 activity associated with the disease susceptibility haplotype 8.1 AH (HLA-A1, B8, DR3), compared with the disease resistance haplotype 7.1 AH. BAT1 is also located very close to the TNFα gene which McKiernan *et al,* 2000 have already shown an association with viral clearance in this cohort, however the significance of

the TNF308 polymorphism was lost on multiple regression analysis in this cohort. In our multiple regression analysis DQB1*0201 came out as the most significant, with a slightly lower p value than BAT1D, however the likelihood ratio statistics for each term in the final model weren't very different, making it difficult to say with any certainty which is more important. Certainly both are significant in the final model, implying independent effects, as reiterated by the fact that when only DQB1*0201 positive individuals were included the BAT1D polymorphism was no longer significant, and vice versa when only the BAT1D positive individuals were looked at. Wong has suggested for other diseases that BAT1 forms part of a haplotype with DQB1*0201, however this data suggests that they are independent of each other.

DDX3 has not only been shown to interact with the HCV core protein but also to be regulated by it (You *et al* 1999, Owsianka *et al* 1999, Mamija *et al* 1999). Expression of DDX3 has also been shown in one study to be upregulated in those with hepato-cellular carcinoma secondary to HCV (Huang *et al* 2004) and in another to be up-regulated only in those with hepato-cellular carcinoma secondary to hepatitis B virus (Chang *et al* 2006). We examined 4 SNPs in this gene and found no correlation with clearance or severity of HCV infection.

The final gene examined in this study was *DDX6/RCK* which is on chromosome 11 and whose expression has been shown to be altered both by the presence of HCV infection and also by subsequent interferon therapy. We failed to show an association with any of the polymorphisms looked at in this gene and HCV persistence or severity.

This study examined a total of 16 SNPs in 4 different DEAD box genes in a homogenous HCV infected population and found one SNP in the BAT1 gene to be associated with HCV persistence. This is the first time this association has been reported and it is in a population, all be it small, with little if any confounding factors. This gene, particularly given its key role in RNA transport and its interaction with other viruses warrants further investigation in relation to its potential role in HCV viral persistence. The next steps would be to assess this gene for impact in a larger HCV infected co-hort and in those with varying genotypes, followed by functional studies.

Chapter 6

6.1. Conclusion

This study investigated the impact of a total of 28 different SNPs on 9 different genes, affecting different aspects of the immune system, on the out-come of Hepatitis C viral infection on 233 women. An additional 50 women were genotyped for CCR5, CCR2 and RANTES polymorphisms. As acute Hepatitis C is usually sub-clinical it often goes undetected. This makes assessing the role of an individuals' immune response in relation to viral clearance often not possible, as its sub-clinical course means that more often than not the acute infection goes undetected. This cohort of women therefore presents a relatively unique opportunity to assess immune genetic variations in a significant number of people who have been exposed to, but spontaneously cleared the virus. In this study I have used the group who cleared the virus spontaneously to act as a control or comparison group for those who remained infected. These women were all ethnically of Irish origin, they were screened for any other type of liver disease, and those who were found to have any potential confounding factors were excluded from the study. One characteristic of this cohort is that their rate of progression of fibrosis is extremely slow, with the majority having only very mild disease, therefore potentially making influence of genetic polymorphisms on severity of fibrosis more difficult to demonstrate. A second undoubted difficulty is that this patient group is of a fixed number and is too small to detect genetic effects of small magnitude. In this regard, it should be noted that negative associations may not be definitive either and may simply reflect a lack of power to detect subtle effects. Both the innate and adaptive immune response and the interplay between the two is very important in host response to HCV, I therefore investigated polymorphisms that could potentially influence both arms of the immune response.

This study showed an association between viral clearance and those who carried the *CCR5* Δ32 mutation and within the DRB1*03011 negative group (previously shown to be associated with more severe inflammation), *CCR5*Δ32 heterozygotes had significantly lower inflammatory scores than the *CCR5*WT/WT group. There are now several papers published examining the impact of the CCR5Δ32 mutation on HCV infection, and whilst no other paper has found it to be associated with HCV viral clearance, Hellier *et al* (2003) and Wald *et al*

(2004) both show it to be associated with decreased hepatic inflammation, as I did in a sub-set of the cohort studied here. A study by Ajuebor *et al* (2005) examining the impact of the CCR5Δ32 mutation on inflammatory response within the mouse model exposed to Concanavalin A hepatitis shows that NKT cells from CCR5 deficient mice are resistant to apoptosis and have increased production of IL-4. It is easy to hypothesize how decreased apoptosis of NKT cells could lead to increased viral clearance in the acute stage. The CCR5Δ32 mutation has also now been reported to be associated with increased viral clearance of Hepatitis B virus, with the presence of CCR5Δ32 heterozygosity halving one's chances of remaining chronically infected with HBV (Thio *et al* 2007). CCR5 inhibitors are already at the trial phase for treatment of HIV. However some of these trials have had to be stopped prematurely as a result of hepato-toxicity. As many HIV patients are co-infected with HCV it is very important to have a full understanding of the impact of the CCR5 mutation on HCV, both from a potential treatment and from a potential side-effects point of view.

A polymorphism in the *BAT1* gene was also found to be significantly associated with HCV viral persistence; this is the first study to demonstrate this association. As mentioned the *BAT1* gene is located in the highly polymorphic MHC region, making its' individual influence more difficult to discern from neighbouring genes in high LD and it may be that it forms part of a 'HCV disease haplotype', influencing disease outcome. This uncontroversial concept has already been proposed by Wong *et al* (2003) for its potential role in diabetes and autoimmune disease susceptibility. The next step would be to do functional studies on BAT1, particularly looking at its ATPase hydrolysis activity in those with the BAT1D polymorphism, and influence of the polymorphism on TNF alpha activity.

Two polymorphisms in *TLR8* were associated with less severe fibrosis, whilst one in *TLR7* was associated with more severe inflammation and one in *TLR8* less severe inflammation. Dolganiuc *et al* recently showed that TLRs 7 and 8 were upregulated in HCV patients' monocytes, with mRNA levels significantly higher in T lymphocytes of HCV infected patients compared to controls, which is supportive of the findings in this thesis that these TLRs may influence severity of HCV inflammation or fibrosis (Dolganiuc *et al* 2007). It is important to next consider functional studies on TLR 7and 8, specifically focussed on secreted mediators impacting on fibrotic activity.

Research into the immune response to HCV is further hampered by the lack of a small animal model, however using the hepatocyte line Huh-7, a TLR7 ligand, SM360320, was recently shown to inhibit HCV viral replication via two pathways. The first was an IFN dependent pathway and the second, a probable IFN independent pathway, thought to be mediated by direct activation of an antiviral response in hepatocytes through the Toll receptor (Lee *et al* 2006). As mentioned in the introduction, studies in mice involving the TLR7 ligand isatoribine, have shown it to limit viral infection by stimulation of the innate immune response. This lead to Horsmans *et al* (2005) initiating a 'proof of concept' clinical study in which varying doses of intra-venous isatoribine were given to 32 patients with chronic HCV infection, which resulted in a significant drop in viral load. These patients were also shown to have increased expression of the IFN responsive genes, *ISG15* and *OAS*, suggesting that isatoribine worked via stimulation of the host immune response rather than direct viral suppression Although the numbers in this study are very small, I think these findings are potentially very exciting in the ongoing search for more effective treatment for HCV infection, particularly amongst those with HCV genotype 1 who have, at best, a 50:50 chance of clearing HCV with the current 'gold standard' of treatment.

Many recent papers have identified TLR and DEAD box genes, in particular the DEAD box gene *RIG-1* (retinoic acid inducible gene – 1), as being the two key pathways involved in the identification of and subsequent immune response to viral infection. As mentioned, TLR7 and 8 are found in the endosome and therefore probably recognize products of viral degradation. RIG-1 is located in the cytoplasm. Stimulation of both these pathways results in the production of cytokines such as IFN and NF-Kb.

RIG-1 is located close to *DDX6/RCK* on chromosome 11 and signals IFN production in response to several RNA viruses, including HCV. It has been shown to limit HCV RNA replication by signalling the initiation of host defence, and it has also been shown to be a target of the NS3 HCV protein in an attempt by HCV to prevent triggering of the innate immune response and thereby inhibit host viral defence. In a study by Breiman *et al* (2005) looking at IFN production in Huh-7 cell lines and HCV, IFN production was shown to be suppressed in the HCV replicon cells compared with the non-HCV cells. This study also showed that in the absence of NS3/4A, IFN production was activated by RIG-1, but the addition of NS3/4A led to its complete inhibition. A genome-wide scan of almost 25,000 putative functional SNPs by Huang *et al* (2006) showed a missense SNP in the Dead box gene *DDX5* to be associated with significantly increased hepatic fibrosis. I have shown that BAT1 plays an important role in the immune response to HCV and I feel that this area

warrants further research, as this is the first time that this association has ever been demonstrated. In the next instance I would consider that focusing on different SNPs in DDX6 and also widening the search to include SNPs in the *RIG-1* and *DDX5* genes could potentially be very exciting future work in the further elucidation of the interplay between HCV and the immune system.

In conclusion, this thesis has shown increased rates of spontaneous HCV viral clearance hin those with the CCR5Δ32 mutation and decreased inflammation in a sub-set of this cohort. It is the first study to show an association between degree of hepatic inflammation and fibrosis and polymorphisms in the *TLR7* and *8* genes. It is also the first to show any association between polymorphisms in the DEAD box gene *BAT1* and HCV persistence, an association which appears independent of DQB1*0201 and the other MHC polymorphisms already looked at in this cohort. This is a very important finding which needs further evaluation with functional studies and assessment amongst a larger HCV population, and also amongst other HCV genotypes.

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Appendix 1:

Abbreviations:

HCV - hepatitis C virus

TLR – toll like receptor

BAT - HLA-B-associated transcripts

ORF - open reading frame

UTR - untranslated regions

PTB - polypyrimidine tract-binding

IRES - internal ribosomal entry site

HVR - hypervariable region

RNA – ribonucleic acid

ISDR - interferon sensitivity determining region

RT-PCR – reverse transcriptase polymerase chain reaction

DC – dendritic cells

CD cells – 'cluster of differentiation' cells

Th – T helper

NK cells – natural killer cells

MHC - major histocompatibility complex

IFN- interferon

TNF – tumour necrosis factor

MIP - macrophage inflammatory protein

RANTES - regulated upon activation, normally T cell expressed and secreted

IL- interleukin

PRR - pattern recognition receptors

APCs - antigen presenting cells

LD – linkage disequilibrium

HLA – human leucocyte antigen

BMI – body mass index

CCR5 – chemokines receptor 5

CCR2 – chemokines receptor 2

LIL- liver infiltrating lymphocytes

HIV- human immunodeficiency virus

PAMP - pathogen-associated molecular patterns

LRR - leucine rich repeats

TIR- toll like IL- 1 receptor

mRNA – messenger ribonucleic acid

ssRNA – single stranded ribonucleic acid

CMV – cytomegalovirus

OAS – oligoadenylate synthetase

ISG15 – interferon-stimulated gene 15

ATP - adenosine triphosphate

DNA - deoxyribonucleic acid

SKI2 - Superkiller 12P

tRNA – transfer ribonucleic acid

SNP – single nucleotide polymorphism

ELISA – enzyme linked immunoassay

RIBA - recombinant immunoblot assay

HAI - hepatic activity index

dNTP - Deoxyribonucleotide triphosphate

RFLP - Restriction fragment length polymorphism

SDS - sequence detection system

htSNPs – haplotype tagging SNPs

NCBI – National centre for biotechnology information

ALT - alanine aminotransferase

MCHS - Multicentre Haemophilia Cohort Study

LCM - lymphocytic choriomeningitis

MHV - mouse hepatic virus

CTL - cytotoxic lymphocytes

HEK - human embryonic kidney cells

NF-κB – nuclear factor kappa B

VSV - vesicular stomatitis virus

PBMCs - peripheral blood monocytes

SiRNA – small interfering ribonucleic acid

HBV – hepatitis B virus

RIG-1 - retinoic acid inducible gene – 1