University of Southampton

Investigation of the $CD4^+$ T Lymphocyte Responses to Hepatitis C Virus Infection: Cytokine Production and its Relationship to Fibrosis and Inflammation

By

Corinne Brooks

A thesis submitted to the University of Southampton for the degree of Doctor of Philosophy

School of Medicine

Supervisors: Dr William Rosenberg and Professor Freda Stevenson

May 2002

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

SCHOOL OF MEDICINE

Doctor of Philosophy

INVESTIGATION OF THE $CD4^+$ T LYMPHOCYTE RESPONSES TO HEPATITIS C VIRUS INFECTION: CYTOKINE PRODUCTION AND ITS RELATIONSHIP TO FIBROSIS AND INFLAMMATION

By Corinne Lucy Brooks

Hepatitis C virus (HCV) infection is a major global healthcare problem with around 170 million people infected worldwide. Only 15% of those infected clear HCV infection spontaneously. Chronic HCV (CHC) infection is associated with progressive liver damage which is believed to be immune mediated. The rate of progression of the liver damage associated with CHC is variable and difficult to predict in individuals.

This study investigated the phenotype and magnitude of the $CD4^+$ T lymphocyte response to HCV to see if there was any correlation with the severity of the resultant liver disease. In addition, genetic markers which could influence the $CD4^+$ T lymphocyte response were examined for correlation with severity of liver disease. Finally, the effect of the HCV specific $CD4^+$ T lymphocyte response on the effector cells of hepatic fibrosis, hepatic stellate cells, was investigated.

Genomic DNA from CHC patients was tested for the presence of the allele HLA DQB1*0301, a Major Histocompatibility Complex class II molecule which has been shown to be over-represented in individuals with evidence of spontaneous resolution of acute infection. There was no correlation between frequency of this allele in our population and severity of CHC related liver disease. However, analysis of clinical information gathered from these patients confirmed that age, duration of infection and excess alcohol intake were all risk factors for more severe liver disease. In addition, severity of fibrosis correlated positively with severity of inflammation; supporting the hypothesis that fibrosis is the result of chronic inflammation in CHC.

Functional studies of the $CD4^+$ T lymphocyte response in HCV infection confirmed that HCV lymphocyte proliferative responses are different between CHC patients and those with evidence of spontaneous resolution of HCV infection. There were no differences in the HCV specific proliferative responses between groups of CHC patients discordant for severity of liver disease. However, HCV specific Interferon-γ secretion was more commonly seen in patients with severe liver disease. $CD4^+$ T lymphocyte responses to non-HCV recall antigens were identical between all groups.

Serial measurements of HCV specific $CD4^+$ T lymphocyte function during anti-viral therapy for HCV failed to demonstrate any differences between the patients who eventually achieved sustained viral clearance and those who did not. However, there was a trend for viral clearance being associated with a Th2 polarised pre-treatment HCV specific $CD4^+$ response, which could be switched to a Th1 dominant response. The rate of viral clearance from the serum was 100% discriminatory between the groups.

Novel assays to measure changes in expression of ICAM-1 by activated human hepatic stellate cells (HSC) were developed throughout the course of the study. These assays were used to investigate the effect of HCV stimulated $CD4^+$ conditioned media on human HSC. Evidence of HCV specific $CD4^+$ responses was found in 50% more CHC patients than had been demonstrated using conventional assays. Some, but not all of the effects on HSC could be attributed to IFN-γ. There was heterogeneity demonstrated between the responses of HSC from different donors to the same stimuli, as well as heterogeneity in the nature and magnitude of the HCV-specific $CD4^+$ responses from different individuals.

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Acknowledgements

Firstly, I would like to express my thanks to Dr William Rosenberg who initially conceived the project and inspired me to undertake the research. Without his help, there would be no project, no funding and no PhD. He has continued to encourage throughout the last 3 ½ years, and been an excellent supervisor and colleague.

I would also like to thank Professor Freda Stevenson has always been supportive when I have approached her for advice and direction. Her expertise has undoubtedly added to the quality and breadth of the research.

Elizabeth Sanders in her capacity as post-doctoral fellow has been invaluable for her technical expertise and immunological knowledge. As a very good friend, she has helped to make the more difficult times easier.

I must thank Tracy Lancaster for all of her efforts with the HLA typing. Without her continued input, chapter 3 would not exist.

Joanna Guy has been invaluable in her help with obtaining samples from patients. Her cheery disposition is also a pleasure to behold.

Fiona Thompson began this project as a BSc student, and taught me many basic techniques which formed the mainstay of the lymphocyte work. Thank you Fiona.

Dr Lindsay Murphy was a great help with the hepatic stellate cell work, both in isolation of cells and development of assays. Without her input, I would have struggled to complete the work in good time.

I would like to mention Professor Michael Arthur, who I consider responsible for my presence in the liver group. He inspired me to become a gastroenterologist and to endeavour to return to the laboratory as a post-graduate student. Professor John Iredale must be thanked for similar reasons, and his unfailing enthusiasm.

I would like to thank the rest of Dr Rosenbergs research team and the liver group for their help with day to day research, and for many, the donation of their blood as controls.

The HCV patients, have all also donated blood unfailingly, and sometimes frequently. I would like to express my gratitude to them.

The Wellcome Trust have been most generous in funding this research, and I would like to express my whole-hearted thanks for the opportunity they have provided for me

Last, but not least, I must thank Dr Jeremy Brooks, for everything else.

Chapter 1

Chapter 1

Introduction

1.1 The Liver – Structure

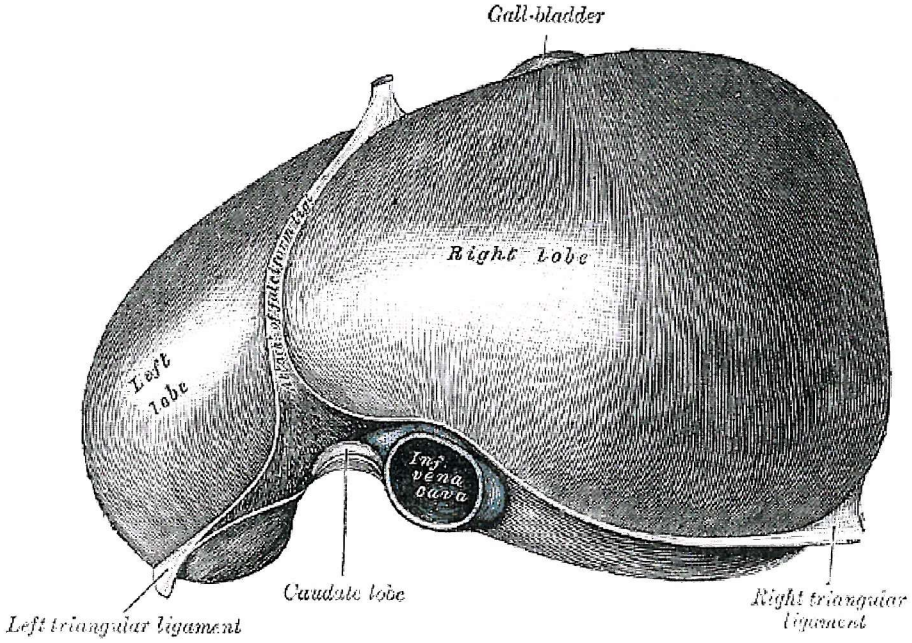
The liver is the largest visceral organ. It is usually situated in the right upper quadrant of the abdominal cavity under the diaphragm, protected by the overlying lower ribs.

1.1.1 Embryology

The embryological origin of the liver is the endodermal epithelial lining of the foregut. It appears during the third week of gestation as the hepatic diverticulum, which proliferates out into the septum transversum in cords. The connection between the hepatic diverticulum and the foregut becomes the bile duct and develops a ventral growth, giving rise to the gall bladder and cystic duct. The epithelial liver cords mingle with the umbilical and vitelline veins to form the hepatic sinusoids. Further differentiation of the liver cords forms the liver parenchyma and bile duct epithelium, whilst the haemopoietic, Kupffer and connective tissue cells are derived from the mesoderm of the septum transversum *(Sadler 1985)*.

1.1.2 Macroscopic Anatomy

The adult human liver weighs between 1.2 and 1.6kg depending on size and sex. The blood supply is derived from the hepatic artery (30%) and the portal vein (70%). Functionally, the liver is divided into 8 segments, each with its own blood supply. Historically, the liver was described as comprising 4 lobes, demarcated by surface anatomy; the left, right, caudate and quadrate lobes (Figure 1.01) *(Gray 1995)*.
The porta hepatis, or hilus of the liver, contains the portal vein, the right and left branches of the hepatic artery, the right and left hepatic ducts and the sympathetic and parasympathetic nerve fibres. It lies on the postero-inferior surface of the liver, with the hepatic lymph nodes.
The hepatic artery and portal vein divide into right and left branches, supplying their respective lobes. The vessels divide, progressively becoming smaller as they move out into the liver lobules. The final common pathway of these vessels is in the hepatic sinusoids through which blood is conveyed to the central vein.



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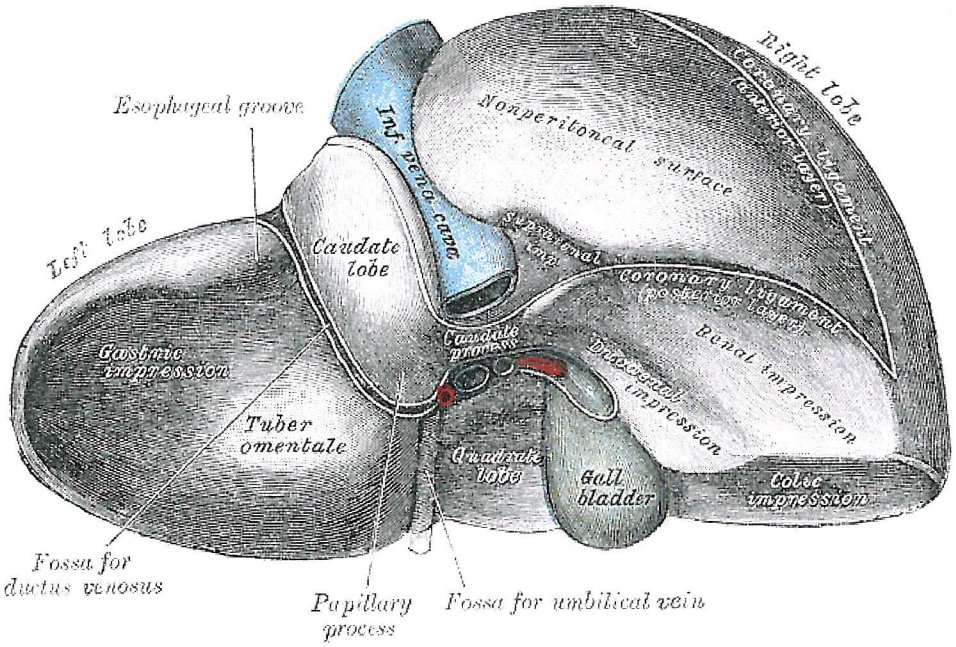

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Figure 1.01 Surface anatomy of the liver as viewed from the superior aspect (a) and the posterior/inferior aspect (b). *(Reproduced from Grays Anatomy Online)*

The sympathetic and parasympathetic nerve supply of the liver is derived from the coeliac plexus. There is a large hepatic branch of the anterior vagal trunk passing directly to the liver.

Bile drains from the liver via the right and left hepatic ducts into the common hepatic duct. The common hepatic duct is joined by the cystic duct, from the gall bladder, to form the common bile duct.

The venous drainage of the liver is via the right and left hepatic veins directly into the inferior vena cava. The lymph drainage is predominantly to the nodes at the porta hepatis, which drain into the coeliac nodes [2].

1.1.3 Microscopic Anatomy

The liver comprises a series of lobules each drained by a central vein. Portal tracts containing a portal vein, bile ductule and hepatic artery ring the central vein. Blood from the portal tract drains to the central vein through a network of sinusoids running between the hepatocytes. The sinusoids are lined by a specialised fenestrated endothelium, allowing the hepatocyte brush border to come into contact with blood across the space of Disse.

The space of Disse is filled with a low-density matrix rich in laminin, type IV collagen and heparan sulphate proteoglycans, and is populated by fat-storing hepatic stellate cells (Figure 1.02) [3].

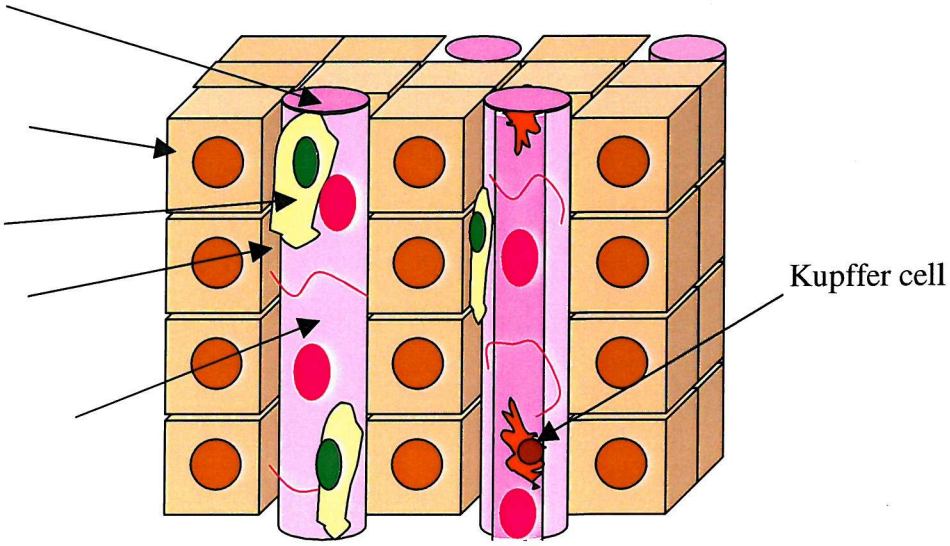

Figure 1.02 Microscopic anatomy of the sinusoid

1.2 The Liver – Function

The functions of the liver are many and complex, but there is a large functional reserve. Liver failure results in the loss of glucose homeostasis, protein synthesis and detoxification mechanisms. The anhepatic state is not compatible with survival. A schematic overview of the most important metabolic pathways of the liver is shown in figure 1.03.

1.2.1 Protein Metabolism

The liver tightly controls the level of circulating protein by balancing protein synthesis and degradation. It synthesises most of the proteins in circulation, with the notable exception of γ-globulins. The rich supply of amino acids required for this task is derived from intestinal absorption and muscle turnover.

Around half of the circulating protein is albumin and the normal liver synthesises 10-12 g per day. Albumin is important for maintaining plasma oncotic pressure and the transportation of water-insoluble substances such as bilirubin, hormones and drugs. Other important proteins synthesised in the liver are clotting factors, components of the complement system, and the carrier proteins, transferrin and caeruloplasmin.

The liver utilises transamination and oxidative deamination to degrade amino acids and form ammonia. The kidney then converts this to urea for excretion *(Millward-Sadler 1992; Harrison and Braunwald 2001).*

1.2.2 Carbohydrate Metabolism

Glucose homeostasis is principally controlled in the liver by insulin, catecholamines, glucagon and growth hormone. The mechanisms used for this include glycogenesis, glycogenolysis, glycolysis and gluconeogenesis.

In the fasting state, glycogenolysis and gluconeogenesis are used to maintain glucose levels. Hepatic glycogen stores are 70-80g and glucose requirements are 150g/day, this means that glycogen stores are exhausted within one day of fasting. Gluconeogenesis requires amino acid precursors, such as alanine, which is derived from the catabolic products of muscle breakdown. During the post-prandial state, the liver directs alanine and other amino acids to the periphery for incorporation into muscle protein *(Millward-Sadler 1992; Harrison and Braunwald 2001).*

1.2.3 Lipid Metabolism

The major sources of fatty acid in the liver are the diet and adipose tissue. In addition the liver synthesises some fatty acids from acetate. These fatty acids may be incorporated into triglyceride, cholesterol and phospholipids, or oxidised to form carbon dioxide or ketone bodies.

Triglycerides are exported from the liver after conversion to lipoproteins. The liver is the major site of production of very low density lipoprotein and the principal site of catabolism of low density lipoprotein. It is probably also involved in the catabolism of high density lipoprotein. In addition, remnants of chylomicrons are degraded by the liver.

Cholesterol occurs naturally in the diet, but the majority is synthesised in the liver. The liver esterifies free cholesterol with fatty acids. Cholesterol within the liver is used in the manufacture of bile acids. The metabolism of cholesterol is complex, and linked with that of fatty acids, triglycerides, lipoproteins and bile. Many enzymes are involved in this complex process *(Millward-Sadler 1992; Harrison and Braunwald 2001)*.

1.2.4 Formation of Bile

Bile consists of water, bile acids, electrolytes, cholesterol, bilirubin and phospholipids. Bile acids are produced from cholesterol and function principally as detergents. Bilirubin is formed from the breakdown of haem containing proteins, especially haemoglobin. The liver conjugates bilirubin with glucuronic acid to render it water soluble for active excretion in bile *(Millward-Sadler 1992; Harrison and Braunwald 2001)*.

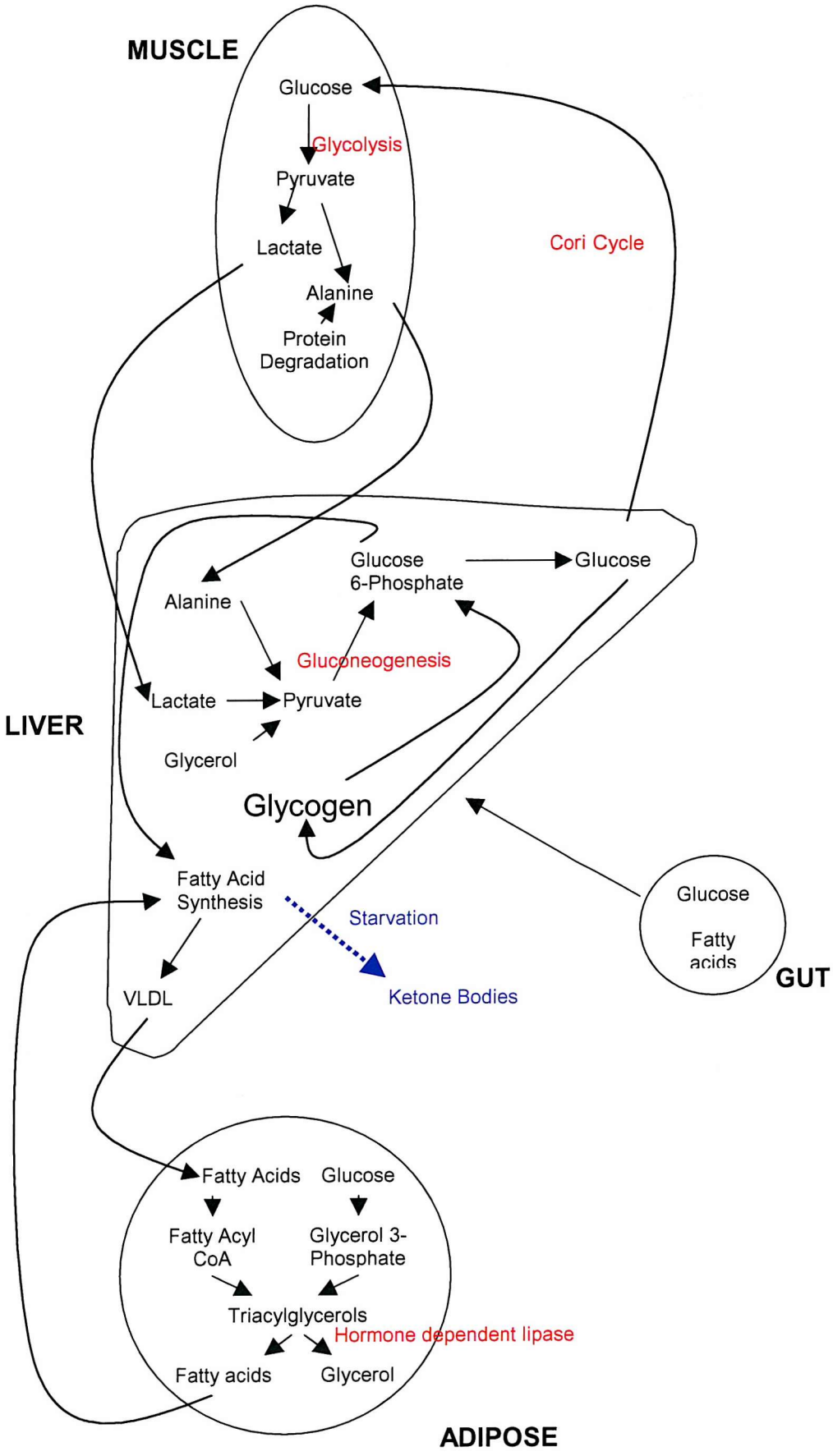

Figure 1.03 Schematic representation of metabolic pathways in the liver

1.2.5 Hormone and Drug Inactivation / Detoxification

Many drugs and toxins are water soluble, making them easy to excrete in bile and urine. Conversely, lipid soluble drugs accumulate in the body unless metabolised by the liver. Hepatic "first pass" metabolism of drugs carried in the portal blood is significant in reducing the effects of certain drugs and bacterial toxins from the gut. The liver has multiple metabolic pathways to inactivate and excrete potentially hazardous molecules. Albumin functions as a transport protein to carry many of these water insoluble compounds. The first phase of this process is a chemical alteration of the compound, and the second phase renders it water soluble for excretion. Phase 1 of metabolism can make a compound more toxic, but this effect can also be harnessed pharmacologically to activate or increase the potency of a prodrug.

One of the most important enzymatic systems utilised for drug clearance is the cytochrome P_{450} group of enzymes. These are a family of enzymes found principally in the mitochondria and liver microsomes and are used for the synthesis of bile salts and steroid hormones. The action of this enzyme system is to hydrolyse compounds using NAPDH, with the resulting hydroxyl group providing conjugation sites for glucuronation, acetylation or the addition of a sulphate molecule. Drugs, proteins and polycyclic aromatic hydrocarbons are all hydroxylated by P_{450} to these reactive epoxides, but some carcinogens are activated by this mechanism to a chemically reactive form. Cytochrome P_{450} enzymes are inducible, and their activity will vary between individuals.

Hormones are metabolised through similar mechanisms, but can also influence these mechanisms themselves *(Millward-Sadler 1992; Harrison and Braunwald 2001).*

1.3 The Liver – Component Cells and Their Functions

1.3.1 Hepatocyte

The most numerous cell in the liver is the hepatocyte. It is epithelial in origin and is the cell responsible for the specialised metabolic functions of the liver *(Millward-Sadler 1992; Harrison and Braunwald 2001).*

1.3.2 Kupffer Cell

The Kupffer cell is an intra-hepatic macrophage. It is found in the sinusoids and responds to liver injury by producing inflammatory and chemo-attractant cytokines. It scavenges debris and the products of cell death *(Millward-Sadler 1992; Harrison and Braunwald 2001).*

1.3.3 Sinusoidal Endothelial Cell

The sinusoidal endothelial cell is fenestrated and lies on the extracellular matrix of the Space of Disse. Evidence from the rat suggests that it can function as an antigen presenting cell, promoting tolerance to new antigens *(Millward-Sadler 1992; Knolle 1998; Knolle 1999; Harrison and Braunwald 2001).*

1.3.4 Pit Cell

This cell is a natural killer cell found in the space of Disse *(Millward-Sadler 1992; Harrison and Braunwald 2001).*

1.3.5 Hepatic Stellate Cell

The stellate cell is a rounded fat-storing cell, rich in vitamin A containing droplets. In normal liver, the hepatic stellate cells are found in the low density basement membrane matrix of the space of Disse. When activated, the cells lose their fat droplets and become elongated and myofibroblast–like, producing large amounts of collagen and matrix modifying enzymes. The activated cells proliferate and migrate throughout the liver, apparently becoming more resistant to apoptosis. It is these cells which are believed to be the predominant producers of the high density matrix and fibrillar collagens seen in hepatic fibrosis and cirrhosis *(Millward-Sadler 1992; Harrison and Braunwald 2001).*

1.4 The Liver – Disease States

The liver is susceptible to injury by many factors: viruses, bacteria, toxins and drugs, alcohol, autoimmune disease, ischaemia, obstruction of bile flow, metabolic disturbance, inborn errors of metabolism, and malignant processes. The nature of the insult will produce a specific injury, and therefore a variable clinical picture.

1.4.1 Acute Liver Injury

Acute liver injury is defined as liver damage which either completely resolves or causes liver failure within 6 months of onset. It is most commonly seen with viruses, toxins, drugs and ischaemia. In this case there is massive hepatocyte death and a rapid loss of metabolic and synthetic capacity of the liver. There is an accumulation of metabolic waste products, reduction in synthetic proteins such as clotting factors and albumin, and a failure of glucose homeostasis. The capacity of hepatocytes to regenerate will result in a full recovery, if the patient survives the acute illness *(Millward-Sadler 1992; Williams 1996).*

1.4.2 Chronic Liver Injury

Chronic liver disease is defined as liver damage which persists beyond 6 months of onset. It is most commonly seen with viruses, alcohol, autoimmune disease, biliary obstruction, and inborn errors of metabolism. The specific nature of the insult will dictate the precise histological changes, which often include gradual hepatocyte loss and inflammatory cell infiltrate. The final common pathway in the pathology of these conditions is fibrosis, caused by abnormal collagen deposition leading to distortion of the vasculature and loss of inter-cellular relationships. It is this disruption that interferes with the function of the hepatocytes, leading to a slow failure of liver function and reduction in hepatocyte numbers. There is a gradual fall in serum albumin and clotting factors, disturbance in plasma lipids, a rise in bilirubin and increasing metabolic disturbance leading to mental disorientation. In addition, the fibrotic bands cause a rise in sinusoidal flow pressure transmitted to the portal vein and leading to portal hypertension. The resultant varices in combination with low platelets from splenomegaly and abnormal clotting may lead to catastrophic gastrointestinal bleeding *(Millward-Sadler 1992).*

1.5 Liver Fibrosis

Fibrosis is the final common pathway of all forms of chronic liver injury, regardless of the original insult. Although inflammatory cell infiltration is a common finding in many chronic liver diseases, it is not universal i.e. haemochromatosis, α-1 anti-trypsin deficiency, Wilson's Disease *(Millward-Sadler 1992)*.

Normal extra-cellular matrix consists of defined molecules which are precisely organized and act as scaffolding for cells. It directs polarization, migration, proliferation, survival and differentiation. The normal liver has 2 types of extra-cellular matrix, a low density basement membrane-like matrix in the sinusoidal sub-endothelial space, and a dense, interstitial-type extra-cellular matrix (ECM) in the peri-portal areas, around the central veins and in the liver capsule. The low density ECM consists of; fibrillar collagens I, III and V, microfibrillar collagen VI, basement membrane collagens IV and XVIII, traces of collagen XIV and the proteoglycans decorin, fibronectin, tenascin-C, laminin, nidogen, heparin sulphate proteoglycans and others. The high density ECM contains less of the basement membrane components and more of the fibrillar collagens.

The process of liver fibrosis results in a qualitative change in the ECM around the periportal and perisinusoidal areas, with up a ten fold increase in collagens and non-collagenous components. At the same time, the fenestrations in the sinusoidal endothelium and the brush border on the hepatocytes are lost. The change in the ECM of the sinusoids is effectively a transformation from low to high density, and is believed to compromise hepatocyte function and further activate hepatic stellate cells *(Schuppan 2001)*.

The ECM is not an inert structure to support the cells. The components can bind bioactive mediators, giving locally high concentrations of certain growth factors, cytokines and chemokines i.e. TGF-β and PDGF. The matrix can be important in presenting the active site of growth factors to cells, allowing binding and internalization. In addition, the matrix itself can interact with integrin receptors on the cell surface of cells, initiating cascades of intra-cellular signaling. In these ways, the exact composition of the ECM can have profound effects on the level of activation, differentiation and proliferation of cells, and the nature of any resultant matrix remodelling *(Schuppan 2001)*.

The cells believed to be principally involved in matrix turnover, and therefore

fibrosis, are the hepatic stellate cells (HSC) located in the hepatic sinusoidal space of Disse. (Figure 1.04) In their quiescent state they are full of vitamin A rich fat droplets, but as they become activated they lose these droplets and take on a myofibroblast-like phenotype *(Friedman 1993; Friedman 1996)*(Figure 1.05). The triggers for activation include, toxins, cholestasis, viruses and auto-immunity, via activation of endothelial cells, liver epithelial cells, mononuclear cells and platelets. There is much evidence that implicates HSC in the role as the common pathway for liver fibrosis regardless of the nature of the liver injury; HSC synthesise and secret large amounts of collagen and other ECM components; collagen expression in situ in damaged livers is localized to the HSC; there is a positive correlation between amount of fibrosis and HSC numbers in damaged livers; inhibition of HSC results in lower levels of fibrosis. In addition to secreting collagens and other matrix components, HSC also have been shown to produce both matrix metalloproteinases and tissue inhibitors of these metalloproteinases. This implies that HSC are involved in matrix production, remodeling and resolution of fibrosis, if this occurs *(Bataller and Brenner 2001; Benyon and Arthur 2001)*.

Activation of HSC can be broadly divided into 2 phases, initiation and perpetuation. The initiation phase is where paracrine influences render the HSC responsive to cytokines, chemokines and growth factors, whilst perpetuation results from the effects of these stimuli maintaining an activated phenotype. The initiation phase results in a change in transcriptional activation, the activation of signaling molecules and early induction of structural genes, the likely paracrine stimuli resulting in this phase come from Kupffer cells, endothelial cells, hepatocytes or ECM itself. The perpetuation phase results in proliferation of HSC, increased contractility, fibrogenesis, production of chemokines, matrix degradation and retinoid loss. The paracrine and autocrine stimuli involved in this phase are likely to be PDGF, TGF-β, endothelin-1, MCP-1 and changes in ECM *(Friedman 1996)*.

There is emerging evidence that HSC are involved in resolution of fibrosis, and can undergo apoptosis as the fibrosis resolves. However, there appear to be mechanisms in activated HSC which render them resistant to apoptosis and may influence the likelihood of resolution of fibrosis once the cascade is activated *(Bataller and Brenner 2001; Iredale 2001; Murphy 2002)*.

The molecular mechanisms resulting from HSC activation are complex and not

yet fully elucidated, but there are pathways that can activate, exacerbate or inhibit fibrogenesis *(Friedman 1993; Friedman 1996; Friedman 1999; Iredale 2001)*.

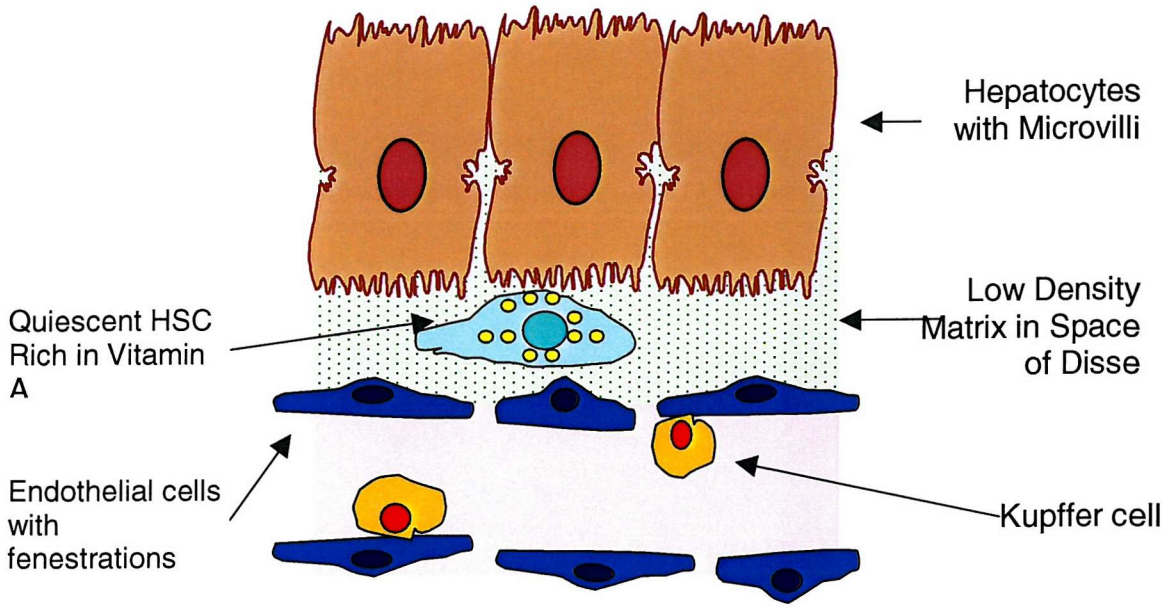

Figure 1.04 Normal Microanatomy of the Hepatic Sinusoid

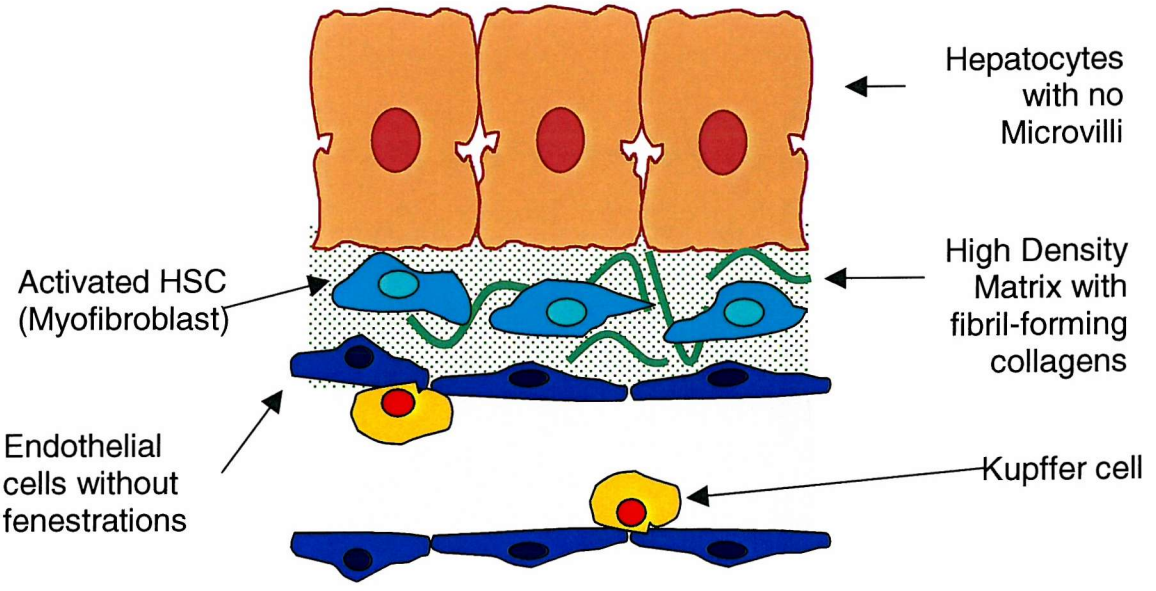

Figure 1.05 Microanatomy of the hepatic sinusoid in fibrosis

1.6 The Discovery of the Hepatitis C Virus

Following the characterisation of hepatitis A and hepatitis B, and the exclusion of these as the cause of post-transfusion hepatitis in many cases, it became clear that there was likely to be another causative agent implicated in these cases. It was known that this agent could be transmitted to chimpanzees. In addition, filtration studies had shown that it was less than 80nm, and sensitivity to organic solvents suggested that it had a lipid envelope. Intensive investigation had failed to demonstrate viral antigens or specific antibodies, and this was thought likely to be due to low circulating titres of virus. Michael Houghton and his team at the Chiron Corporation investigated this agent and identified a cDNA clone derived from the blood borne non-A non-B viral hepatitis genome using a strategy that relied heavily on molecular techniques. Briefly, infectious chimpanzee plasma was ultracentrifuged to ensure pelleting of a small virus, and nucleic acid was recovered from the pellet. The nucleic acid was denatured, as the nature of the viral genome was unknown, and then random primers were used to initiate reverse transcription and synthesise cDNA from both RNA and DNA. The derived cDNA library was cloned in bacteriophage λgt11 and expressed in bacteria giving around 10^6 recombinant phage, allowing efficient expression of cDNA encoded polypeptides. The resulting polypeptides were screened with serum from a chronic non-A non-B hepatitis patient as a presumed source of anti-viral antibodies. A positive cDNA clone was identified. Further characterisation proved that the cDNA clone would not hybridise to human or chimpanzee DNA, to RNA from non-infected chimpanzee liver or to nucleic acid pellets from uninfected chimpanzee serum. The hybridisation of this clone to nucleic acid pellets from infected chimpanzee serum was disrupted by ribonuclease, but not deoxyribonuclease, suggesting that it was hybridising to an exogenous RNA molecule in the infected serum. Only one of the strands of the cDNA clone could hybridise, so it was surmised that the viral RNA was single stranded. Northern blot analysis of infectious serum identified the viral RNA as having a size of 5000-10000 nucleotides. Finally, to confirm that this cDNA sequence represented an RNA virus closely associated with non-A non-B hepatitis, the polypeptide was expressed in bacteria and used for immunoblotting. All infected patients and infected chimps sera reacted with the polypeptide, whilst healthy controls did not. In addition, healthy chimps developed antibodies to the polypeptide after

exposure to the infectious agent of non-A non-B hepatitis. The new viral agent was called hepatitis C virus *(Choo 1989).*

The discovery of hepatitis C virus in 1989, and subsequent antibody testing revealed the causative agent in over 90% of previously designated non-A non-B post transfusion hepatitis and the majority of cases of community acquired non-A non-B hepatitis. Screening of donated blood for HCV infection has been widespread since 1991 *(Garson 1992; Crawford 1994).*

1.7 Hepatitis C – The virus

1.7.1 Structure and Function

The hepatitis C virus is a positive stranded RNA virus of the hepacivirus genus. It is around 9.4kb in length and has a highly conserved 5' end which is thought to encode the internal ribosomal entry site. The virus replicates in the cytoplasm of host cells using its own RNA dependent RNA polymerase in combination with the hosts own protein manufacturing apparatus. Translation produces a single polypeptide of 3000 amino acids, which is subsequently cleaved by viral and host proteases into structural and non-structural proteins. (figure 1.06, table 1.01) The viral envelope is formed by the insertion of viral glycoproteins into portions of host cell membrane; this contains the nucleocapsid proteins and viral genome *(van Doorn 1994; Major and Feinstone 1997; Rosen and Gretch 1999).*

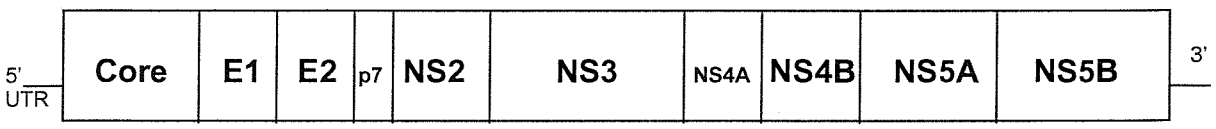

Figure 1.06 Structure of the HCV Polyprotein (Adapted from Rosen et al.1999)

Region / Protein	Nucleotide Location	Amino Acid Location	Functions
5' UTR	1-341		Initiation of translation, ?replication
Core	342-857	1-191	Structural, ?encapsulation of viral RNA
E1	915-1490	192-383	Structural, receptor binding, ?cell entry
E2	1491-2579	384-746	Structural, receptor binding, ?cell entry
E2p7	1491-2768	384-809	Not Known ?precursor or structural
P7	2580-2768	747-809	Not Known
NS2	2769-3419	810-1026	Part of the NS2-3 protease
NS3	3420-5312	1027-1657	Part of the NS2-3 protease, serine protease, helicase, ATPase
NS4A	5313-5476	1658-1711	Co-factor for NS3 serine protease activity
NS4B	5477-6257	1712-1972	?Replicase component
NS5A	6258-7600	1973-2420	?Replicase component
NS5B	7601-9374	2421-3011	RNA dependent RNA polymerase
3' UTR	9375-9621		?Replication ?Packaging of viral genome

Table 1.01 Function of HCV Genome and Proteins (Adapted from Major *et al.*1997)

The RNA dependent RNA polymerase lacks a proof reading capacity, resulting in nucleotide mis-incorporation. Many of these errors are lethal to the virus but others do not even affect amino acid translation. Certain nucleotide substitutions may lead to changes in the amino acid sequence that confer survival advantage to the virus, either by improving replication efficiency or allowing immune evasion through epitope changes. Thus a single host will be infected with multiple quasi-species of the virus, usually with one dominant strain *(van Doorn 1994; Major and Feinstone 1997; Rosen and Gretch 1999)*. The 5' end of the genome is highly conserved, and the predicted structure of this area is shown in figure 1.07.

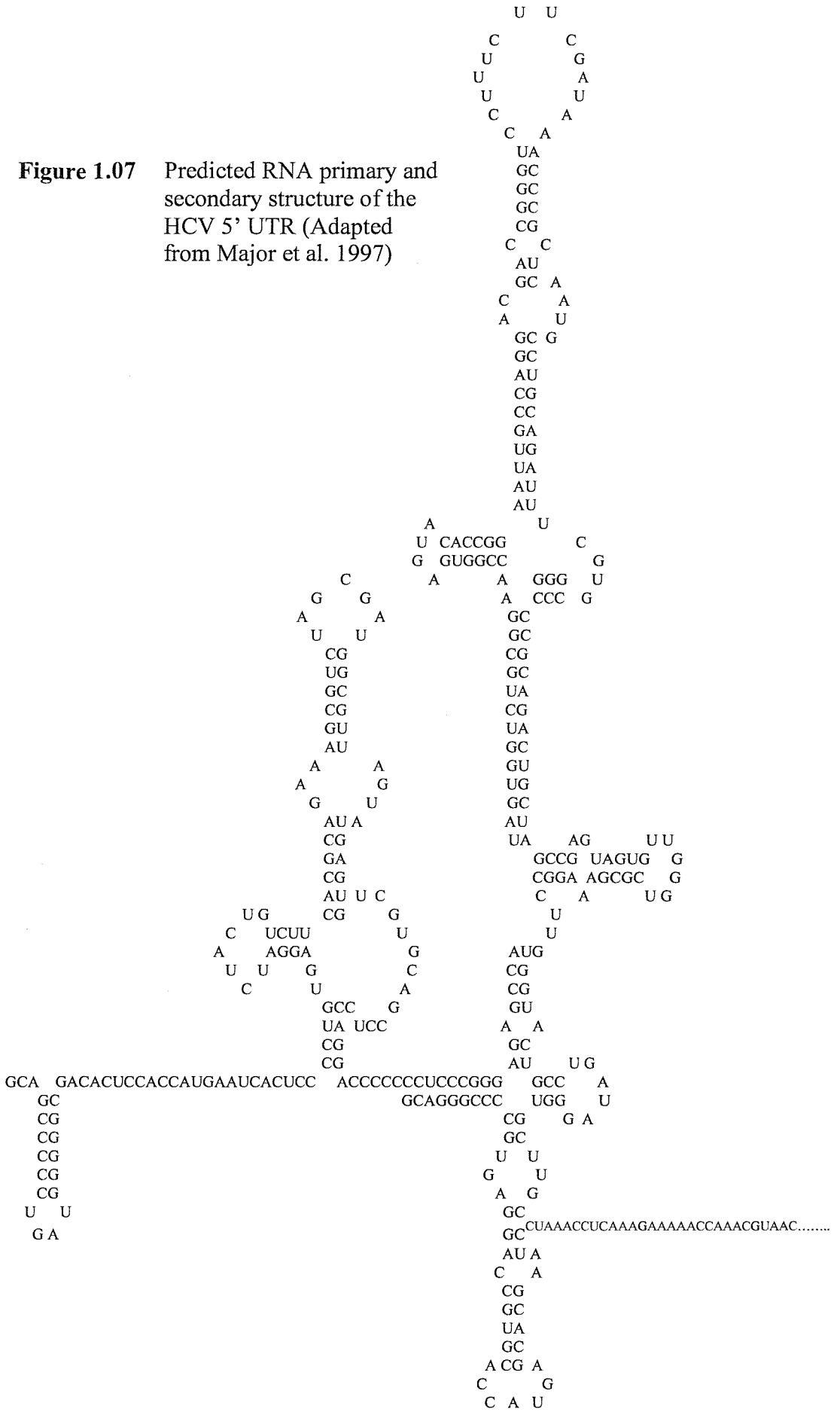

Figure 1.07 Predicted RNA primary and secondary structure of the HCV 5' UTR (Adapted from Major et al. 1997)

1.7.2 Phylogenetics

Genetic variation caused by mutation in one host will cause the formation of quasispecies. Over decades and continents these genetic variations are significant, and lead to the formation of distinct viral genotypes recognised by sequences in the core, E1 and NS5B regions. There are currently 6 major genotypes, each with various sub-types (figure 1.08) *(Simmonds 1997; Robertson 1998; Rosen and Gretch 1999).* Viral genotype does not correlate with the outcome of host infection in either acute or chronic disease, but does influence susceptibility to anti-viral therapy *(Simmonds 1996; Simmonds 1997).*

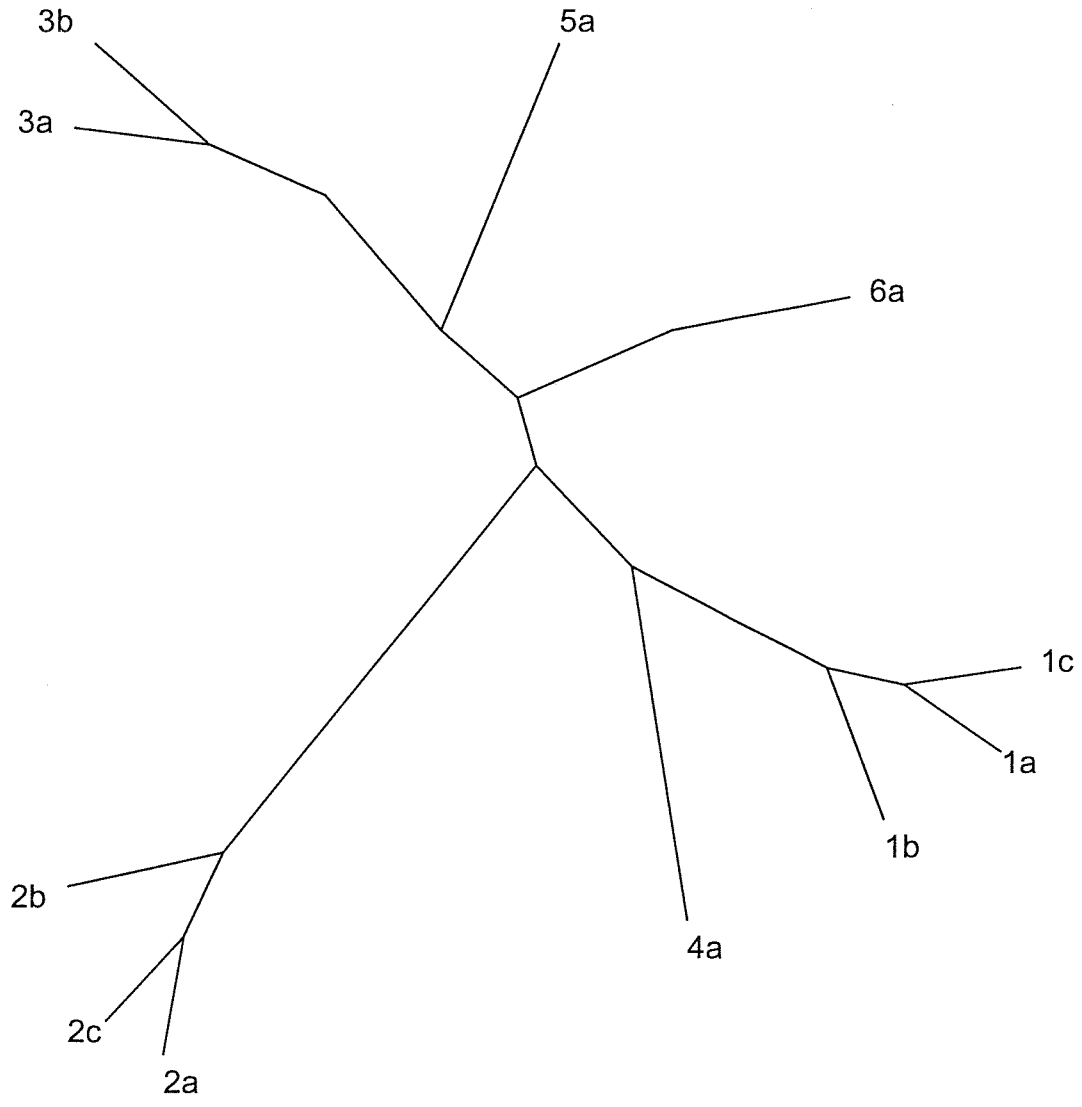

Figure 1.08 Phylogenetic Tree of Hepatitis C Virus (Adapted from Robertson et al. 1998)

1.7.3 Epidemiology

Hepatitis C establishes a chronic infection in 85% of exposed individuals and is the commonest cause of chronic liver disease worldwide. It is the leading indication for liver transplantation in the Western world. Global estimates suggest that 170 million people have chronic infection, 4 million in the United States of America. Prevalence of HCV positive serology in the United Kingdom is 0.1-1% of the population. Infection is often silent, and the majority of affected individuals are not aware that they have the virus [18, 25-30].

Viral genotypes 1, 2 and 3 are ubiquitous, whilst 4, 5 and 6 are generally restricted to defined geographical areas (Figure 1.08) [22, 29, 31].

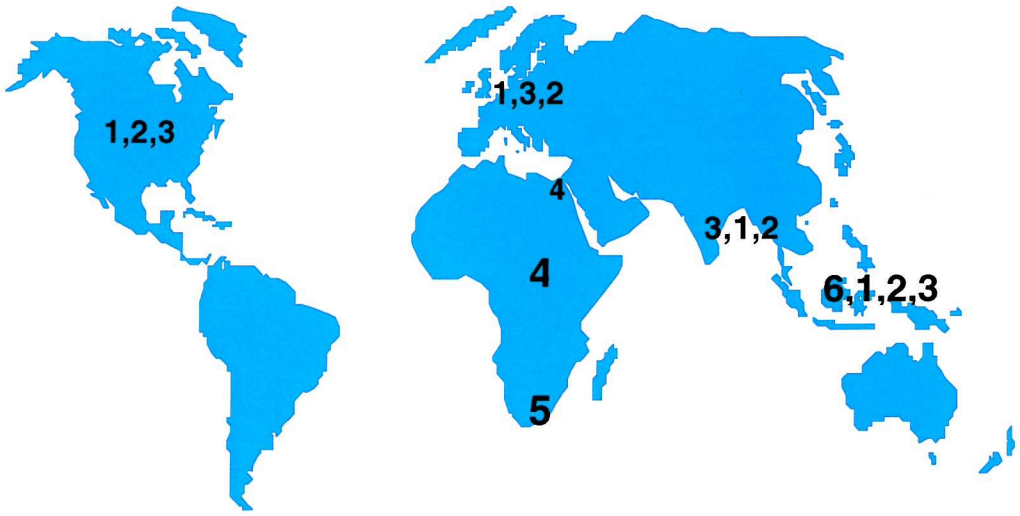

Figure 1.09 Geographical distribution of HCV genotype

1.8 Hepatitis C - The Disease

1.8.1 Routes of Infection

Hepatitis C is a parenterally transmitted virus. The commonest route of infection varies with the geographical region studied *(MacDonald 1996; Simmonds 1996; Shah 1997)*.

In adults in the UK, the commonest risk factor for transmission of hepatitis C is intra-venous drug use (IVDU). It is estimated that 80-90% of intravenous drug users are positive for anti-hepatitis C antibodies, suggesting almost universal exposure in this population *(Crawford 1994; Di Bisceglie 1998; Rosenberg 1999)*.

Following IVDU, the next most common risk factor for HCV infection is exposure to blood or blood products (clotting factors, platelets, immunoglobulins etc.). Since 1991, blood donors in the UK have been screened for evidence of HCV infection and the prevalence of HCV infection in UK blood donors is around 0.1%. *(Garson 1992)*The risk of HCV infection from blood transfusion prior to 1991 was low, but immunoglobulins and clotting factors produced by pooling serum from multiple donors carried a much higher risk. Almost 100% of UK haemophiliacs have evidence of HCV exposure from infected factor VIII *(MacDonald 1996; Simmonds 1996)*.

IVDU and transfusion may account for the majority of HCV infection in the UK, but there are several other well-recognised risk factors with less well-defined transmission rates. These include: vertical transmission (delivery and breast-feeding) which carries around a 7% transmission rate at the time of delivery; sexual contact (heterosexual and homosexual); tattooing and body piercing; occupational exposure (needlestick etc.) *(Rosenberg 1999; Conte 2000; Yee 2000)*.

Early studies of risk factors for HCV transmission revealed that 40% of cases had an unidentifiable route of transmission. Later studies have shown that there is no identifiable route of transmission in only 15-20% of cases. The best results however follow a careful history taken by an expert (e.g. HCV specialist nurse) who the patient trusts. In these circumstances, the percentage of patients in whom it is still not possible to identify the route of infection can be as low as 5%. The commonest reason for this drop in unknown risk factors, is that the patient will admit to at least one episode of intra-venous drug use. The 5% in whom no risk can be identified may be considered to have had nosocomial transmission, although proving nosocomial transmission is very difficult in HCV infection *(Rosenberg 1999)*.

1.8.2 Diagnosis

The primary diagnostic test for hepatitis C infection is the presence of anti-HCV antibodies. These antibodies can be detected by commercially available third generation enzyme-linked immunoassays (ELISA), with false positives excluded using a supplementary recombinant immunoblot assay (RIBA). Both of these tests use recombinant viral proteins to detect HCV specific antibodies in the serum. The sensitivity and specificity of these assays is generally high (>95%), but does depend on the nature of the study population. In a low risk group, such as blood donors, there is a high false positive rate using ELISA, whereas this is a very reliable and accurate screening tool in high risk populations, such as intra-venous drug users. In addition, these antibody dependent tests are less sensitive and specific (around 75-80%) in immuno-compromised populations, such as haemodialysis patients. There is no universally accepted 'Gold Standard' test for HCV, and the sensitivity and specificity of all the tests depends upon the population studied and nature of the test chosen as the gold standard. In all of these diagnostic tests there is a window of 4-6 weeks between infection and development of detectable anti-HCV antibodies *(Holland 2000; Tobler 2000; Colin 2001)*.

Hepatitis C viraemia is detected in serum using HCV specific primers in a polymerase chain reaction (PCR). There are various commercially available systems for HCV PCR, and many institutions have developed their own in-house assays. The sensitivity of these assays is generally very good, down to a level of viraemia of 40-50 copies of virus per ml. There is a window from infection to positive PCR of 1-4 weeks. Recently, reports have suggested that patients who have resolved HCV infection acutely can lose detectable levels of anti-HCV antibodies over years. Numbers of such cases are difficult to quantify, and may confound the calculated rate of spontaneous resolution of infection *(Lee 2000; Takaki 2000)*.

1.8.3 Acute Infection

Acute hepatitis C infection is usually sub-clinical with jaundice manifest in less than 20% of patients. HCV RNA is usually detectable in the serum within 1-4 weeks of infection, and detectable HCV specific antibodies develop over the next 4-6 weeks. Acute infection produces a mild hepatitis, reflected in a modest transaminase rise, which can be as high as 50x the upper limit of normal. Bilirubin may rise to

clinically detectable levels in around 15% of cases, and clotting factors remain adequate. There are only very rare reports of fulminant hepatic failure from acute hepatitis C infection, and these are not unequivocally HCV related.
Spontaneous and sustained virological clearance occurs in around 15% of infected individuals. Clinical symptoms are more frequent in those who successfully clear the virus but may be mistaken for a flu-like illness. The group with a clinically detectable illness associated with their infection (jaundice or flu-like symptoms) will acutely resolve their infection in around 50% of cases *(Di Bisceglie 1998)*.

1.8.4 Chronic Infection

85% of HCV infected individuals develop chronic infection. The majority of these patients are unaware of their acute infection, and it is only with the development of chronic liver disease that they begin to develop specific symptoms. Some individuals may have initially cleared the virus from their blood, but persistent viraemia recurs within 6 months. Once chronic infection is established the annual rate of spontaneous clearance is negligible.
Chronic infection may give non-specific symptoms of fatigue, malaise and vague abdominal pains. Frequently, the diagnosis of chronic HCV infection is made when routine blood tests reveal a raised alanine transferase (ALT). A persistently raised and fluctuating ALT is often the only indication of chronic infection, and is easily over-looked. However, in many cases the ALT will be normal, sometimes even in cases with significant liver damage.
The diagnosis of chronic hepatitis C infection can only be made if there is a suspicion of exposure to the virus, as it is only under these circumstances that the appropriate diagnostic tests will be requested. Once the diagnosis has been made, and there has been an estimation of the duration of infection, then assessment can be made of the severity of any resultant liver damage.
Chronic infection is associated with positive anti-HCV antibodies in the serum together with persistently detectable HCV RNA by PCR *(Hoofnagle and di Bisceglie 1997; Di Bisceglie 1998)*.

1.8.5 Assessment of Severity of Liver Damage

Acute HCV infection is rarely clinically apparent and serological liver function tests are used to assess severity. Biochemical and haematological markers monitor the severity of the hepatitis, whilst PCR is used to identify virological responses. Liver biopsy is seldom required. Sustained viral clearance is not associated with progressive liver disease. Any hepatocellular damage associated with the acute hepatitis will resolve completely within 3-6 months if there is sustained viral clearance.

Chronic HCV infection is usually silent and biochemical and haematological markers are often unhelpful. The full blood count profile is usually normal, with a normal white cell count. Liver function tests can be normal, but may show a mild elevation in ALT (up to 10x upper limit of normal). The liver function tests are often completely normal in the presence of mild liver damage, but can still be normal in advanced liver disease.

In CHC, clinical history and examination may reveal the duration of infection and clinical evidence of chronic liver disease. Screening for other causes of liver damage will help to assess the contribution of chronic HCV infection to any co- or pre-existing liver pathology. In some cases it is clear from this basic assessment that the patient has established cirrhosis *(Alter 1992; Rodger 2000)*.

The gold standard for assessing the severity of HCV dependent liver damage is histology. Liver tissue can usually be safely obtained by percutaneous, trans-jugular or intra-operative biopsy. It is then fixed, stained and sectioned for examination by an experienced liver histopathologist. There are several validated scoring systems used for the assessment of liver biopsy material. Scores are assigned using standardised definitions of severity for each of several features seen in chronic hepatitis C. Degree of lymphocyte infiltration of the portal tracts and parenchyma, inflammatory cell recruitment and necrosis, hepatocyte damage with evidence of apoptosis and fibrosis are all included in most of these systems. Scoring systems such as these allow intra- and inter-patient comparisons to be drawn *(Knodell 1981; METAVIR 1994; Ishak 1995; Bedossa and Poynard 1996)*.

If cirrhosis is already established, assessment of the patient is clinical and follows the same criteria as other causes of end-stage liver disease i.e. Child-Pugh Grading *(O'Grady 2000)*.

1.8.6 Progression of Liver Disease in Chronic Hepatitis C Infection

The outcome of chronic hepatitis C (CHC) infection is unpredictable. In some patients it appears to be a very benign condition whilst it produces aggressive liver damage in others. There are some factors favouring a good prognosis (female sex, young age at infection and low alcohol intake), but these are not absolute. Early studies suggested that the route of acquisition of the virus may influence the outcome of chronic infection, with a large inoculum from a blood transfusion being associated with more rapidly progressive liver disease. However, these studies are flawed as there are many confounding factors, such as the age and co-morbidity of patients.

Broadly, CHC patients can be divided into thirds by the severity of the resultant liver disease: a third will develop only mild liver damage with little evidence of progressive disease; a third will progress slowly to cirrhosis over the course of 20-40 years; a third will have aggressive liver disease with cirrhosis within 10-20 years, and an increased risk of hepatocellular carcinoma (Figure 1.10). Although the pathology in the liver in CHC is characterised by inflammation and fibrosis, it is progressive fibrosis which causes the most serious complications. This is because fibrosis is associated with portal hypertension and progressive liver failure; the commonest cause of HCV related death. Inflammation without progressive fibrosis in CHC may be associated with multiple symptoms, but does not cause liver failure *(Di Bisceglie 1998; Poynard 2000; Saadeh 2001).*

There is no evidence that the virus is directly cytopathic, and it is generally believed that the liver damage is immune mediated. Despite early reports to the contrary it is apparent that viral genotype does not affect the outcome of disease. Careful epidemiological studies have shown that these earlier misperceptions that genotype 1 was associated with a worse prognosis were explained by longer duration of infection in the genotype 1 patients in the samples *(Simmonds 1997; Huang and Koziel 2000).*

1.8.7 Other Complications of CHC

A serious complication of CHC is hepatocellular carcinoma (HCC), which occurs at a rate of 3-10% per year in the presence of cirrhosis. If small and solitary it is sometimes possible to resect the tumour, or remove the liver and undertake orthotopic liver transplantation. In some instances, medium sized tumours can be managed with chemo, radio frequency or ethanol ablation, sometimes in combination with

systemic chemotherapy. Often the tumours are large or multi-focal when they are detected, and then the only treatment is supportive or palliative. The ideal is early diagnosis, and so screening of cirrhotics with liver ultra-sound scanning and serum estimation of α-feto-protein is recommended every 3-6 months. However, evidence of the benefit of such screening does not exist *(Alberti 1999)*.

More common than HCC are the usual complications of any cirrhosis or end-stage liver disease. Portal hypertension with variceal haemorrhage, coagulopathy, encephalopathy and metabolic disturbance are regularly seen.

Chronic hepatitis C infection may produce complications in the absence of cirrhosis. These can be troublesome to both patient and physician, and include mixed cryoglobulinaemia, glomerulonephritis and Sjögrens syndrome *(Cacoub 1999)*.

Complication	**Effects of Complication**
Non-Cirrhotic	
Cryoglobulinaemia	Rashes and arthralgia
Glomerulonephritis	Renal failure
Sjögrens syndrome	Dry mouth and eyes
Hypothyroidism	Weight gain, lethargy, cold intolerance, constipation
Cirrhotic	
Portal hypertension	Varices, variceal haemorrhage and ascites
Hypoalbuminaemia	Ascites and peripheral oedema
Metabolic disturbance	Encephalopathy and hypoglycaemia
Coagulopathy	Bruising and prolonged bleeding
Hyperbilirubinaemia	Clinical jaundice
Hepatocellular carcinoma	

Table 1.02 Major Complications of Chronic hepatitis C infection

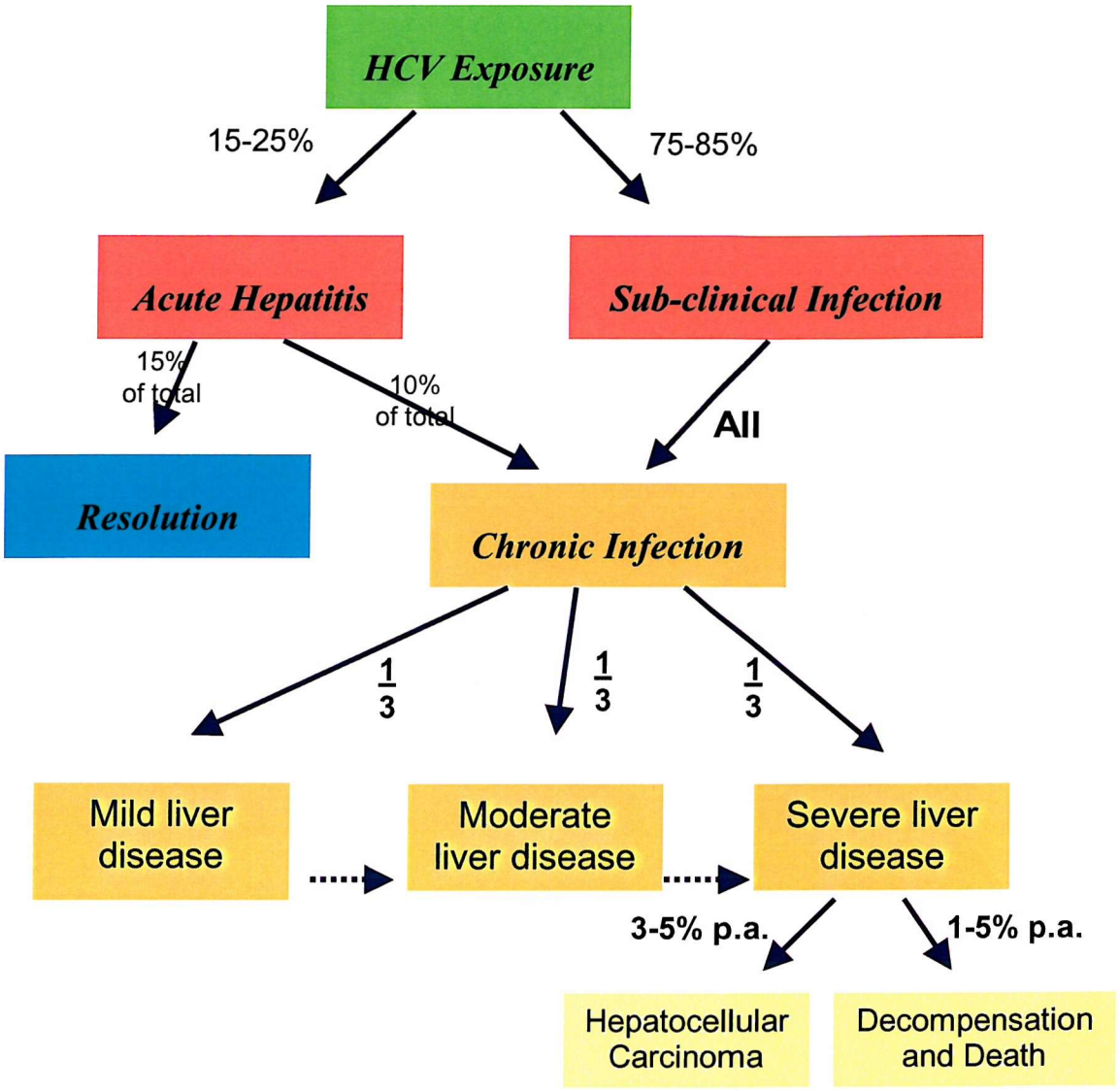

Figure 1.10 The clinical course of HCV infection

1.9 The Immune Response

1.9.1 Basic Components of the Antigen Specific Acquired Immune Response

The innate immune system has evolved the capacity to destroy invading organisms using many components; natural killer cells, natural antibodies, cytokines, a complex system of lytic proteins called the complement cascade and many others. Evolution has produced pathogens with sophisticated mechanisms which avoid destruction through these non-specific defenses. In response, there has been selection pressure on mammals to adapt to these developments with increasingly complex and specific solutions.

The nature of the acquired immune response is antigen specific. There are 3 classes of molecules used in antigen recognition; immunoglobulins, major histocompatibility complex molecules and T cell antigen receptor molecules. Of all these classes of molecule, immunoglobulins can display the most diversity in specificity and bind with the greatest discrimination and avidity *(Roitt 1997)*.

1.9.1.1 Immunoglobulins and the Humoral Response

If the complement system does not detect a pathogen, then activation of the cascade will not occur. Many organisms have evolved to be unrecognisable as pathogens to the innate immune system. However, these organisms may still be sensitive to the lytic properties of the complement cascade, NK cells and macrophage opsonisation if exposed. Adaptive mechanisms have evolved in parallel with these pathogens to provide specific solutions. In one such mechanism, immunoglobulins, or antibodies, specifically bind pathogen derived antigen and act as a "flag" to activate innate immune responses.

B lymphocytes are the effector cells of the humoral immune response. They are the only cells to manufacture antibody, which they express on their surface. Antibody expressed in the B cell membrane acts as the B cell receptor, whilst the majority of antibody produced is secreted into serum. Immunoglobulins from a single B cell will share the same amino acid sequence in their variable region, which is at the antigen combining site. There are around 10^7-10^9 different immunoglobulin molecules expressed by each individual.

Antibodies are molecules formed from four polypeptides, 2 heavy (55-70kD) and 2 light (24kD) chains, bound together by di-sulphide bonds. The antibody combining sites are formed by the juxtaposition of the variable domains of the light and heavy chains. The remainder of each of the light and heavy chains comprises a constant region. Antibody polypeptides are characterized by repeating homologous sequences of 110 amino acids which fold into a 2 layer β-pleated sheet, the immunoglobulin domain. (Figure 1.11) This feature recurs in all molecules in the immunoglobulin gene superfamily. Light chains have one variable and one constant domain, whilst the heavy chain has one variable and three or four constant domains. The antigen combining site is responsible for interaction with antigen, whilst the constant regions mediate most of the biological functions of antibodies by interacting with cells and complement. The carboxy terminal of the heavy chain is used to anchor the antibody to the B cell membrane in membrane-bound molecules.

Each B cell maturing in the bone marrow exploits recombination of genes, producing an exclusive selection, which forms a unique protein sequence at the variable region, or antigen combining site.

During B cell maturation, RAG1 and RAG2 are proteins expressed during pro-B and pre-B stages in periods of low mitotic activity. These proteins mediate recombination of the immunoglobulin heavy and light chain genes. Recombination of these genes leads to diversity in Complementarity Determining Regions (CDRs), achieved by unique combinations of the V, D and J gene segments in the variable domains. The greatest number of loci is in the V (or variable) segment of the gene, followed by the D (or diversity) segment and finally the J (or joining) segment (around 51, 27 and 6 potentially functional loci respectively). There is a lot more genetic material present in these regions, but large segments of DNA do not seem to code for potentially functional loci, and their presence and function is poorly understood. The most variability is found in the D segment as this can exploit frame shifts to produce new combinations of functioning polypeptides. Using these recombination mechanisms, there is a potential for an antibody repertoire of around 10^8 unique antigen specificities. Further diversity is created by the addition or removal of nucleotides at the junctions between the V,D and J segments, termed junctional diversity which can increase the antibody repertoire to around 10^{11} unique specificities.

In the variable domains of the light and heavy chains there are 3 highly divergent stretches each of around 10 amino acids long, termed the hypervariable segments. When the antibody is assembled, the three hypervariable segments of the light and heavy chains are brought together in a three dimensional space, supported by the more constant regions of the immunoglobulin domain, to form the antigen binding surface. This surface is complementary to the bound antigen, and the hypervariable regions are therefore also termed complementarity determining regions (CDRs), and are numbered 1, 2 and 3. CDR3 (in the D segment) shows the most diversity.

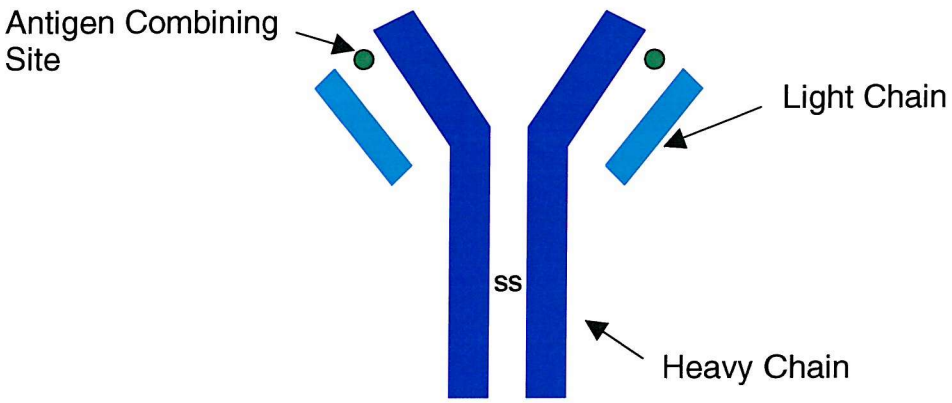

Figure 1.11 Structure of an immunoglobulin molecule

Invading organisms express proteins on their surface that may be bound by the receptor antibodies on the B cell surface. If the antigen is bound in an antigen combining site with a 'good fit', then this immunoglobulin signals the B cell to divide. The immunoglobulins expressed on the surface of naïve B cells are so-called "natural antibodies" as they have not been produced in response to a particular pathogen, but are the product of random genetic recombination.
Following proliferation signals, the progeny of a single B cell will exclusively produce antibody with the same specificity as the parent cell. However, in a germinal centre with proliferating B cells, point mutations will occur in the variable regions of the secreted immunoglobulin. These point mutations are all seen in the VDJ segment encoded portion of the variable domain. Mutations will produce antibodies with either more or less affinity for the antigen than the parent cell immunoglobulin. This

phenomenon is called "somatic mutation" and leads to selective predominance of cells producing antibody with high affinity. Selective predominance of the B cells producing antibody of the highest affinity is dependent on three factors; a dendritic cell, a B cell and a T cell. Antigen presentation by a dendritic cell will allow the B cell to engage if there is high affinity of the antibody expressed on the B cell surface. However, T cell help using co-stimulatory molecules such as CD40L is necessary to allow differentiation and proliferation in the activated B cell. Lack of any of these factors will not result in B cell proliferation or differentiation into a plasma cell for secretion of antibody.

Following proliferation some of the daughter cells will become memory cells with the ability to activate more quickly upon further antigen challenge, whilst the others differentiate into plasma cells producing large quantities of antibody to release into the serum. This antibody will bind antigen, thus activating complement and aiding phagocytosis.

The biological activity of the heavy chain constant domains varies with class or isotype. Progeny of the activated B cell may switch antibody class to an isotype with a more appropriate biological effect. e.g. IgA (with α heavy chain) in circumstances where mucosal immunity is required. This phenomenon is called "class switching" *(Roitt 1997; Abbas 2000).*

1.9.1.2 Cell Mediated Immunity and the T cell receptor

If an invading organism can infiltrate host cells without causing cell death, it may be able evade the humoral immune response. Viruses are obligate intracellular parasites, as they need the host's metabolic apparatus to replicate. Other pathogens, such as Mycobacteria and Leishmania, replicate within cells to take advantage of the protection they afford. The host cell processes pathogen proteins to generate small antigenic fragments which are displayed on the cell surface.

T lymphocytes mature in the thymus and respond to antigen encountered on the surface of infected host cells. T cells are characterized by an antigen specific receptor, the T cell receptor, that will recognise an epitope when it is in the binding cleft of a complementary surface molecule (Major Histocompatibility Complex or Human Leukocyte Antigen Molecule) on a host cell. T cell receptors can bind to such receptors on infected cells and professional antigen presenting cells, i.e. dendritic cells. Antigen presenting cells can also take up exogenous viral protein from the serum and process it for presentation to T cells, to prime naïve cells or augment an existing response *(Banchereau and Steinman 1998)*.

The T cell receptor (TCR) is a formed as a heterodimer of 2 molecules of the immunoglobulin gene superfamily, covalently joined by di-sulphide bonds. There are 2 possible pairings, α and β chains or γ and δ chains, of which the α/β TCR forms at least 95% of the T cell population, and all of the CD4 and CD8 positive T cells. The following description is related to these α/β TCRs. Analogous with antibody, the TCR molecules have a variable domain responsible for antigen recognition comprising V, D and J segments, and a constant domain for membrane binding to the T cell. There is no D segment on the Vα chain. There are 3 complementarity determining regions (CDRs) on each chain of the TCR, but there is an extra region in the V domain on the β chain responsible for binding super-antigens (e.g. enterotoxins). The CDR3 on both chains of the TCR at the V-(D)-J junctions contain random nucleotide additions and therefore have the most sequence variability. The TCR is clonally distributed, so T cells with different antigen specificities will express different TCRs. T cells do not undergo somatic mutation *(Moss 1992)*.

The juxtaposed variable domains of the α and β chains form the part of the TCR that recognises peptide-MHC complexes. Each TCR is restricted by MHC molecule

and peptide antigen. The CDR1 and CDR2 (and sometimes CDR3) recognise the MHC complex, whilst the CDR3 contacts the peptide. The TCR complex / MHC peptide complex bond is low affinity, and the likely role of accessory molecules is to stabilize this bond and allow initiation of biological processes.

The TCR is non-covalently linked to an invariant proteins CD3 and ζ to form the TCR complex. It is the invariant proteins in the TCR which are responsible for signal transduction to the T cell following antigen recognition. In addition, there are closely associated accessory molecules, CD2, CD4, CD8 and C28 which transduce a signal in concert with the TCR complex to fully activate the cell *(Moss 1992; Roitt 1997; Abbas 2000)*.

There are 2 major subsets of T lymphocyte:

a) T helper cells

T helper (Th) cells use MHC class II molecules as the complementary surface antigen presenting molecule for their T cell receptor and mainly recognise antigen processed by the exogenous pathway. If an appropriate epitope is presented to a Th cell in the binding cleft of a class II molecule, then a T helper cell will secrete cytokines in response. The pattern of the cytokines produced will depend on many factors: the type of Th cell (naïve or memory), the existing cytokine environment, co-stimulatory molecules. The cytokines produced by the Th cell will preferentially promote either cytotoxic T cell responses or an antibody response. In addition, there may be clonal expansion of the Th cell and recruitment of other immune and inflammatory cells.

The maturation of an antigen stimulated Th cell involves terminal differentiation to a phenotype that will be reproduced in all its daughter cells. The pattern of cytokine production defines the Th cell sub-type, each combination promoting a specific balance in the resultant immune response. Th1 cells primarily produce IL-2 and IFN-γ, which promote cell mediated immunity and cytotoxic T cells. Th2 cells, in contrast, produce IL-4, 5, 6, 10, 13 and TNF-α, which are favourable to supporting the humoral response. The cytokine profiles of these two Th subsets are mutually inhibitory, making a switch from cell mediated to humoral immunity or *vice versa* a rare occurrence once dominance is established.

In Th cells the defining accessory molecule is CD4. The CD4 molecule is invariable, and therefore conserved between individuals of a given species but unable to

specifically recognise variable ligands such as antigens. It is a membrane bound monomer of the immunoglobulin gene superfamily, with 4 extracellular immunoglobulin-like domains, a transmembrane portion and a 38 amino acid cytoplasmic tail. The CD4 protein binds to the non-polymorphic β2 domain of the MHC class II molecule and helps to stabilize the TCR/peptide/MHC complex, whilst transducing a signal to the T cell to promote activation *(Abbas 1996; Mosmann and Sad 1996; Waldrop 1998; Abbas 2000)*.

b) Cytotoxic T cells

Cytotoxic T (Tc) cells use MHC class I molecules as the complementary surface antigen presenting molecule recognised by their T cell receptor and thus recognise antigen processed via the endogenous pathway. If an epitope is presented to a Tc cell in the binding cleft of a class I molecule, then the cytotoxic T cell will secrete interferon-γ and initiate apoptosis in the host cell using Fas/FasL interactions and perforin mediated mechanisms. Antigen stimulated Tc cells can also undergo clonal expansion.

Recently, subsets of Tc cells have been described that are analogous to Th cell subsets. These have been designated Tc1 and Tc2 cells, and the cytokine profiles are similar to those of the Th cells. Thus, in addition to Th1 support of the cytotoxic T cell response, the Tc subsets can feedback to the $CD4^+$ cells through cytokine mediated promotion or inhibition of Th1 and 2 cells.

Tc cells express the invariable accessory molecule CD8. This is either a heterodimer of di-sulphide linked α and β invariant chains or a homodimer with α chains. Each has a single extracellular immunoglobulin domain, a transmembrane portion and a 25 amino acid intracellular tail. The CD8 molecule binds to the non-poymorphic α3 domain of the class I MHC molecule, and acts to stabilize the TCR bond to the peptide MHC complex and transduce an activating signal to the T cell *(Mosmann and Sad 1996; Lalvani 1997; McMichael and O'Callaghan 1998; Vukmanovic-Stejic 2000)*.

1.9.1.3 Human Leukocyte Antigens (The Major Histocompatibility Complex)

Most cells have surface expression of Human Leukocyte Antigen (HLA) class I molecules, and specialised cells also express class II. The HLA molecules are coded for on chromosome 6, although the beta$_2$-microglobulin chain of the class I is coded on chromosome 15. Each molecule consists of an alpha and beta chain linked by non-covalent bonds.

The function of the HLA complex is to present peptide fragments on the surface of the cell for surveillance by the immune system. Proteins from within the cell and those endocytosed from the extracellular space are broken down for recycling into peptides and then amino acids. Some of these peptides are packaged into the binding cleft of suitable MHC molecules and transported to the cell surface. T cells with the appropriate specificity recognise the peptide as either a 'self' or 'foreign' epitope, and activate if infection is suspected *(Abbas 2000)*.

a) MHC class I

The MHC class I molecules are used for the presentation of intracellular peptides. The molecules consist of a β_2-microglobulin polypeptide non-covalently bound to an alpha polypeptide chain with 2 peptide binding domains, an immunoglobulin-like domain, a transmembrane domain and a cytoplasmic tail. (Figure 1.12)

There are around 20 class I genes on chromosome 6, but there are 3 classic or class 1a genes which are of the most immunological importance: HLA-A, B and C.

The 2 chains of the class I molecule are manufactured separately and brought together by chaperone proteins in the endoplasmic reticulum (ER). The dimers are then bound to suitable degraded peptides at the point they enter the ER assisted by "transporters associated with antigen-processing" (TAP) molecules. Each class I molecule has a unique shape to the binding cleft and will have variable binding affinities for different peptides. The heavy chains are then combined with the β2 microglobulin and peptide and transported to the cell surface for presentation as an MHC peptide complex (figure 1.13).

Presentation of the peptide products of invading organisms will lead to recognition by peptide and HLA specific cytotoxic T cells. Activation of these cytotoxic mechanisms

should lead to the destruction of infected cells. Specialised antigen presenting cells can process extra-cellular proteins and peptides through the MHC class I system for priming of cytotoxic T lymphocytes *(Klein and Sato 2000; Klein and Sato 2000).*

b) MHC class II

The MHC class II molecules are most commonly found on activated T lymphocytes, dendritic cells, B cells, macrophages and thymic epithelial cells, although interferon-γ can induce other cell types to express these molecules. The class II molecules are used to present peptides derived from extracellular protein endocytosed by the cell for processing.

In contrast to class I, the alpha and beta polypeptide chains in the class II molecules each have similar domains: a peptide binding domain, an immunoglobulin-like domain, a transmembrane domain and a cytoplasmic tail. (Figure 1.12) There are multiple class II genes on chromosome 6, and the designation of the molecules is given by three letters: the first (D) indicates the class, the second (M,O,P,Q,R) indicates the family, and the third (A or B) indicates the chain – alpha or beta. The individual gene is represented by an arabic numeral, and the allelic variant of this denoted by a further number following an asterisk i.e. HLA DQB1*0301.

The alpha and beta chains are manufactured in the ER and an invariant chain is placed in the peptide binding groove to prevent premature binding. The molecule is then transported to the endosome for combination with a peptide with suitable binding affinity, following removal of the invariant chain. The class II / peptide complex is then transported to the cell surface where it can interact with T helper lymphocytes (figure 1.14) .

Activation of the T cell will occur if the T cell receptor recognises the epitope as foreign and can bind with sufficient affinity *(McFarland 1999; Klein and Sato 2000; Klein and Sato 2000).*

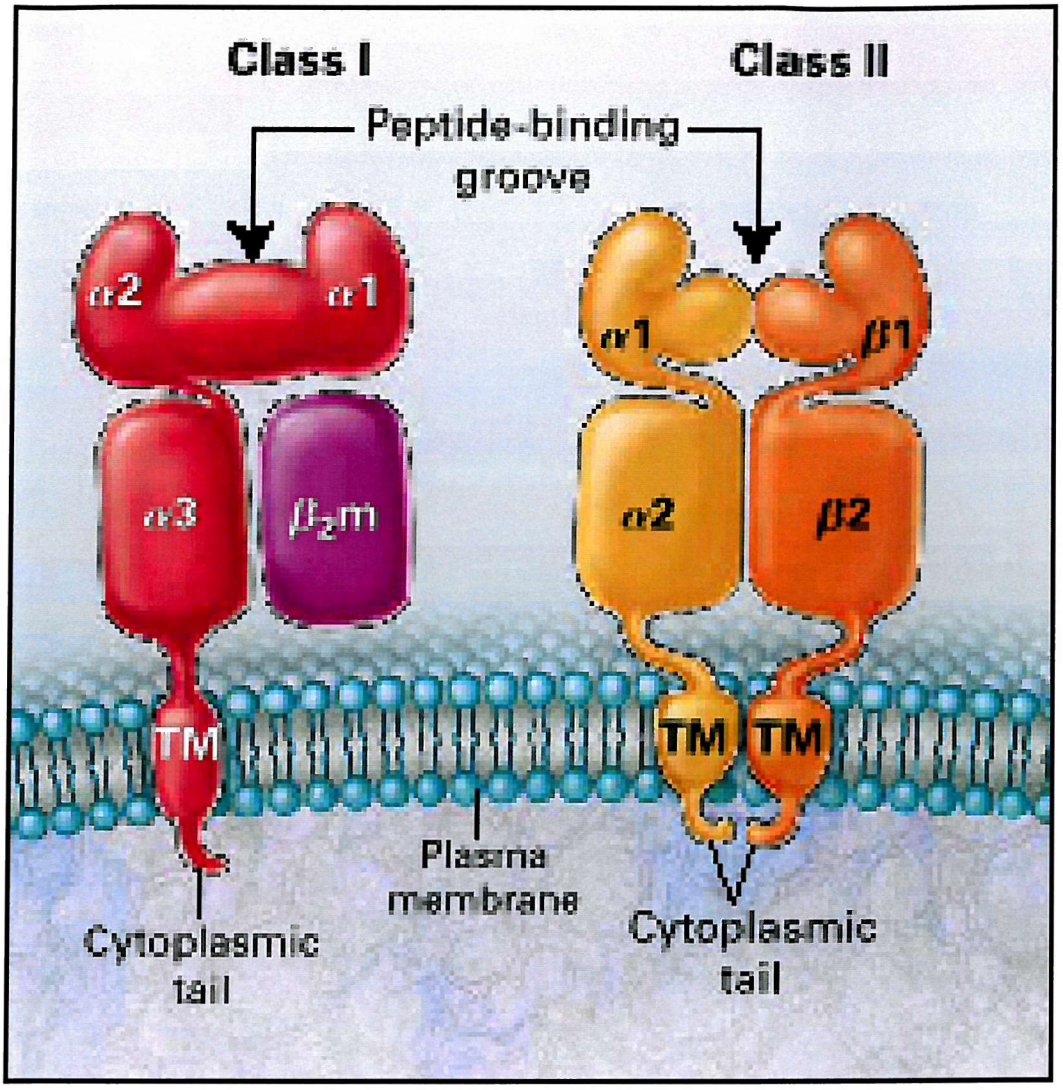

Figure 1.12 Structure of HLA Complex molecules *(Reproduced from Klein, NEJM 2000)*

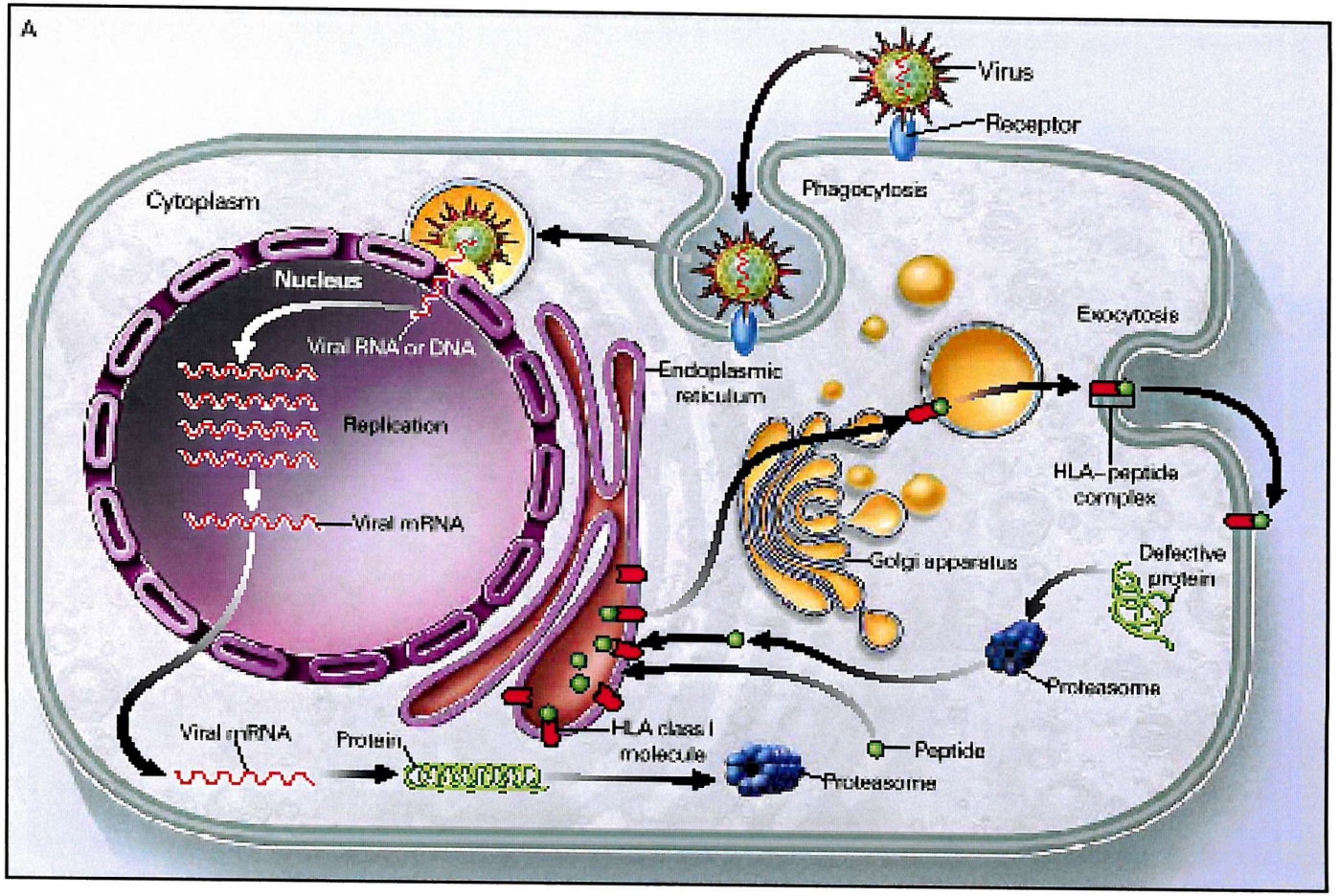

Figure 1.13 MHC class I antigen processing *(Reproduced form Klein, NEJM 2000)*

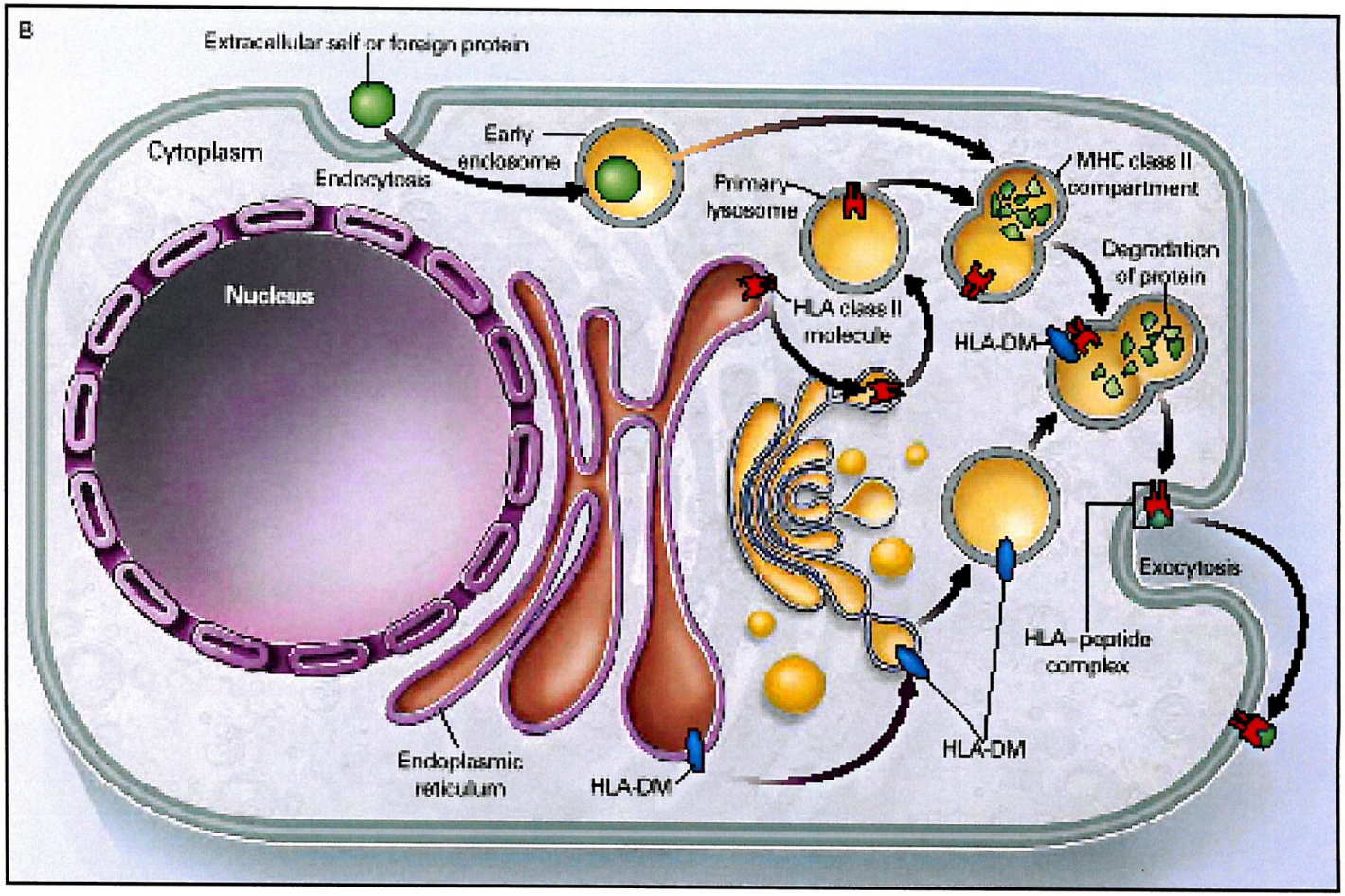

Figure 1.14 MHC class II antigen processing *(Reproduced form Klein, NEJM 2000)*

1.10 The Immune Response in Hepatitis C

Acute hepatitis C virus (HCV) infection is spontaneously cleared by immune mechanisms in 15% of patients. The 85% who fail to clear the virus are left with chronic infection. A persistent immune response to the virus is believed to be responsible for the resultant liver damage. The small percentage exposed to HCV who spontaneously clear the virus, and the heterogeneity of the severity of liver disease in chronic infection, suggests that the immune response to HCV varies enormously between individuals *(Rehermann 1999)*.

1.10.1 The B Cell Response in HCV Infection

Following acute infection with hepatitis C virus the body mounts a humoral response, regardless of the eventual outcome. The first detectable antibodies are to the structural proteins of the virus (nucleocapsid and envelope), followed much later in the infection by antibodies to one or more of the immunogenic non-structural proteins. The antibodies are not neutralising or protective. A patient who has cleared the virus following acute infection, will not necessarily by protected upon further viral challenge. Similarly, attempted neutralisation of a viral inoculum by pre-culture with HCV specific antibodies will not prevent infection.

The mechanisms behind the inability to produce neutralising antibodies to HCV are not clear. It may be that the virus mutates to avoid antibody neutralisation, or that the viral proteins responsible for cellular invasion are non-immunogenic, allowing the virus to reach intra-cellular sanctuary with impunity *(Farci 1994; Shimizu 1994; Farci 1996; Geissler 1997; Chen 1999)*.

Anti-HCV antibodies are detectable in all patients with chronic infection, at higher levels than HCV RNA negative individuals. There is some evidence to suggest that if the infection has been resolved spontaneously, detectable antibodies can disappear from the serum. This is difficult to investigate and, if true, may have skewed the epidemiological data for the rate of spontaneous resolution of infection *(Takaki 2000)*. It is possible that the early activation of the antibody response may be protective in some individuals, but there is no evidence to support this at present. Indeed, previous spontaneous resolution of HCV infection with residual anti-HCV antibodies does not necessarily protect from future HCV challenge, suggesting that the antibody response alone may not be sufficient to combat acute infection.

1.10.2 T Cell Responses in HCV Infection

1.10.2.1 $CD4^+$ T Cells in HCV Infection

Antigen specific proliferation and cytokine production have been used as the primary markers of Th ($CD4^+$) lymphocyte reactivity. For these assays, peripheral blood mononuclear cells are isolated and then cultured in the presence or absence of recombinant HCV proteins. The cytokine production is assessed using either flow cytometry, enzyme linked immuno-spot assay or enzyme linked immunosorbent assay of conditioned cell supernatants. Proliferation data is retrieved from parallel cultures to the cytokine assays, using tritiated thymidine uptake as a surrogate marker of new DNA formation. Similar experiments have been conducted using intra-hepatic lymphocytes, but these studies are generally flawed due to the need to expand T cells numbers using mitogen stimulation prior to antigen specific assays *(Minutello 1993; Bertoletti 1997)*.

Using these techniques, it has been shown that during the first 4 weeks of infection it is possible to detect significant $CD4^+$ responses in most patients. These responses are most often directed against the non-structural proteins NS3 and NS4, and core antigen. Most studies of cytokine profiles reveal a predominance of interferon-γ production. After 4 weeks, most studies demonstrate a divergence in magnitude and breadth of the responses amongst those that achieve and maintain viral clearance and those developing chronic infection. There are a group of patients who initially appear to have cleared the virus, but the viraemia returns after several months, associated with a gradual loss of HCV specific $CD4^+$ responses *(Diepolder 1995; Diepolder 1997; Gerlach 1999; Naoumov 1999)*.

Viral clearance is associated with vigorous, sustained proliferative responses to multiple hepatitis C proteins and MHC class II restricted epitopes. These responses are maintained for many months after the acute infection, and for years in some individuals. The $CD4^+$ response associated with viral clearance is of a Th1 phenotype. Chronic infection is characterised by a gradual loss of HCV specific $CD4^+$ proliferative responses. Within 6 months of acute infection, there are often no detectable $CD4^+$ responses using standard assays. If responses are maintained, they tend to be less vigorous than in resolved infection, and directed against at most

one HCV antigen. This is a HCV specific effect as Th responses to other antigens, such as influenza and tetanus, are maintained *(Diepolder 1995; Diepolder 1997; Cramp 1999; Gerlach 1999; Naoumov 1999; Pape 1999).*

Although HCV specific Th cell proliferation is lost as chronic infection becomes established, there is often still a significant cytokine response. Surprisingly, it has not been possible to demonstrate clear and convincing differences in cytokine production induced by HCV antigens between those with chronic and resolved infection. It is likely that persistent antigen driven cytokine production by $CD4^+$ cells in the livers of patients with chronic hepatitis C infection may contribute to the inflammatory cell recruitment and hepatocellular damage seen in this disease. However, it has been difficult to obtain liver infiltrating $CD4^+$ lymphocytes in significant numbers to allow this hypothesis to be properly investigated *(Minutello 1993; Bertoletti 1997; Dumoulin 1997; Nuti 1998).*

1.10.2.2 $CD8^+$ T cells in HCV Infection

Antigen specific cytotoxicity and cytokine production are the standard methods for monitoring $CD8^+$ T cell activity in hepatitis C infection. Cytotoxicity is usually measured by quantifying the ability of $CD8^+$ cells to kill antigen loaded, HLA matched B cells. This is assessed at different ratios of cytotoxic to target cells and titration of peptide antigen, and most often uses radio-labeled chromium release as the read-out. Flow cytometry can be used to examine the number of cells expressing perforin on the cell surface, a marker of cytotoxic potential. Interferon-γ production is assessed using enzyme linked immuno-spot assay or flow cytometry.

It is difficult to obtain $CD8^+$ cells in significant numbers from the liver although there is a body of literature using mitogenic expansion of intra-hepatic $CD8^+$ cells followed by antigen specific assays *(Nelson 1997; Giuggio 1998).* Mitogenic stimulation of these intra-hepatic cells prior to use in assays has cast doubt upon their validity, and so peripheral blood mononuclear cells are now most often used for these studies.

HCV specific $CD8^+$ cells are at low frequency in peripheral blood and clonal expansion is required to have enough cells to study. Interleukin-2 and 7 are used to stimulate epitope specific cells to proliferate in the presence of an appropriate MHC class I restricted peptide. Bulk cultures are expanded using these methods and then

feeder cells loaded with the appropriate peptide are also used to increase clonal numbers further. Many attempts at clonal expansion will be unsuccessful as it is impossible to predict with certainty which epitopes will be recognised by a given individual *(Hiroishi 1997)*.

Patients who have spontaneously resolved infection with HCV, have strong cytotoxic responses to multiple HCV epitopes. In addition, they produce interferon-γ in response to the same peptides. Specific cytotoxicity can be demonstrated at relatively low effector to target cell ratios *(Christie 1998)*. A study by Chang *et al.* recently suggested the opposite of this, that $CD4^+$ responses were maintained after viral clearance, but that memory $CD8^+$ HV specific cells were lacking *(Chang 2001)*. Analogous with the $CD4^+$ responses in chronic hepatitis C infection, cytotoxic responses in the $CD8^+$ cells are weak or absent in the patients who have not achieved sustained virological clearance. Cytotoxic responses are not only weaker, requiring higher effector to target cell ratios, but they are directed against fewer epitopes then in resolved acute infection. This inadequate cytotoxicity appears to be virus specific, as responses to other antigens is maintained i.e. EBV and influenza virus. Interestingly, there is little difference in the number of cells that will produce interferon-γ between the two groups of patients, suggesting that there are virus specific cytotoxic T cells in chronic hepatitis C, but that they have impaired capacity to kill. As with Th cells, this may mean that there are cytokine producing cells in the liver of chronically infected patients contributing to inflammatory cell recruitment and hepatocellular damage *(Liaw 1995; Hiroishi 1997; Koziel and Walker 1997; Nelson 1997; Giuggio 1998; Jackson 1999)*.

Finally, there have been some studies to suggest that the core protein of HCV has immunomodulatory effects. Preliminary work has shown that the virulence of vaccinia viruses in mice can be increased by the addition of an HCV core protein construct into the vaccinia genome. It is thought that the reason for this increased virulence is through failure of induction of cytotoxic T cells in the presence of the HCV core protein, although the mechanism for this is not clear *(Large 1999)*.

1.10.2.3 Dendritic Cells in HCV Infection

There are clear differences in the Th and Tc responses in chronic hepatitis C compared with spontaneously resolved HCV infection *(Christie 1998; Cramp 1999; Takaki 2000)*. The apparent deficiency in these responses appears to be virus specific and does not represent a global immunosuppression. There is evidence that HCV core protein is immunomodulatory, but this is not significant in all cases, as 15% of exposed individuals can clear the virus without treatment *(Large 1999)*. It is possible therefore, that the underlying problem in chronic hepatitis C is with antigen presentation, and dendritic cells are the most important cells involved in this process. Much of the investigation of dendritic cell function to date has used cultured dendritic cells. These are isolated from peripheral blood and cultured for 2 weeks in the presence of interleukin 4 and granulocyte macrophage colony stimulating factor (GM-CSF) to preferentially mature resting dendritic cells and inhibit granulocyte maturation prior to using them in functional assays. The functional assays are designed to look for augmentation of antigen specific Th responses in the presence of antigen pulsed, irradiated dendritic cells, or to look at dendritic cell ability to stimulate allogeneic T cells in a mixed leukocyte reaction. It has been shown by several groups that the allostimulatory capacity of dendritic cells from CHC patients is impaired when compared with healthy controls *(Kanto 1999; Bain 2001)*. This impaired allostimulatory capacity is only seen in CHC patients with on-going infection, as normal allostimulatory capacity appears to be restored following anti-viral treatment to achieve viral clearance. This phenomenon may be due to restoration of a normal maturation response in dendritic cells to standard maturation stimuli *(Auffermann-Gretzinger 2001)*.

There is evidence to suggest that dendritic cells themselves are subject to infection with HCV virus *(Bain 2001)*. In addition, there is evidence that expression of HCV proteins (HCV core-E2) by murine dendritic cells, will reduce their capacity for allostimulation of T cells in a mixed leukocyte reaction (when compared with dendritic cells transfected with a non-HCV protein containing plasmid) *(Hiasa 1998)*. Histological evidence shows that there are many dendritic cells within the HCV infected liver, and that these cells are in close contact with CD8+ T cells and hepatocytes. They are found around the portal tracts and out in the liver

parenchyma beyond the limiting plate. This evidence supports a leading role for dendritic cells in the organization and recruitment of the inflammatory response in the liver in CHC *(Galle 2001)*.

There are obvious limitations to using cultured rather than freshly isolated dendritic cells and the results of assays using fresh cells are eagerly awaited.
It is likely that dendritic cell function is impaired in chronic hepatitis C infection, but whether this is due to an existing fault in the host cells, or a virus specific effect is unclear. There could be abnormalities in antigen processing for presentation, dendritic cells maturation or in co-stimulation during dendritic cell / T cell interactions. Work in this field is emerging and not yet conclusive.

1.10.2.4 HLA Associations

The HLA complex is used to present epitopes from invading organisms to the immune system. Other viral infections have clear HLA associations with outcome of infection, and similar associations have been sought in hepatitis C infection *(Thursz 1995)*.
HLA alleles have predetermined peptide binding specificities, and some alleles may be better at binding HCV derived epitopes for presentation on the cell surface. This may lead to different T cell responses between individuals, and a variable chance of clearing the infection acutely.
In hepatitis C, there do not appear to be any MHC class I associations with disease outcome, but there are some class II candidates. The class II allele DQB1*0301 has been shown to be over-represented in those individuals who have spontaneously cleared infection when compared with the background population. This finding has been reproduced in several populations with different genetic backgrounds, and it is therefore likely that this allele is truly protective from chronic infection with hepatitis C. Another possible allele involved with protection from chronic infection is HLA DRB1*1101, although evidence for this is less conclusive *(Tibbs 1996; Alric 1997; Diepolder 1999; Thursz 1999; Tillmann 2001)*.

1.11 Mechanisms of Immune Evasion in HCV

Hepatitis C virus has employed many diverse mechanisms to avoid elimination by the host immune system. An attractive hypothesis in immune evasion is believed to be escape mutation. The HCV RNA dependent RNA polymerase lacks a proof-reading capacity, leading to multiple mistakes in the replication process. Many errors will be fatal, meaning that the resultant proteins are not functional and are unable to sustain viral replication, but a small proportion of the mutations will be successfully incorporated into the viral genome. Through this gradual process of mutation it is possible for the virus to positively select mutations that are less well recognised by the immune system, thus increasing chances of survival. In any chronically infected individual it is possible to identify multiple quasispecies of the virus, representing a gradual drift away from the sequence of the original infecting virions. There will usually be a dominant strain of the virus, but this can quickly change if the immune system adapts *(Cooreman and Schoondermark-Van de Ven 1996; Christie 1999; Rosenberg 1999).*

In addition to the evolution of epitopes not recognised by the immune system, there may be a further advantage to mutation. It is believed that some HCV epitopes can have an amino acid substitution at a critical point that does not interfere with HLA binding, but induces anergy in a T cell when the T cell receptor binds to the peptide / MHC complex. The effect of this would be to prevent T cell mediated cytotoxicity and reduce further inflammatory cell recruitment *(Rosenberg 1999; Wang and Eckels 1999).*

There is some evidence that the core protein of hepatitis C is immunomodulatory. Core protein expressed in a recombinant vaccinia virus (rVV) construct will render the rVV much more virulent to mice. This is thought to be because it somehow inhibits the cytotoxicity of $CD8^+$ T cells. This effect is specific to the core protein of HCV and is not seen with other structural or non-structural HCV proteins *(Large 1999).*

Preliminary data emerging in the literature suggests that HCV may have an effect on dendritic cells, interfering with antigen presentation and T cell priming and activation. Mechanisms involved in these pathways are not yet clear *(Kanto 1999; Auffermann-Gretzinger 2001; Bain 2001; Sarobe 2002).*

Using the signaling pathway of the TNF-α superfamily receptors, HCV seems to

be able to make infected cells resistant to apoptosis. This is not specifically immune evasion, but does help to promote viral persistence *(Marusawa 1999)*.
Utilising all of these mechanisms, and others yet to be elucidated, HCV seems to be very successful at ensuring that it can evade the hosts natural immune responses.

1.12 The Immune Response and Fibrosis

Infiltration of the liver by inflammatory cells can cause massive hepatocyte damage, but in chronic hepatitis C infection the necrosis and apoptosis are usually low grade. In HCV the most troublesome pathological finding in the liver is the abnormal matrix deposition, or fibrosis, associated with the inflammatory lesion. As the disease progresses it is the degree of fibrosis that dictates the clinical outcome, and many of the complications that ensue *(1994; Ishak 1995; Di Bisceglie 1998; Poynard 2000)*.
There is evidence in non-hepatic fibroblast systems that there can be direct cell to cell interactions between lymphocytes and fibroblast-like cells *(Rezzonico 1998)*. To date, there is no definitive evidence that this occurs in the liver with hepatic stellate cells (HSC), but activated HSC do express molecules that would facilitate such interactions, i.e. ICAM-1, class II MHC, CD40 *(Hellerbrand 1996; Bataller 2000; Schwabe 2001)*.
Some cytokines are implicated in modulating the activation and proliferation of HSC *(Friedman 1996; Friedman 1999; Maher 1999)*. Thus, the phenotype of the immune response may influence the degree of fibrosis. Manipulation of the Th1/Th2 balance in animal models of liver fibrosis has confirmed this theory, although the system is very complex and multifactorial. In addition, introducing exogenous cytokines can affect the outcome of such experimental models *(Wynn 1995; Baroni 1996; Shi 1997)*.
Generally, cytokines can be grouped as pro- or anti-fibrotic in their effects on fibro- and myofibroblasts *(Friedman 1999)*(Table 1.02).

Pro-fibrotic Cytokines	Anti-fibrotic Cytokines
Transforming Growth Factor-β1 Tumour Necrosis Factor-α* Interleukin-4, -6, -13 Platelet Derived Growth Factor Epidermal Growth Factor	Interleukin-10 Interferon-α, –β, –γ Tumour Necrosis Factor-α*

Table 1.03 The effects of cytokines on fibroblasts and myofibroblasts

*Tumour necrosis factor-α (TNF-α) appears on both sides of the table, as it has both pro- and anti-fibrotic properties. The direct effect of TNF-α is to reduce collagen synthesis, yet it is a major mediator of the inflammatory response, stimulating production of pro-fibrotic cytokines. Also, TNF-α can induce apoptosis of fibrogenic and inflammatory cells. Thus, the fibro-active effect of TNF-α is dependent on the type of cell that is the dominant responder, and how this cell responds *(Friedman 1999)*.

It is clear that there are multiple mechanisms through which fibrogenesis can be initiated by the immune system. Immune responses may be one of the triggers for HSC activation, or they may just play a role in modulating activation once it has occurred.

1.13 Manipulation of the Immune Response

The immune response in acute hepatitis C appears to dictate the outcome of the infectious challenge. In chronic disease, the immune response may be responsible for the severity of the resultant liver damage. Careful manipulation of the immune system in both acute and chronic infection may therefore be advantageous *(Rehermann 1999)*.

1.13.1 Cytokines

It is recognised that a vigorous Th1 response is associated with viral clearance, and that certain cytokines will favour the development of a Th1 phenotype i.e. interleukin-12 and interferon-γ *(Woitas 1997; Koziel 1999; Rehermann 1999)*. In addition, Th1

cytokines favour an anti-fibrotic effect in the liver, whilst a Th2 response is pro-fibrogenic *(Friedman 1999)*.

The only cytokine regularly used in the treatment of HCV infection is interferon-α, and this is mainly recognised for its anti-viral effects rather than any bias it may exert on the polarisation of the Th response. An apparent consequence of this treatment in chronic disease is the anti-fibrotic action, which is probably due to a direct effect on the cells involved in matrix remodeling *(Sobesky 1999; DuFour and Kaplan 2000; Poynard 2000)*.

Theoretically, exogenous IL-12 would promote a Th1 response and aid viral clearance, whilst IL-10 would dampen the immune response, encouraging viral persistence but reducing liver fibrosis in chronic disease. A pilot study and a small randomised control trial of 2 doses of IL-10 in chronic hepatitis C, did show noramlisation of ALT, and improvement of inflammation and fibrosis on liver histology after 3 months treatment. As predicted, there was no change in viral RNA levels *(McHutchison 1999; Nelson 2000)*. Currently, there is a lack of well-constructed trials investigating these effects.

1.13.2 Vaccines

Vaccination allows manipulation of the immune response by selective priming. This may be used for prevention of infection in HCV negative individuals, or to aid viral clearance once persistence is already established.

Unfortunately, there is not an effective vaccine for HCV at present although this is an area of intense investigation. Studies of cytotoxic T cells will allow selection of epitopes that reliably induce potent responses for inclusion in a vaccine.

Traditional vaccine strategies involve inoculation with an antigen, collection of antigens or whole pathogen in an attenuated form. This is effective if the epitopes included in the vaccine will result in the development of protective immunity. In HCV infection, there are no universal antigens which will provide such a response in all individuals, and other strategies must therefore be investigated *(Cooreman and Schoondermark-Van de Ven 1996; Rosenberg 1999)*.

DNA vaccines hold promise for future HCV vaccine development. In this case, the host is inoculated with a plasmid containing DNA coding for the epitopes of interest. The plasmid is taken up into muscle cells, which then translate the coded

peptide(s) and manufacture them through the cells own system. This mimics the actual production of the viral epitopes in an infected individual and can allow more efficient priming of the immune response. The advantage of the DNA vaccine system is that other DNA fragments can be included with the viral epitopes of interest. This will allow inclusion of either an "alert" signal for the immune response (a well recognised immunogenic epitope unrelated to HCV), or perhaps a cytokine such as IL-12 which would serve to bias the priming of the immune response to Th1 *(Geissler 1997; Ishioka 1999; Vidalin 1999).*

1.14 Treatment in Chronic HCV Infection

1.14.1 Supportive

HCV infection is usually silent, and the diagnosis is often made as an incidental finding during investigation for other or non-specific symptoms. As a result, most patients with chronic HCV infection do not receive any specific treatment. If they have slowly progressive liver disease, then they will probably die with HCV rather than as a result of its complications. More aggressive disease usually presents at the time that cirrhosis is already established, with a complication such as variceal bleeding. Even patients with known HCV infection are often managed with supportive therapy only, and specific anti-viral therapy is currently only available to a minority *(Di Bisceglie 1998; Alter 1999; Davis 2000).*

Supportive therapy in chronic HCV is dependent on the severity of the liver disease. Mild disease usually requires monitoring only, with a liver biopsy done at various intervals depending on the rate of progression. More severe disease is managed according to the resultant complications i.e. banding of varices, maintaining good nutrition, draining ascites *(Yousuf 1992; Saadeh 2001).*

1.14.2 Interferon-α Therapy

Interferon-α (IFN) is currently the mainstay of treatment for HCV infection, acute and chronic. Although it is a cytokine, its major effect is as a very potent anti-viral agent. As a sole agent in chronic infection, the efficacy of IFN 3MIU thrice weekly is around 15% sustained virological clearance. Around double this figure are negative for viraemia at the end of treatment, but relapse occurs within the subsequent 6

months in half. Despite these disappointing figures, there are other benefits in those receiving treatment, such as normalisation of ALT and improvement in liver fibrosis *(DuFour and Kaplan 2000; Shindo 2001)*. However, if HCV infection is identified in the acute phase and treated promptly with IFN therapy, then sustained viral clearance can be achieved in up to 90% of patients *(Jaeckel 2001)*.

Recently, the addition of a polyethylene glycol (PEG) moiety to the IFN molecule has allowed the pharmacokinetics of the drug delivery to be greatly improved. PEG is inert in the body, and is only very slowly cleared by the liver and kidney. The addition of the PEG moiety effectively makes the whole molecule "slow release", as it is cleared from the injection site more slowly than free IFN and then eliminated much more slowly. The addition of the PEG moiety does not affect the ability of the active sites of IFN to bind. These compounds can now be given once weekly, and do not have such dramatic peaks and troughs in serum IFN levels. This means that there are no IFN free periods when the virus levels can recover. Results for efficacy of PEGylated IFNs are encouraging, with around 30% sustained virological clearance and improved anti-fibrotic potential *(Heathcote 2000; Zeuzem 2000)*. Another potential explanation for this improved efficacy is that PEGylation may alter the tissue distribution of interferon by enhancing uptake in the liver, possibly in Kupffer cells *(Yamaoka 1994)*.

There is a problem with IFN resistance in HCV infection, coded for by a mutation on the NS5 viral protein. This mutation is most commonly found in HCV genotype 1, and patients with genotype 1 infection have the poorest response rates to all IFN based therapies *(Simmonds 1997)*.

1.14.3 Interferon-α in combination with Ribavirin

Ribavirin is a nucleoside analogue with a potentially broad range of anti-viral activity. It was originally developed for use in HIV infection, but early promise was not fulfilled in this area. Ribavirin as a sole agent in chronic hepatitis C infection is ineffective *(Lee 1998; Zoulim 1998)*.

Ribavirin in combination with IFN therapy achieves sustained virological response rates of around 40%. Response rates vary according to age, sex and viral genotype, and the young female patient with genotype non-1 may have up to an 80% chance of sustained virological clearance with this combination of drugs *(McHutchison*

1998; Poynard 1998).

As the response rates to IFN have improved following PEGylation, so the response rates to combination therapy using PEGylated IFN have also improved. The sustained virological response rates with this combination are around 55-56% *(Fried 2001; Manns 2001; Manns 2001).* As with standard combination therapy, HCV genotypes 2 and 3 are more sensitive to treatment and youth and female sex are favourable predictors of response.

It is interesting that although ribavirin is a nucleoside analogue with anti-viral properties, it is not through these mechanisms that it exerts its effects in chronic hepatitis C infection. It is not fully understood, but evidence suggests that ribavirin increases IL-1 levels and biases the immune response towards the more favourable Th1 phenotype, and this is its major effect in the treatment of HCV *(Hultgren 1998; Ning 1998).*

There has been some investigation of the $CD4^+$ response during treatment with combination therapy. Cramp et al. studied *in vitro* T cell responses in $CD4^+$ cells during treatment and found that they were enhanced by treatment with combination IFN and ribavirin therapy. In this study, those who eventually achieved sustained virological clearance developed enhanced responses similar to those seen following spontaneous acute viral clearance. These enhanced responses were sustained following suspension of treatment *(Cramp 2000).*

1.14.3 Transplantation

In severe HCV related liver disease, the anti-viral therapies are often not well tolerated as they can have serious side effects. In this case the treatment is supportive whilst the complications are manageable. Unfortunately many patients with HCV related cirrhosis die of their disease, but a few will be offered liver transplantation. HCV is the leading indication for liver transplantation in the western world, but graft numbers are limited and therefore not available to all. There is extensive physical and psychological testing to ensure that a patient is fit for the rigors of an orthotopic liver transplant and the subsequent immunosuppressive therapy *(McCaughan 1993; Sherlock 1994; Di Bisceglie 1998).*

Following liver transplantation for HCV related cirrhosis, the graft will invariably become infected with the virus. The immunosuppression given to prevent graft

rejection will have the unfortunate side effect of accelerating the HCV related liver damage in the new graft. IFN therapy cannot be used following liver transplantation as it may induce graft rejection, and so post-transplant hepatitis C is managed supportively. The ultimate effect of the HCV infection in the new liver will be to cause a recurrent cirrhosis, but hopefully several years with a good quality of life will pass before this happens *(Sherlock 1994; Rosen and Martin 2000).*

Chapter 2

Chapter 2

Materials and Methods

2.1 Identification and Classification of Subjects

2.1.1 Recruitment of Subjects

2.1.1.1 Healthy Controls

a) Cellular Assays:

Healthy controls for the cellular assays were identified from volunteers within the research unit and medical school. A brief history was obtained to ensure that they had a low risk of hepatitis C or HIV infection, and no other contra-indication to donating up to 60ml of blood. All subjects gave verbal informed consent and understood the nature of the assays to be undertaken. It was felt unethical to test for evidence of hepatitis C or HIV exposure.
All phlebotomy was undertaken in a designated clinical area with resuscitation equipment available.

b) Genetic Testing:

Healthy controls for the genetic susceptibility study were identified from a pool of local blood donors. A cohort of 300 hepatitis C negative volunteers were consented and bled by the blood transfusion service. This cohort of patients serves as an indicator of the frequency of Human Leukocyte Antigen (HLA) markers within our local population.

2.1.1.2 Chronic Hepatitis C Subjects

Subjects with chronic hepatitis C infection were identified and recruited to the studies via the hepatology out-patients clinics. The patients were bled either at the time of attendance to the clinic, or when they were admitted to the ward for clinical

assessment by liver biopsy. Each patient was verbally consented, given a written information sheet and asked to sign a printed consent form before they were recruited into the study. No coercion or financial incentive was offered. All HCV RNA positive patients attending the clinic were considered, and approached only if felt appropriate by the attending physician.

An additional cohort of patients with mild liver disease was identified in the Cambridge Hepatology Out-patients clinic by Dr Graeme Alexander. These patients were consented and recruited to hepatitis C genetics studies in accordance with local ethical committee rules. Dr Alexander kindly agreed to their inclusion in the present study and provided genomic DNA from each patient together with appropriate clinical details.

Patients in the treatment monitoring study were recruited after they had been identified for treatment of chronic HCV.

2.1.1.3 Acute Resolved Hepatitis C Subjects

Healthy controls with evidence of HCV exposure, but no chronic infection were identified via several routes. As the spouse of a chronically infected patient, after referral to the hepatology out-patient clinic for assessment or when attending the haematology clinic for management of haemophilia. All of these patients were HCV antibody positive but HCV RNA negative on at least 2 occasions. The acute resolved patients underwent the same consent procedure as those with chronic HCV infection.

2.1.2 Classification of Chronic Hepatitis C Patients

2.1.2.1 Liver Biopsy

Most of the chronic hepatitis C patients involved in the study had a liver biopsy either at the time of inclusion to the study, or within the last 5 years. The liver biopsies were taken for clinical assessment by either a percutaneous, transjugular or intra-operative route. The samples were fixed in formalin, paraffin embedded and submitted for histological assessment. An experienced liver histopathologist examined each of the liver biopsies.

A few chronic HCV patients did not have a liver biopsy because they had a

significant contra-indication to liver biopsy ie. coagulopathy and thrombocytopaenia. These patients had a clinical diagnosis of severe liver disease, or cirrhosis (Child-Pugh B or C).

2.1.2.2 Histological Scoring System for Liver Biopsy

The Southampton Pathology Department has developed a liver biopsy scoring system for use on local specimens, the IPA score. It is a simplified 3 point system based on the Metavir score *(Bedossa and Poynard 1996)*. There has been a period of validation for this scoring method. The manuscript to publish this data is currently in preparation by Dr. EJ Williams and Dr. GH Millward-Sadler.
The IPA score is assessed using standard haematoxylin and eosin stained sections, in combination with a reticulin stain to assess architecture. Each of the 3 components is scored on a scale from 0-3, with 0 representing no detectable changes.

The "I" score is a measure of "Inflammation", or portal tract cellularity. It assesses the number chronic inflammatory cells infiltrating the portal tract and interface hepatitis.

The "P" score is a measure of "Parenchymal Damage". It assesses the degree of inflammatory infiltration of the liver lobule, with accompanying hepatocellular damage, particularly necro-inflammatory foci and apoptotic bodies.

The "A" score is a measure of "Architectural" disturbance. It assesses the amount of abnormal fibrotic tissue.

The final score is represented as the 3 individual scores given in strict IPA order ie. 3,3,2.

2.1.2.3 Severity of Liver Disease

For the purposes of the present studies, the patients were classified with mild, moderate or severe chronic hepatitis C disease. The final classification depends upon the total of their IPA score when all 3 components are summed.

IPA 0-3 = Mild disease

IPA 4 = Moderate disease

IPA 5-9 = Severe disease

This combined classification has been substituted for stratification according to individual components of the score where appropriate. e.g. for fibrosis:

I score 0-1 = Mild fibrosis

I score 2 = Moderate fibrosis

I score 3 = Severe fibrosis

2.1.3 Gathering of Information

Clinical information on all subjects was gathered by direct questioning. Additional details on the hepatitis C patients were obtained from the medical records and computerised pathology record systems. The patients all gave consent to examination of their records for information relevant to the study.

2.1.3.1 Healthy Controls

Age and racial origin was recorded in all healthy controls. For the cellular studies, the subjects were excluded if they thought they might have been exposed to HCV infection. The blood transfusion cohort was known to be HCV antibody negative.

2.1.3.2 Hepatitis C Positive Subjects

Information Recorded:

Age and racial origin was noted. In addition, the following information was recorded when available:

Route and duration of infection

Alcohol intake, including current or previous periods of excess consumption

Previous and current treatment

Concurrent medical conditions

Liver biopsy IPA score and evidence of other liver pathology

Liver biochemistry, including ALT, Albumin and bilirubin

Blood clotting

Serum iron and total iron binding capacity

Result of HIV testing (positive results excluded patients from the study)

2.1.3.3 Database

The information gathered on the hepatitis C positive subjects was entered into an access database for analysis. The database was designed to allow identification of study sub-groups according to any of the criteria collected. The database held all of the demographic information, together with the results of the genetics study.

2.2 Human Leukocyte Antigen (HLA) Typing

2.2.1 Collection and Transportation of Blood

Venous blood for HLA typing was taken in an appropriate clinical area using aseptic technique using universal precautions. The blood was collected in commercially prepared tubes containing either sodium citrate or EDTA to prevent clotting (Vaccutainer).

The blood tubes were labelled for identification and transferred to the category 2 laboratory in a sealed polythene bag. If blood was transferred from outside Southampton General Hospital, the sealed bag was placed in a sealed hard plastic container, which was clearly marked to indicate the infectious nature of the contents. The blood tubes were placed vertically in a rack in a safe area for 24 hours to allow settling of the blood cells into a red cell base, overlying white cell rich buffy coat layer and serum layer.

2.2.2 Genomic DNA Extraction and Purification

A rapid salting out technique was used to extract DNA from the whole blood samples.

2.2.2.1 Materials

Red cell lysis buffer {0.32M Sucrose, 1% Triton X-100, 5mM $MgCl_2.6H_20$, 12mM Tris-HCl; pH7.5}

5x Proteinase K buffer {0.375M NaCl, 0.12M EDTA; pH 8.0}

10mg/ml Proteinase K (Sigma) in TE Buffer

6M NaCl

70% Ethanol

10% SDS

TE buffer {10mM Tris-HCl, 1mM EDTA; pH 7.5}

2.2.2.2 Extraction Method

1 ml of blood, including the white cell enriched buffy coat layer, was harvested from the standing EDTA or citrate sample. It was vortexed thoroughly with an equal volume of red cell lysis buffer. The residual cellular debris was centrifuged at 13000rpm for 5 minutes, washed in double distilled water (ddH_2O) and then centrifuged again. The cell pellet was then resuspended in 220µl ddH_2O, 40µl 10% SDS, 80µl 5x Proteinase K buffer, 30µl Proteinase K 10mg/ml and incubated at 55^0C for 30-60 minutes.

To precipitate out the unwanted debris, 200µl of 6M NaCl was added to the mixture, mixed thoroughly and then centrifuged at 13000 rpm for 5 minutes. The supernatant was then carefully decanted into a clean eppendorf. 1 ml of absolute ethanol was added to the supernatant, causing the DNA to precipitate out. The DNA was then pelleted by centrifugation and then washed and centrifuged again in 70% ethanol. Finally, the pellet was dissolved in ddH_2O by gently heating in a water bath to 37^0C for 30-60 minutes. DNA concentration and purity was evaluated using mass spectrometry. The DNA was then stored at -20^0C until required for further evaluation.

2.2.3 HLA Typing using Sequence Specific Primers for Polymerase Chain Reaction (SSP-PCR)

HLA typing using an SSP-PCR technique was undertaken by a colleague within the laboratory using samples I had collected and classified. In particular I was interested in the MHC class II allele, HLA DQB1*0301, and its relationship with viral clearance and severity of chronic infection.

2.2.3.1 Method

Briefly, SSP-PCR was performed using purified genomic DNA and allele specific primer pairs. A small amount of DNA was added to a reaction mix, containing a pair of control primers from a conserved DRB1 intron sequence and an allele specific pair of primers from the allele of interest. After DNA amplification by PCR, the products were resolved on an ethidium bromide prestained gel. The gel was then examined under ultraviolet light. The presence of one band indicated that the control had amplified successfully, and a second that the allele of interest was also present.

2.2.3.2 Analysis of Results

Results were analysed by several methods. The frequency of HLA DQB1*0301 was compared between the general local population and subjects with evidence of HCV exposure to look for increased disease susceptibility. In addition, patients with chronic infection and those with evidence of previous acute resolved infection were compared to reveal any protection from chronicity. Finally, allele frequency was compared between chronic HCV patients discordant for severity of disease to reveal any over-representation in mild disease.

2.3 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

2.3.1 Collection and Transportation of Blood

2.3.1.1 Materials

Heparin — Preservative free porcine intestinal heparin at 2500IU/ml in sterile phosphate buffered saline (*Sigma*)

2.3.1.2 Method

Venous blood for isolation if PBMC was taken in an appropriate clinical area using aseptic technique using universal precautions. 10-60ml of blood was collected into a pre-heparinised syringe (1250IU heparin per 60ml of blood) using a 22G needle. The blood was gently mixed to avoid clotting, labelled, and the needle exchanged for a

protective rubber cap. The blood was transported to the category 2 laboratory in a sealed hard plastic box.

2.3.2 Density Gradient Centrifugation

2.3.2.1 Materials

RPMI 1640	Cell culture medium, containing 2mM L-Glutamine, 80IU/ml Penicillin, 80IU/ml Streptomycin (Gibco Life Technologies)
Human AB Serum	(BioWhittaker)
Lymphoprep	Ficoll (Nycomed)

2.3.2.2 Method

The heparinised blood was diluted 1:1 with pre-warmed RPMI 1640 medium (37^0C) within 4 hours of collection. The blood mixture was then carefully layered onto 10ml of Lymphoprep in a 50ml falcon tube, maintaining a clear interface. The falcon was then centrifuged at 1650rpm for 30 minutes (brake off). The mononuclear cells were harvested from the interface between the Lymphoprep and serum using a plastic Pasteur pipette. The cells were washed twice in RPMI, each wash with a progressively slower centrifuge step to remove contaminating platelets.
Finally, the cells were resuspended in RPMI containing 5% human AB serum (complete medium) and counted using a haemocytometer. Cell concentration was adjusted to $2x10^6$/ml.

2.4 Lymphocyte Proliferation Assays

2.4.1 Optimisation of Conditions

2.4.1.1 Materials

96 well plate	MHVB S4510 Hydrophilic (Millipore)
Complete medium	RPMI/L-Glutamine/Penicillin/Streptomycin + serum
Human AB serum (HAB)	(BioWhittaker)

Foetal Calf serum (FCS)	(Gibco Life Technologies)
Freshly isolated PBMC	See *Density Gradient Centrifugation*
Phytohaemagglutinin	(Sigma)
HCV proteins	Recombinant Core, NS3, NS4, NS5, Helicase in buffer (Mikrogen, Germany)
HCV peptides	Core, NS3 in Dimethylsulphoxide (manufactured in house)
Influenza A antigen	
Tetanus Toxoid protein	(NIBSC)
Tritiated Thymidine	Methyl ^{3}H Thymidine (Amersham)
Vacuum manifold	For washing 96 well plates (Millipore)
Phosphate Buffered Saline	
Methanol	
Scintillant	
MicroBeta Plate Reader	(Wallac)

2.4.1.2 Method

Freshly isolated PBMC in complete medium at $2x10^6$ cells/ml were plated onto a 96 well plate, 100µl per well. The complete medium contained 5% FCS, 10% FCS, 5% HAB or 10% HAB. Each condition was repeated in triplicate. 100µl of assay medium was added to each well, consisting of complete medium with either no additive, phytohaemagglutinin, protein carrier buffer, DMSO, protein or peptide. Proteins were used at a final concentration in the wells of 1µg/ml, peptides at 20µg/ml and phytohaemagglutinin at 45µg/ml. The plate was covered and cultured in 100% humidity and 5% CO_2 at 37^0C for a variable period of time (2-8 days). Tritiated Thymidine was added for the last 16 hours of culture. Each well on the plate was then washed 3 times with phosphate buffered saline on the vacuum manifold, and fixed with 200µl of methanol for 10 minutes. The plate was washed a further 3 times with methanol and then dried. 20µl of scintillant was then added to each well and Beta emission was counted on the plate reader.

Stimulation index was calculated as a ratio of counts per minute in the presence of antigen over counts per minute with the appropriate control ie protein carrier

buffer for proteins and DMSO for peptides.
Conditions were optimised for length of culture period and concentration and nature of added serum.

2.4.2 Final Protocol for Lymphocyte Proliferation Assays

Following optimisation of conditions for the lymphocyte proliferation assay the final protocol used 5% human AB serum for the protein and 5% FCS for the peptide assays. The plates were cultured for 4-6 days prior to addition of tritiated thymidine. For some assays, in addition to phytohaemagglutinin as a positive control, influenza antigen and tetanus toxoid were also used. For the whole protocol see *Optimisation of lymphocyte proliferation assay.*

2.5 $CD4^+$ Enrichment / Depletion of PBMC for Lymphocyte Proliferation Assays

2.5.1 MACS bead positive selection of $CD4^+$ Cells

2.5.1.1 Materials

Freshly isolated PBMC	See *Density Gradient Centrifugation*
MACS buffer	PBS, 0.5% Bovine Serum Albumen, 2mM EDTA
MACS CD4 Microbeads	Miltenyi Biotech
MS^+/RS^+ Positive Selection column	Miltenyi Biotech
MACS Separator magnet	Miltenyi Biotech
RPMI complete medium	5% human AB serum in RPMI with glutamine, plus antibiotics

2.5.1.2 Method

Freshly isolated PBMC were washed and pelleted. The pellet was then resuspended in a small volume of MACS buffer as per the manufacturers instructions. CD4 MACS beads were incubated with the PBMC for 15 minutes at 6-12^{0}C. The cells were then washed in MACS buffer.
The positive selection column was placed in the magnetic field and primed with

MACS buffer and the cells were then loaded on in appropriate aliquots. The CD4 negative fraction was collected in a falcon tube from beneath the column.
When the column had finished draining the negative cells, it was removed from the magnetic field and the positive cells captured within the column were flushed out into a fresh falcon tube using MACS buffer.
Both fractions of cells were then washed and resuspended in RPMI complete medium at 2 million cells per ml for use in standard lymphocyte proliferation assays or supernatant collections.

2.5.2 FACS Analysis to Confirm Purity of $CD4^+/CD4^-$ Populations

2.5.2.1 Materials

Freshly isolated PBMC
Freshly isolated CD4 enriched and depleted fractions from MACS separation

FACS buffer	HBSS, 2% Foetal calf serum, 1% sodium azide
APC labeled anti-CD4	Becton and Dickinson
APC labeled IgG	Becton and Dickinson
PBS	

2.5.2.2 Method

The different cell fractions were washed in FACS buffer, then incubated in the dark for 30 minutes at 4^0C with 5µl of APC labeled anti-CD4 or isotype negative control IgG. The cells were washed again in FACS buffer, then resuspended in PBS. FACS analysis was undertaken on a FACScalibur flow cytometer, using FL4 channel to record the percentage of positively staining cells in each fraction.

2.6 Enzyme Linked Immuno-Sorbent Assays

All of the Enzyme Linked Immuno-Sorbent Assays (ELISAs) were undertaken using commercially available matched antibody pairs for a sandwich ELISA. Each assay was optimised for background staining and sensitivity.

Assay material was supernatant harvested from PBMCs established in parallel identical cultures to those in the lymphocyte proliferation assays. The supernatants were harvested at various time-points, often limited by the number of cells available to assay. The supernatants were harvested and stored at -20^0C until the ELISAs were undertaken. ELISAs using hepatic stellate cell supernatants were collected and stored at the time that the stellate cells were harvested for RNA extraction.
To avoid repeated freeze-thawing, all of the ELISAs on a given sample were undertaken in parallel.

2.6.1 Interleukin 2 (IL-2)

2.6.1.1 Materials

Capture Antibody	Mouse anti-human IL-2 mono-clonal antibody (R and D Systems)-used at 4μg/ml in PBS
Detection Antibody	Mouse Biotinylated anti-human IL-2 polyclonal antibody (R and D)-used at 25ng/ml in Assay Diluent
IL-2 Standard	Recombinant Human IL-2 (R and D Systems)-used at 2000pg/ml in Sample Diluent, with progressive 1:2.5 dilutions to 8.2pg/ml for the standard curve

Harvested supernatants
MaxiSorb ELISA plate (Nunc)
AnalR water (used to make all solutions)
Phosphate Buffered Saline (PBS)
Tris Buffered Saline (TBS-20mM Trizma Base, 150mM Sodium Chloride)

Blocking buffer	1% Bovine Serum Albumin (BSA), 5% Sucrose in PBS
Sample Diluent	10% FCS in RPMI 1640
Assay Diluent	0.1% BSA, 0.05% Tween 20 in TBS
Wash Buffer	0.05% Tween 20 in PBS

Streptavidin Horse Radish Peroxidase (Streptavidin HRP)-used at 1:3300 in Assay Diluent

Substrate Solution	1ml Tetramethylbenzidine Free base 1mg/ml in DMSO, plus 9ml Phosphate-Citrate buffer (0.05M Na_2HPO_4, 0.025M Citric Acid), plus 2μl Hydrogen Peroxide

Stop Solution 1.8N Sulphuric acid (H_2SO_4)

2.6.1.2 Method

100µl of capture antibody was pipetted into each of the 96 wells of the ELISA plate. The plate was sealed and incubated at room temperature overnight. Using an automated ELISA plate washer (Wolf Laboratories), the wells were aspirated and washed with wash buffer 3 times. Excess moisture was tapped out of the plate prior to the addition of 300 µl of Blocking buffer to each well. The plate was sealed and incubated for 1 hour at room temperature and then washed 3 times as before. 100 µl of assay supernatant or standard was loaded into each well, all conditions were repeated in triplicate (a known volume of sample diluent was added to achieve a final volume of 100 µl per well if limited supernatant was available). The plate was sealed and incubated at room temperature for 2 hours and then washed 3 times as before. 100µl of detection antibody was added to each well. The plate was sealed and incubated at room temperature for 2 hours then washed 3 times as before. 100µl of Streptavidin HRP was added to each well. The plate was sealed and incubated at room temperature for 30 minutes and then washed 3 times as before. 100µl of Substrate solution was added to each well. The plate was placed in the dark for 10-30 minutes to allow the colour reaction to develop. Before the negative on the standard curve began to change colour, the reaction was stopped by the addition of 50µl of stop solution. The optical density of each well was then determined using a microtiter plate reader set to 450nm with a reference filter at 570nm.
A typical plate layout is shown in figure 2.01.

A sample standard curve for IL-2 ELISA is shown in figure 2.02.

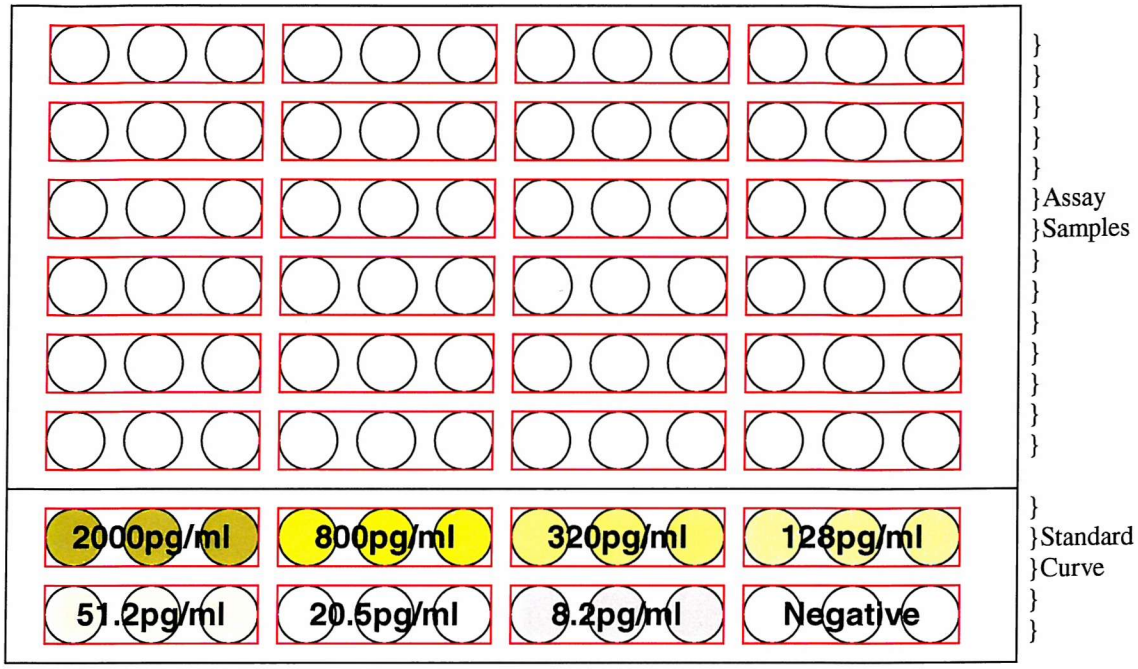

Figure 2.01 Standard plate lay-out for cytokine ELISA of PBMC supernatant

2.6.2 Interleukin-4 (IL-4)

2.6.2.1 Materials

Capture Antibody	Mouse anti-human IL-4 mono-clonal antibody (R and D Systems)-used at 4µg/ml in PBS
Detection Antibody	Mouse Biotinylated anti-human IL-4 polyclonal antibody (R and D)-used at 12.5ng/ml in Assay Diluent
IL-2 Standard	Recombinant Human IL-4 (R and D Systems)-used at 2000pg/ml in Sample Diluent, with progressive 1:2.5 dilutions to 8.2pg/ml for the standard curve

Harvested Supernatant

For remainder of assay materials see *IL-2 Materials* section

2.6.2.2 Method

Method is identical to that for *IL-2*

A sample standard curve for IL-4 ELISA is shown in figure 2.03.

2.6.3 Transforming Growth Factor-beta (TGF-β)

2.6.3.1 Materials

Capture Antibody	Mouse anti-human TGF-β mono-clonal antibody (R and D Systems)-used at 4μg/ml in PBS
Detection Antibody	Mouse Biotinylated anti-human TGF-β polyclonal antibody (R and D)-used at 25ng/ml in Assay Diluent
IL-2 Standard	Recombinant Human TGF-β (R and D Systems)-used at 2000pg/ml in Sample Diluent, with progressive 1:2.5 dilutions to 8.2pg/ml for the standard curve

Harvested supernatant

MaxiSorb ELISA plate (Nunc)

AnalR water (used to make all solutions)

Phosphate Buffered Saline (PBS)

Tris Buffered Saline (TBS-20mM Trizma Base, 150mM Sodium Chloride)

Blocking buffer	5% Tween 20, 5% Sucrose in PBS
Sample Diluent	0.1% FCS in RPMI 1640
Assay Diluent	0.1% Bovine Serum Albumin (BSA), 0.05% Tween 20 in TBS
Wash Buffer	0.05% Tween 20 in PBS

Streptavidin Horse Radish Peroxidase (Streptavidin HRP)-used at 1:3,300 in Assay Diluent

Substrate Solution	1ml Tetramethylbenzidine Free base 1mg/ml in DMSO, plus 9ml Phosphate-Citrate buffer (0.05M Na_2HPO_4, 0.025M Citric Acid), plus 2μl Hydrogen Peroxide
Stop Solution	1.8N Sulphuric acid (H_2SO_4)

2.6.3.2 Method

Method is identical to that for *IL-2*

A sample standard curve for TGF-β ELISA is shown in figure 2.04.

2.6.4 Interferon-gamma (IFN-γ)

2.6.4.1 Materials

Capture antibody	Mouse anti-human IFN-γ monoclonal antibody (Biosource International)-used at 1μg/ml in coating buffer A
Detection antibody:	Mouse biotinylated anti-human IFN-γ polyclonal antibody (Biosource International)-used at 0.4mg/ml in Assay Diluent
IFN-γ Standard	Recombinant Human IFN-γ (Biosource International)-used at 2000pg/ml in Sample Diluent, with progressive 1:2.5 dilutions to 8.2pg/ml for the standard curve

Harvested supernatants

MaxiSorb ELISA plate (Nunc)

AnalR water (used to make all solutions)

Coating buffer A	NaCl 8g/l, $Na_2HPO_4.2\ H_2O$ 1.42g/l, $KH_2PO_4$0.2g/l, KCl 0.2g/l
Blocking buffer	1% Bovine Serum Albumin (BSA) in coating buffer A
Sample Diluent	10% FCS in RPMI 1640
Assay Diluent	0.1% Tween 20 in Blocking buffer
Wash Buffer	0.1% Tween 20 in NaCl 9g/l

Streptavidin Horse Radish Peroxidase (Streptavidin HRP)-used at 1:3,300 in Assay Diluent

Substrate Solution	1ml Tetramethylbenzidine Free base 1mg/ml in DMSO, plus 9ml Phosphate-Citrate buffer (0.05M Na_2HPO_4, 0.025M Citric Acid), plus 2μl Hydrogen Peroxide
Stop Solution	1.8N Sulphuric acid (H_2SO_4)

2.6.4.2 Method

100μl of capture antibody was pipetted into each of the 96 wells of the ELISA plate. The plate was sealed and incubated at 4^0C overnight. Using an automated ELISA plate washer (Wolf Laboratories), the wells were aspirated and washed with wash buffer 3 times. Excess moisture was tapped out of the plate prior to the addition of 300 μl of Blocking buffer to each well. The plate was sealed and incubated for 2

hours at room temperature and then washed 3 times as before. 100 µl of assay supernatant or standard was loaded into each well, all conditions were repeated in triplicate (a known volume of sample diluent was added to achieve a final volume of 100 µl per well if limited supernatant was available). 50µl of detection antibody was also added to each well. The plate was sealed and incubated on an orbital shaker at room temperature for 2 hours and then washed 3 times as before. 100µl of Streptavidin HRP was added to each well. The plate was sealed and incubated at room temperature for 30 minutes and then washed 3 times as before. 100µl of Substrate solution was added to each well. The plate was placed in the dark for 10-30 minutes to allow the colour reaction to develop. Before the negative on the standard curve began to change colour, the reaction was stopped by the addition of 50µl of stop solution. The optical density of each well was then determined using a microtiter plate reader set to 450nm with a reference filter at 630nm.

A sample standard curve for IFN-γ ELISA is shown in figure 2.05

2.6.5 Interleukin 10 (IL-10)

2.6.5.1 Materials

Capture Antibody	Mouse anti-human IL-10 mono-clonal antibody (R and D Systems)-used at 3µg/ml in PBS
Detection Antibody	Mouse Biotinylated anti-human IL-10 polyclonal antibody (R and D)-used at 380ng/ml in Assay Diluent
IL-10 Standard	Recombinant Human IL-10 (R and D Systems)-used at 2000pg/ml in Sample Diluent, with progressive 1:2.5 dilutions to 8.2pg/ml for the standard curve

Harvested supernatants

MaxiSorb ELISA plate (Nunc)

AnalR water (used to make all solutions)

Phosphate Buffered Saline (PBS)

Tris Buffered Saline (TBS-20mM Trizma Base, 150mM Sodium Chloride)

Blocking buffer	1% Bovine Serum Albumin (BSA), 5% Sucrose in PBS
Sample Diluent	10% FCS in RPMI 1640

Assay Diluent	0.1% BSA, 0.05% Tween 20 in TBS
Wash Buffer	0.05% Tween 20 in PBS

Streptavidin Horse Radish Peroxidase (Streptavidin HRP)-used at 1:3300 in Assay Diluent

Substrate Solution	1ml Tetramethylbenzidine Free base 1mg/ml in DMSO, plus 9ml Phosphate-Citrate buffer (0.05M Na_2HPO_4, 0.025M Citric Acid), plus 2µl Hydrogen Peroxide
Stop Solution	1.8N Sulphuric acid (H_2SO_4)

2.6.5.2 Method

Method is identical to that for *IL-2*

2.6.6 Monokine Activated by Interferon-γ (MIG)

2.6.6.1 Materials

Capture Antibody	Mouse anti-human MIG mono-clonal antibody (R and D Systems)-used at 2µg/ml in PBS
Detection Antibody	Goat Biotinylated anti-human MIG polyclonal antibody (R and D)-used at 50ng/ml in Assay Diluent
MIG Standard	Recombinant Human MIG (R and D Systems)-used at 2000pg/ml in Sample Diluent, with progressive 1:2.5 dilutions to 8.2pg/ml for the standard curve

Harvested supernatants

MaxiSorb ELISA plate (Nunc)

AnalR water (used to make all solutions)

Phosphate Buffered Saline (PBS)

Tris Buffered Saline (TBS-20mM Trizma Base, 150mM Sodium Chloride)

Blocking buffer	1% Bovine Serum Albumin (BSA), 5% Sucrose in PBS
Sample Diluent	10% FCS in RPMI 1640
Assay Diluent	0.1% BSA, 0.05% Tween 20 in TBS
Wash Buffer	0.05% Tween 20 in PBS

Streptavidin Horse Radish Peroxidase (Streptavidin HRP)-used at 1:3300 in Assay Diluent

Substrate Solution	1ml Tetramethylbenzidine Free base 1mg/ml in DMSO, plus 9ml Phosphate-Citrate buffer (0.05M Na_2HPO_4, 0.025M Citric Acid), plus 2µl Hydrogen Peroxide
Stop Solution	1.8N Sulphuric acid (H_2SO_4)

2.6.6.2 Method

Method is identical to that for *IL-2*

2.6.7 Monocyte Chemotactic Protein 1 (MCP-1)

2.6.7.1 Materials

Capture Antibody	Mouse anti-human MCP-1 mono-clonal antibody (R and D Systems)-used at 2µg/ml in PBS
Detection Antibody	Goat Biotinylated anti-human MCP-1 polyclonal antibody (R and D)-used at 100ng/ml in Assay Diluent
MCP-1 Standard	Recombinant Human MCP-1 (R and D Systems)-used at 2000pg/ml in Sample Diluent, with progressive 1:2.5 dilutions to 8.2pg/ml for the standard curve

Harvested supernatants

MaxiSorb ELISA plate (Nunc)

AnalR water (used to make all solutions)

Phosphate Buffered Saline (PBS)

Blocking buffer	1% Bovine Serum Albumin (BSA), 5% Sucrose in PBS
Sample Diluent	10% FCS in RPMI 1640
Assay Diluent	1% BSA in PBS
Wash Buffer	0.05% Tween 20 in PBS

Streptavidin Horse Radish Peroxidase (Streptavidin HRP)-used at 1:3300 in Assay Diluent

Substrate Solution	1ml Tetramethylbenzidine Free base 1mg/ml in DMSO, plus

	9ml Phosphate-Citrate buffer (0.05M Na_2HPO_4, 0.025M Citric Acid), plus 2µl Hydrogen Peroxide
Stop Solution	1.8N Sulphuric acid (H_2SO_4)

2.6.7.2 Method

Method is identical to that for *IL-2*

2.6.8 Interleukin 8 (IL-8)

2.6.8.1 Materials

Capture Antibody	Mouse anti-human IL-8 mono-clonal antibody (R and D Systems)-used at 4µg/ml in PBS
Detection Antibody	Goat Biotinylated anti-human IL-8 polyclonal antibody (R and D)-used at 20ng/ml in Assay Diluent
IL-8 Standard	Recombinant Human IL-8 (R and D Systems)-used at 2000pg/ml in Sample Diluent, with progressive 1:2.5 dilutions to 8.2pg/ml for the standard curve

Harvested supernatants

MaxiSorb ELISA plate (Nunc)

AnalR water (used to make all solutions)

Phosphate Buffered Saline (PBS)

Tris Buffered Saline (TBS-20mM Trizma Base, 150mM Sodium Chloride)

Blocking buffer	1% Bovine Serum Albumin (BSA), 5% Sucrose in PBS
Sample Diluent	10% FCS in RPMI 1640
Assay Diluent	0.1% BSA, 0.05% Tween 20 in TBS
Wash Buffer	0.05% Tween 20 in PBS

Streptavidin Horse Radish Peroxidase (Streptavidin HRP)-used at 1:3300 in Assay Diluent

Substrate Solution	1ml Tetramethylbenzidine Free base 1mg/ml in DMSO, plus 9ml Phosphate-Citrate buffer (0.05M Na_2HPO_4, 0.025M Citric Acid), plus 2µl Hydrogen Peroxide
Stop Solution	1.8N Sulphuric acid (H_2SO_4)

2.6.5.2 Method

Method is identical to that for *IL-2*

2.6.5 Calculation of Results

On each ELISA plate there was a standard curve. The method for the standard curve is described above for each cytokine. Each point on the standard curve was calculated as a mean of the three wells in the triplicate. The curve was plotted on a log/log graph with concentration of standard against ratio of optical density of positive over negative wells, using *Microsoft Excel*. The software was then used to generate a best-fit trendline and equation for this line. The mean optical density from each triplicate was used in this equation to calculate the concentration of cytokine in the assay sample. If the samples had been diluted, then the result was multiplied by this dilution factor to calculate the concentration in the original sample.

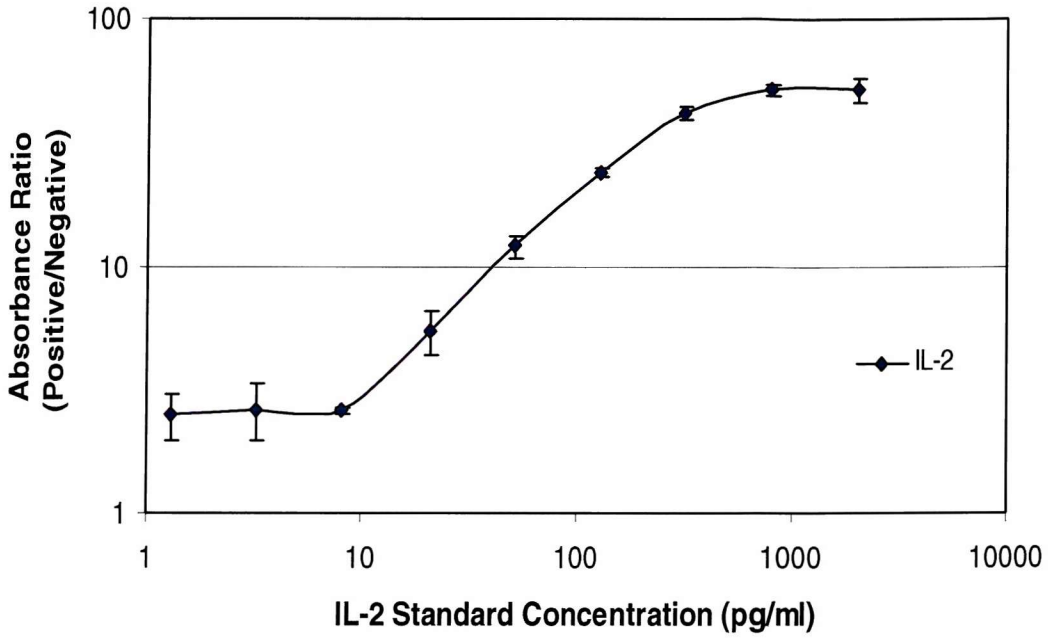

Figure 2.02 Sample standard curve for IL-2 ELISA

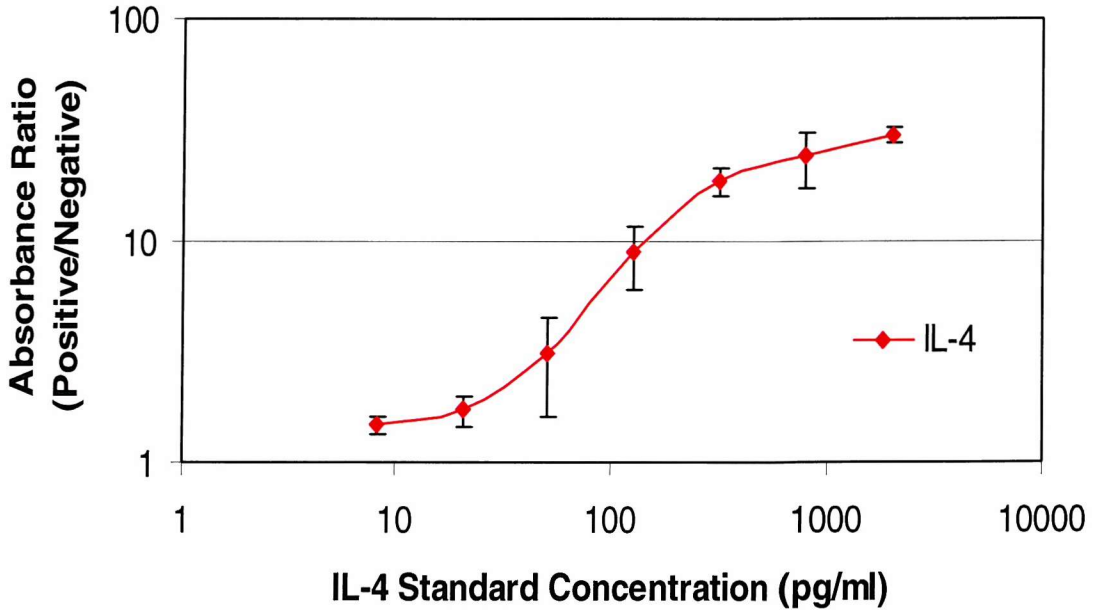

Figure 2.03 Sample standard curve for IL-4 ELISA

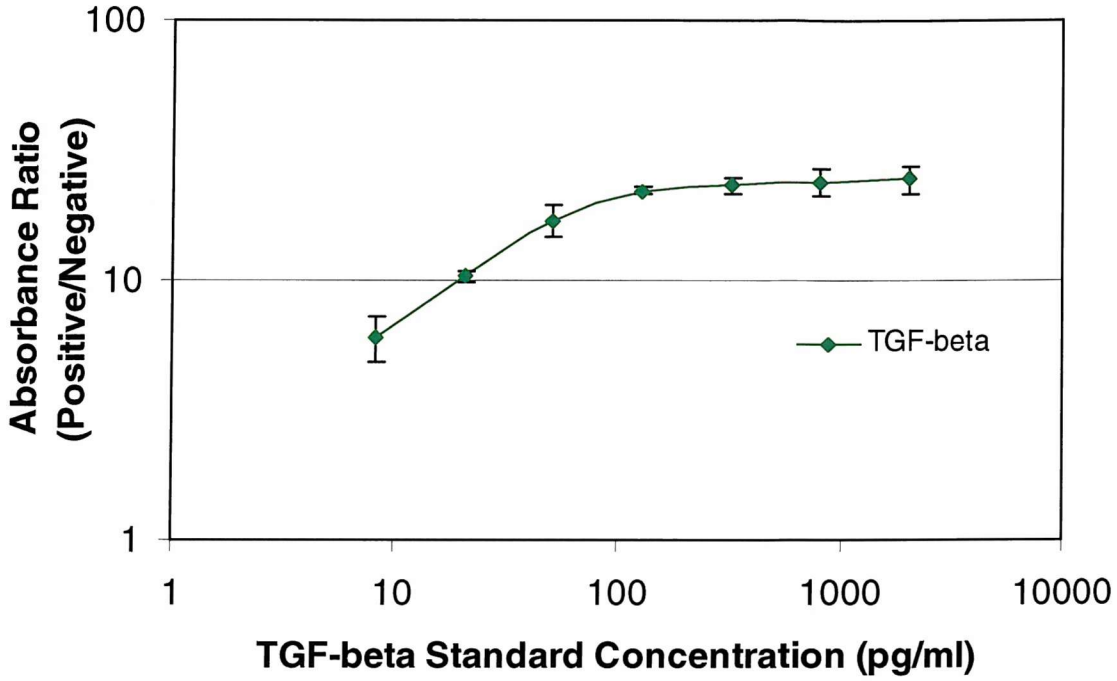

Figure 2.04 Sample standard curve for TGF-β ELISA

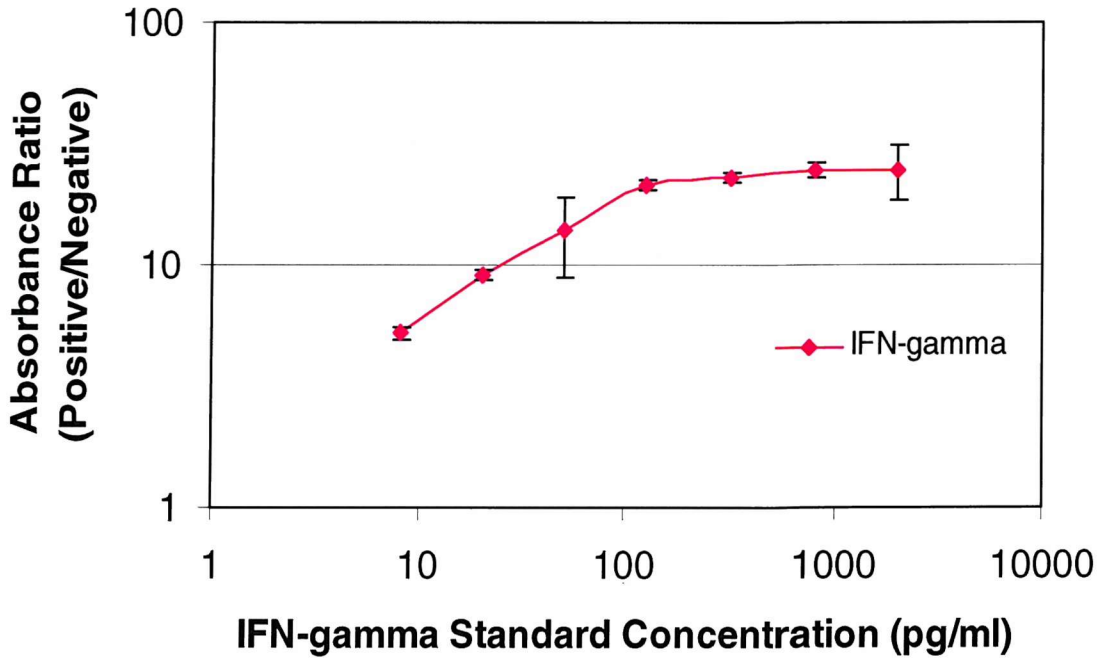

Figure 2.05 Sample standard curve for IFN-γ ELISA

2.7 Fluorescence Activated Cell Scanning (FACS) Analysis of PBMC

FACS analysis was used to look for differences in the responses between chronic hepatitis C patients discordant for disease severity. In addition, FACS analysis was used to follow a cohort of chronic hepatitis C patients as they underwent treatment with PEGylated Interferon-α (Interferon-α with a Poly Ethylene Glycol group) and Ribavirin.

2.7.1 Preparation of Cells for Analysis

2.7.1.1 Materials

Freshly isolated peripheral blood mononuclear cells at 2 x 10^6 cells/ml
Recombinant hepatitis C proteins (Mikrogen)
Influenza antigen
Tetanus toxoid (NIBSC)
Brefeldin A 4mg/ml in RPMI
Cell Dissociation Buffer (Gibco)
FACS Staining Buffer 2% Foetal Calf Serum, 0.1% Sodium Azide in Calcium Free HBSS (Hanks Buffered Salt Solution-Gibco)

2.7.1.2 Method

Freshly isolated PBMCs were cultured in 5% human AB serum at 37^0C in a CO_2 incubator overnight in 2ml aliquots. The next day, 2 of the aliquots were left unstimulated to use as negative controls and set-up tubes for the FACS machine. The remaining aliquots were pulsed for 6 hours with one of the antigens; HCV Core, NS3, NS4, Helicase and Flu were used at 1μg/ml and Tetanus toxoid at 1:10000. For the last 5 hours of culture, Brefeldin A was added to all the aliquots at a final concentration of 2μg/ml (this allows the manufactured proteins to accumulate within the Golgi apparatus).
The cells were then washed in FACS staining buffer and centrifuged at 1200 rpm for

5 minutes. The cell pellet was resuspended in pre-warmed cell-dissociation buffer, and incubated at 37^0C for 15 minutes. The cells were washed again in FACS staining buffer, centrifuged and resuspended in FACS staining buffer. They were then distributed into the FACS tubes ready for staining.

2.7.2 Staining PBMC for Cell Surface Markers and Intracellular Cytokines

2.7.2.1 Materials

FACS Staining Buffer	2% Foetal Calf Serum, 0.1% Sodium Azide in Calcium Free HBSS (Hanks Buffered Salt Solution-Gibco)
4% Paraformaldehyde	In PBS (Sigma)
0.1% Saponin	In FACS staining buffer (Sigma)

Antibodies from Becton and Dickinson

Controls	Mouse Ig G1 FITC labelled	}
	Mouse IgG1 APC labelled	} Negative controls
	Mouse IgG1 PE labelled	} Unknown specificity
	Rat IgG1 PE labelled	}
	Mouse anti-human CD4 FITC labelled	} Positive
	Mouse anti-human CD4 APC labelled	} controls
	Mouse anti-human CD3 PE labelled	}
Cell surface markers	Mouse anti-human CD69 FITC labelled	
	Mouse anti-human CD4 APC labelled	
Intra-cellular Cytokines	Mouse anti-human IL-2 PE labelled	
	Mouse anti-human IL-4 PE labelled	
	Mouse anti-human IFN-gamma PE labelled	
	Rat anti-human IL-10 PE labelled	

2.7.2.2 Method

The unstimulated cells were divided equally between 8 set-up tubes and 4 assay tubes, each to be stained for a different intra-cellular cytokine. Each antigen stimulated cell aliquot was divided equally between 4 assay tubes, one for each cytokine. A typical experimental design is shown in table 2.01:

Tube No.	Set-up/Assay	Antibodies used in staining procedure
1	Set-up	Unstained
2	Set-up	FITC Negative
3	Set-up	APC Negative
4	Set-up	Mouse PE Negative
5	Set-up	Rat PE Negative
6	Set-up	FITC Positive
7	Set-up	APC Positive
8	Set-up	PE Positive
9	Assay-unstimulated	CD69, CD4, IL-2
10	Assay-Core stimulated	CD69, CD4, IL-2
11	Assay-Helicase stimulated	CD69, CD4, IL-2
12	Assay-NS5 stimulated	CD69, CD4, IL-2
13	Assay-PHA stimulated	CD69, CD4, IL-2
14	Assay-unstimulated	CD69, CD4, IL-4

Etc. This pattern is repeated for each cytokine

Table 2.01 Example of experimental design for staining in FACS experiments

To stain the cells, they were centrifuged in the FACS tubes, and the supernatant was flicked out. The cells were then resuspended in FACS staining media either alone or containing cell surface marker antibody, FITC or APC negative control as appropriate to a final volume of 50µl. They were incubated in the dark at 4^0C for 30 minutes. The cells were then washed in FACS staining medium and spun prior to discarding the

supernatant. The cells were resuspended in 50µl 4% paraformaldehyde and incubated at 4^0C for 15 minutes to fix them. After a further wash in FACS staining medium, the cells were permeabilised by the addition of 50µl 0.1% saponin. At this stage the PE negative controls or cytokine antibodies were added as appropriate, and the cells were incubated in the dark at 4^0C for 30 minutes. The cells were washed once more in FACS staining medium and then resuspended in PBS. The cells were kept on ice and in the dark until ready for analysis.

2.7.3 Fluorescence Activated Cell Scanning of PBMCs

2.7.3.1 Materials

Stained and unstained PBMCs (see staining method above)
FACSflow (Becton and Dickinson)
FACScalibur© FACS machine (Becton and Dickinson)
Apple MacIntosh computer with Cell Quest© Software (Becton and Dickinson)

2.7.3.2 Method

The set-up tubes were used in turn to optimise settings on the FACScalibur machine. The machine was adjusted to show the cell population of interest with easy discrimination between each of the fluorescent markers. Quadrants were set to allow identification of positively, negatively and dual stained cells.
The negative control for each fluorescent marker was then recorded, followed by each of the assay tubes in order. The defined quadrants were used to record differences in expression of cell surface markers and cytokine profiles between the individual assay conditions.

2.8 Viral Load Measurement

Hepatitis C viral load was measured using a TAQman-based assay. Stephen Hadfield developed the assay as part of a PhD project, under the supervision of Dr. S Green. The viral load measurements quoted in the results section were undertaken by Stephen Hadfield on samples collected from the treatment monitoring study patients.

2.9 Treatment Monitoring Protocol

Chronic hepatitis C patients who had been approved for treatment with standard Interferon-α and Ribavirin were studied with lymphocyte proliferation assays before, during and after their treatment period (6-12 months). Responses were correlated with their virological response to treatment.

In addition, the immune responses of group of patients being treated with PEGylated Interferon-α and Ribavirin in the context of a clinical trial were studied in more depth. Routine haematology and biochemistry were monitored as part of the clinical trial. The investigation plan is shown in table 2.02:

Week of Treatment	Experiments/Measurements Undertaken
Baseline	Lymphocyte proliferation assay, Viral load, FACS analysis
Week 1	Lymphocyte proliferation assay, Viral load
Week 2	Lymphocyte proliferation assay, Viral load
Week 4	Lymphocyte proliferation assay, Viral load
Week 6	Lymphocyte proliferation assay, Viral load
Week 8	Lymphocyte proliferation assay, Viral load
Week 12	Viral load, FACS analysis
Week 18	Lymphocyte proliferation assay, Viral load
Week 24	Lymphocyte proliferation assay, Viral load, FACS analysis
Week 30	Lymphocyte proliferation assay, Viral load
Week 36	Lymphocyte proliferation assay, Viral load
Week 42	Lymphocyte proliferation assay, Viral load
Week 48	Lymphocyte proliferation assay, Viral load, FACS analysis
Week 72	Lymphocyte proliferation assay, Viral load, FACS analysis

Table 2.02 Schedule for assays undertaken on blood collected from patients during the PEGylated interferon and Ribavirin study

2.10 Generation and Collection of Conditioned Supernatants from PBMC

2.10.1 Isolation of PBMC from whole blood

Heparinised blood was collected from the donors and PBMCs were freshly isolated using the density gradient centrifugation method described in section **2.3**.

2.10.2 PBMC Culture to Condition Supernatants

The freshly isolated PBMCs were counted and established in culture at a concentration of 2 x 10^6 per ml in RPMI medium containing 5% human AB serum, penicillin, streptomycin and glutamine (see section **2.4**, Lymphocyte proliferation assays). The volume of cell suspension per condition was determined by the number of cells isolated. To each aliquot of PBMC suspension was added an equal volume of complete medium containing either recombinant HCV proteins, or appropriate positive and negative controls, to give the same final concentration of test substance as described in section **2.4**. The cells were then cultured for 3 days at 37^0C, in 100% humidity.

2.10.3 Collection of Supernatants

After 3 days in culture, the supernatants were harvested into sterile collection tubes using a sterile plastic Pasteur pipette. These tubes were then centrifuged at high frequency to pellet any cellular debris or contamination. The supernatants were then carefully transferred into fresh sterile collecting tubes, labeled and stored at -20^0C for future use.

2.11 Isolation and Culture of Normal Human Hepatic Stellate Cells

2.11.1 Recruitment of Subjects

Patients admitted for routine liver resection under the care of Professor Primrose were approached for consent to donate for laboratory research any excess normal tissue resected as part of their standard surgical procedure. Each patient was verbally consented, given a written information sheet and asked to sign a printed consent form before they were recruited into the study. No coercion or financial incentive was offered.

2.11.2 Collection of Specimens

The liver resection specimen was collected directly *ex vivo* in a dry container. The specimen was taken to the pathology department for macroscopic dissection by an experienced pathologist. A portion of macroscopically normal liver, not required for diagnostic purposes, was identified and transferred immediately to the tissue culture laboratory. The time taken from removal from the donor, to arrival in the tissue culture laboratory was around 15-25 minutes.

2.11.3 Primary Human Hepatic Stellate Cell Isolation and Culture

2.11.3.1 Materials

Hanks Buffered Saline Solution (HBSS) with Calcium

HBSS Minus Calcium	Gibco / Life Technologies
DNase	Roche Diagnostics (0.25 mg/ml in HBSS with Calcium)
Pronase	Roche Diagnostics (12.5 mg/ml in HBSS with Calcium)
Collagenase	Roche Diagnostics (2.5mg/ml in HBSS with Calcium)
HEPES	Gibco / Life Technologies
Dulbeccos Modified Eagles Medium with Glutamine	Gibco / Life Technologies
Penicillin and Streptomycin	Gibco / Life Technologies (used at a final concentration of 100iu/ml)
Foetal Calf Serum	Gibco / Life Technologies (used at a final

	concentration of 16%)
Optiprep Gradient	Nycomed
Nybolt	Membrane for filtering particulate matter
Heparin (500IU/ml)	Glaxo-Wellcome (used at 10IU/ML in HBSS minus Calcium)

2.11.3.2 Method

All of the solutions were diluted to the working concentration 1 hour prior to the isolation, sterile filtered and pre-warmed to 37^0C.

The sample of liver tissue was placed in a beaker containing 250ml of warmed HBSS minus calcium with heparin to rinse. A 10ml syringe with HBSS was used to flush any visible vessels, in order to remove as many red blood cells as possible. The specimen was then transferred to a fresh beaker of 250ml warmed HBSS minus calcium and chopped into small pieces using a scalpel. It was then mechanically homogenized using a hand mixer for 5-10 seconds.

The "soupy" mixture was poured into a sterile 500 ml bottle, and 40 ml each of pronase and collagenase was added. The bottle was sealed and placed on an orbital shaker at 37^0C for 5-10 minutes, to allow complete digestion of extracellular matrix components.

6ml of DNase solution was added to the liver digest the whole contents were filtered through a nybolt membrane, and washed through with warmed HBSS with calcium, to a final volume of around 400ml.

The cell suspension was divided into 50ml flacon tubes and centrifuged at 400G for 7 minutes. The supernatants were discarded, and each cell pellet resuspended in 2ml DNase solution. Warmed HBSS with calcium was added to 50ml, and the centrifuge step repeated to wash the cells.

Meanwhile, the gradient was prepared by mixing 15.6ml HBSS with calcium and 14ml of Optiprep.

The cell pellets were resuspended in 1 ml of DNase each, and pooled together. They were made up to 44.4ml with HBSS minus calcium and then mixed with the prepared gradient. The resultant mixture was then split between 2x50ml falcon tubes and 2-3 ml of HBSS plus calcium was carefully layered onto the top of each as a cushion.

The falcons were then centrifuged at 1400g for 17 minutes, and allowed to slow down without braking, to avoid disruption of the gradient layering.
The stellate cells were harvested as a cloudy cellular layer from just below the HBSS cushion. The cells were washed once in Dulbeccos Modified Eagles Medium with glutamine (DMEM) and then resuspended in DMEM containing antibiotics and 16% foetal calf serum. The cell suspension was then divided between an appropriate number of plastic tissue culture flasks and placed in an incubator with 5% CO_2, maximum humidity and temperature 37^0C.
The cells were examined daily to ensure that they began to adhere to the plastic and proliferate. Once adherent, after 3-5 days, the medium was be changed on a regular basis until the cells became confluent.

2.11.4 Passage of Human Hepatic Stellate Cells

2.11.4.1 Materials

HBSS Minus Calcium	Gibco / Life Technologies
Dulbeccos Modified Eagles Medium with Glutamine	Gibco / Life Technologies
Penicillin and Streptomycin	Gibco / Life Technologies (used at a final concentration of 100iu/ml)
Foetal Calf Serum	Gibco / Life Technologies (used at a final concentration of 16%)
Trypsin	Gibco / Life Technologies

2.11.4.2 Method

Primary human HSC were examined daily to observe that they became adherent and began to proliferate. This phenomenon was associated with a change in phenotype from rounded globular cells, to elongated myofibroblast-type phenotype, and generally took a total of 2-3 weeks to reach confluence.
When the cells were confluent, the medium was tipped off and the cell layer washed three times with pre-warmed HBSS minus Calcium, which was then tipped off.

The trypsin was diluted 1:10 with HBSS minus calcium, and then enough to just cover the monolayer was added to each tissue culture flask. The flask was placed back in the incubator for 2 minutes, and then checked to see if the cells were becoming detached from the plastic surface. If still adherent, they were returned to the incubator for a further minute before being checked for adherence again. This process was repeated until around 90% of cells were in suspension.

Foetal calf serum (FCS) was then added to the flasks to neutralize the trypsin activity (half the volume of the amount of trypsin that had been used). The cell suspension was harvested into a falcon and washed once in HBSS minus calcium by centrifugation. The cell pellet was then resuspended in an appropriate volume of DMEM containing antibiotics, and 16% FCS.

Cells were split 1:2 or 1:3 depending on how confluent they had been prior to passage. The cells were then cultured in tissue culture flasks or plates depending on experimental intent for the cells.

The same process was applied to passaged HSC if further passage was required.

2.12 Fluorescence Activated Cell Scanning (FACS) Analysis of HSC

FACS analysis of HSC was used to confirm purity of cultures, and look for changes in level of expression of cell surface molecules with cytokine stimulation.

2.12.1 Preparation of Cells for Analysis

2.12.1.1 Materials

Confluent hepatic stellate cells (HSC), primary or passaged

Recombinant human Interferon-γ (IFN-γ) and Tumour necrosis factor-α (TNF-α) R and D Systems

DMEM complete medium (DMEM plus glutamine with antibiotics and 16% FCS)

Cell Dissociation Buffer (Gibco)

HBSS minus calcium Gibco / Life Technologies

FACS Staining Buffer 2% Foetal Calf Serum, 0.1% Sodium Azide in Calcium Free HBSS (Hanks Buffered Salt Solution-Gibco)

2.12.1.2 Method

Confluent HSC were incubated overnight in either fresh DMEM complete medium alone, or DMEM complete medium containing 10ng/ml of either IFN-γ or TNF-α. The cells were then washed in three times in warmed HBSS minus calcium and then incubated for 15 minutes with warmed cell dissociation buffer to cover the monolayer, until the cells were no longer adherent. The cells were harvested into a falcon and washed in FACS staining buffer by centrifugation at 1200 rpm for 5 minutes. The cell pellet was resuspended in FACS staining buffer and then distributed into the FACS tubes ready for staining.

2.12.2 Staining HSC for Cell Surface and Intracellular Markers

2.12.2.1 Materials

FACS Staining Buffer	2% Foetal Calf Serum, 0.1% Sodium Azide in Calcium Free HBSS (Hanks Buffered Salt Solution-Gibco)
4% Paraformaldehyde	In PBS (Sigma)
0.1% Saponin	In FACS staining buffer (Sigma)
Streptavidin Quantum Red	Sigma
Phosphate buffered Saline (PBS)	
Antibodies from Becton and Dickinson	
Controls	Mouse Ig G1 FITC labeled } Negative controls
	Mouse IgG Biotinylated } Unknown specificity
Test antibodies	Mouse anti-human ICAM-1 (CD54) FITC labeled
	Mouse anti-human MHC class II FITC labeled
Antibodies from Ansell	Mouse anti-human ICAM-1 (CD54) Biotinylated
	Mouse anti-human CD40 Biotinylated
Antibodies from Sigma	Mouse anti-human α-Smooth muscle Actin FITC labeled

2.12.2.2 Method

The unstimulated cells were divided equally between set-up tubes and assay tubes, each to be stained for a different marker. The cytokine stimulated cells were distributed between assay tubes, one for each marker. A typical experimental design is shown in table 2.03:

Tube No.	Set-up/Assay	Antibodies used in staining procedure
1	Set-up	Unstained
2	Set-up	FITC Negative
3	Set-up	Quantum Red Negative (biotinylated)
4	Set-up	Permeabilised FITC Negative
5	Assay-Control	ICAM-1 (CD54) FITC
6	Assay-Control	CD40 Quantum Red
7	Assay-Control	MHC class II FITC
8	Assay-Control	α-SMA FITC
9	Assay-Control	α-SMA FITC Permeabilised
10	Assay-cytokine stimulated	ICAM-1 (CD54) FITC
Etc. This pattern is repeated for each stimulating cytokine		

Table 2.03 Example of experimental design for staining in HSC FACS experiments

To stain the cells, they were centrifuged in the FACS tubes, and the supernatant was flicked out. The cells were then resuspended in FACS staining media either alone or containing cell surface marker antibody, FITC or Biotinylated negative control as appropriate to a final volume of 50µl. They were incubated in the dark at 4^0C for 30 minutes. The cells were then washed in FACS staining medium and spun prior to discarding the supernatant. Cells stained with biotinylated antibodies were then incubated in FACS buffer containing Streptavidin QR for 15 minutes at 4^0C in the dark, other cells were simply resuspended in FACS buffer alone. The cells were then

washed in FACS staining medium and spun prior to discarding the supernatant. The cells were resuspended in 50µl 4% paraformaldehyde and incubated at 4^0C for 15 minutes to fix them. After a further wash in FACS staining medium the cells not requiring permeabilisation were resuspended in PBS and stored at 4^0C in the dark until required for FACS analysis. Cells requiring permeabilisation were permeabilised by the addition of 50µl 0.1% saponin. At this stage the permeabilised negative control FITC labeled antibody or anti-α-SMA antibodies were added as appropriate, and the cells were incubated in the dark at 4^0C for 30 minutes. The cells were washed once more in FACS staining medium and then resuspended in PBS. The cells were kept on ice and in the dark until ready for analysis.

2.12.3 Fluorescence Activated Cell Scanning of HSC

2.12.3.1 Materials

Stained and unstained HSC (see staining method above)
FACSflow (Becton and Dickinson)
FACScalibur© FACS machine (Becton and Dickinson)
Apple Macintosh computer with Cell Quest© Software (Becton and Dickinson)

2.12.3.2 Method

The set-up tubes were used in turn to optimise settings on the FACScalibur machine. The machine was adjusted to show the cell population of interest with easy discrimination between each of the fluorescent markers. Quadrants were set to allow identification of positively and negatively stained cells.
The negative control for each fluorescent marker was then recorded, followed by each of the assay tubes in order. The defined quadrants were used to record differences in expression of cell surface markers and cytokine profiles between the individual assay conditions.

2.13 Cellular ELISA for ICAM-1 in Hepatic Stellate Cells

2.13.1 Preparation of Cells for Analysis

2.13.1.1 Materials

Confluent passaged HSC in 96 well tissue culture plates

Harvested conditioned supernatants from PBMC cultures

5% Human AB Serum (Biowhittaker) in RPMI complete medium

Recombinant Human Cytokines from R and D Systems

Interferon-γ (IFN-γ)

Tumour Necrosis Factor-α (TNF-α)

Transforming Growth Factor-β (TGF-β)

Platelet Derived Growth Factor (PDGF)

IL-4, -6, -10, -12, -13

Recombinant human Interferon-α (IFN-α) from Roche Pharmaceuticals

Recombinant HCV proteins from Mikrogen, Germany

HBSS	Gibco Life Technologies
Methanol	Sigma

Phosphate Buffered Saline (PBS)

Bovine Serum Albumin	Sigma

Biotinylated Mouse anti-human ICAM-1 antibody from R and D systems

Biotinylated Mouse anti-human IL-2 antibody from R and D systems

Monoclonal Mouse Anti-IFN-γ antibody from R and D systems

AnalR water (used to make all solutions)

Blocking buffer	1% Bovine Serum Albumin (BSA) in PBS
Assay Diluent	1% BSA in PBS
Wash Buffer	PBS

Streptavidin Horse Radish Peroxidase (Streptavidin HRP)-used at 1:3300 in Assay Diluent

Substrate Solution	1ml Tetramethylbenzidine Free base 1mg/ml in DMSO, plus 9ml Phosphate-Citrate buffer (0.05M Na_2HPO_4, 0.025M Citric Acid), plus 2μl Hydrogen Peroxide

Stop Solution 1.8N Sulphuric acid (H_2SO_4)

2.13.1.2 Method

The confluent HSC in 96 well plates were washed twice with HBSS, and then incubated at 37^0C overnight with either, 5% human AB serum in RPMI complete alone, 5% human AB serum in RPMI complete containing recombinant human cytokines or HCV proteins, or in PBMC conditioned medium.

In interferon-g blocking experiments, the supernatants were pre-incubated with 2ng/ml anti-IFN-γ antibody at room temperature for 30 minutes before the supernatants were applied to the cells.

The plates were then washed three times with HBSS and then 100µl of ice cold methanol was added to each well for 10 minutes to fix the cells. Each well was then washed three times in wash buffer and the fluid residue was flicked out. 300µl of blocking buffer was added to each well and the plate left at room temperature for at least 1 hour.

The blocking buffer was flicked out and 100µl of assay diluent containing biotinylated anti-ICAM-1 at 0.1 mcg/ml was added to each well.(or an irrelevant control, biotinylated antibody-anti-IL-2 at 25ng/ml). The plate was left at room temperature for 1-2 hours.

Each well was then washed 3 times with wash buffer and the excess fluid flicked out. 100µl of assay diluent containing Streptavidin HRP was added to each well and the plate incubated at room temperature for 30 minutes. The plate was then washed 3 times as before. 100µl of Substrate solution was added to each well. The plate was placed in the dark for 10-30 minutes to allow the colour reaction to develop. Before the negative control began to change colour, the reaction was stopped by the addition of 50µl of stop solution. The optical density of each well was then determined using a microtiter plate reader set to 450nm with a reference filter at 570nm.

2.14 Gene Array for Assessment of mRNA Levels in HSC

2.14.1 Preparation of Cells for Analysis

Gene arrays were always undertaken on confluent primary HSC, using 2 parallel small ($24cm^2$) plastic tissue culture flasks. For the IFN-γ experiments, the control flask was exposed overnight to fresh DMEM complete medium with 16% FCS, whilst the test flask had the same medium supplemented with 10ng/ml IFN-γ. For the experiments involving PBMC conditioned media, the control flask was exposed to PBMC media conditioned with protein carrier buffer, whilst the test flask was exposed to PBMC media conditioned by the same donor with recombinant HCV NS3 protein.

2.14.2 Preparation of RNA from HSC

2.14.2.1 Materials

Flasks of confluent HSC stimulated overnight with either control or test media

QIAshredders	QIAgen
RNeasy Minikit	QIAgen

2.14.2.2 Method

The tissue culture flasks were removed from the incubator, and the supernatants poured off and collected for use in ELISAs. The flasks were stood up-ended to drain to dryness. The cells were lysed using the appropriate buffers from the RNeasy minikit (containing β-mercapto-ethanol and Guanadinium Isothiocyanate). The lysates were then passed through QIAshredder columns to homogenize the samples. Total RNA was then collected by following the protocol from the RNeasy minikit, which essentially captures the precipitated RNA in a column, washes it and then elutes it into an eppendorf tube using RNase free water. All of the procedure is undertaken using RNase free solutions and gloves to avoid degradation of the sample. Following RNA extraction, the concentration of RNA in each sample was calculated using a spectrophotometer. The samples were then stored at -20^0C until required for use.

2.14.3 Preparation of Biotinylated cDNA probe from RNA samples

2.14.3.1 Materials

5mcg RNA in water prepared from HSC lysates	
RNase free eppendorf tubes	
GEA Primer Mix	SuperArray Inc
RNase free water	SuperArray Inc
Biotin -16-dUTP	Boehringer Mannhem
MMLV Reverse Transcriptase	Promega
RNase inhibitor	Promega
5x Non-Rad-GEAlabeling Buffer	SuperArray Inc
10x Stop Solution	SuperArray Inc
10x Denaturing Solution	SuperArray Inc
2x Neutralisation Solution	SuperArray Inc

2.14.3.2 Method

For initial annealing, prior to labeling with biotin, each RNA sample was mixed with GEAprimer mix and water to a final volume of 20µl. The mixture was then heated to 70^0C for 2 minutes, then cooled to 42^0C for 2 minutes. 20µl of master labeling mix, preheated to 42^0C for 2 minutes, was added to each sample and the samples were then maintained at 42^0C for 2 hours. (Master labeling reaction mix = 8µl Nonrad-GEAlabeling buffer, 4µl Biotin-16-dUTP, 1µl RNase inhibitor, 2µl MMLV Reverse Transcriptase and 5µl water). The reaction was stopped by adding 5µl of 10x stop solution.

Prior to use in the hybridisation step, the biotin labeled cDNA probe was denatured by the addition of 5µl of 10x denaturing solution to the labeling mix, this was then incubates at 68^0C. for 20 minutes. Finally, 50µl 2x neutralization solution was added for 10 minutes at 68^0C.

The biotin labeled probe was then ready for use in the hybridisation reaction.

2.14.4 Hybridisation of Biotinylated cDNA probe to Customised GeneArray Membranes and Chemiluminescent Detection

2.14.4.1 Materials

Denatured biotin labeled cDNA probe synthesised from HSC RNA

2 GEArray membranes	SuperArray Inc
GEAhyb Hybridisation Solution	SuperArray Inc
Sheared Salmon Sperm DNA	GIBCO BRL

Hybridisation tubes and Hybridisation oven set at 68^0C

GEAblocking Solution	SuperArray Inc
AP-Streptavidin	SuperArray Inc
5x wash Buffer	SuperArray Inc
AP-assay Buffer	SuperArray Inc
CPD-*Star*® Substrate	SuperArray Inc

20x SSC (17.3g NaCl, 88.2g Na_3Citrate.$2H_2O$, up to 1000ml with water, pH 7.0)

20% SDS (200g SDS up to 1000ml with water, heated to 65^0C to dissolve)

Clingfilm

X-Ray Film Kodak

2.14.4.2 Method

Sheared salmon sperm DNA was heated to 100^0C for 5minutes and chilled quickly on ice to denature. The denatured salmon sperm DNA was added to pre-warmed (68^0C) GEAhyb Hybridisation solution (15ml per membrane) to a final concentration of 100mcg DNA/ml. The GEArray membranes were wet with water and each was placed in its own hybridisation tube. 10ml of the salmon sperm DNA /GEAhyb solution was added to each tube and the membranes were pre-hybridised by incubation at 68^0C for 1-2 hours with constant agitation. The pre-hybridisation solution was then poured away, and the pre-prepared biotin labeled cDNA probes were each added to a remaining 5ml aliquot of salmon sperm / GEAhyb solution at 68^0C, and added to the appropriate hybridisation tube. The hybridisation reaction was then carried out by incubation overnight at 68^0C with constant agitation.

The membranes were then washed twice, at 68^0C, with 2x SSC, 1% SDS solution. The two 68^0C washes were repeated with 0.1x SSC, 0.5% SDS solution. The membranes were then blocked with GEAblocking solution at room temperature for 40 minutes, then incubated with AP-Streptavidin (1:5000) in blocking solution for 40 minutes at room temperature. The membranes were then washed 4 times in diluted 5x wash buffer, prior to washing in AP-assay buffer. The membranes were then exposed to CPD-*Star*® Substrate for 2 minutes then blotted to remove excess moisture. The membranes were sealed in Clingfilm and exposed to X-Ray film. A graphics package to assess densitometry of spots produced on the X-Ray film was used to semi-quantitate the assay (normalised to the house-keeping gene β-actin)

Chapter 3

Chapter 3

HLA association study

3.1 Introduction

There are many reported associations between genetic markers and human disease. In particular, HLA molecules are frequent candidates for such associations as they play a pivotal role in adaptive immunity *(Thursz 1995; Klein and Sato 2000; Klein and Sato 2000)*. For example, if an immune response to a given viral epitope is critical for resolution of infection, but this epitope will not fit any of the binding clefts of an individual's HLA repertoire, then this may prevent effective viral clearance.

HLA associations with the outcome of infectious diseases are not absolute. Rather, over- or under-representation of the allele compared with the background frequency in the general population can be detected. For example, in the Gambia the HLA class I allele HLA-B*53, is found at a frequency of 25% in healthy people and patients with mild malaria, but only at 15% in patients with severe malaria, suggesting that it protects against the most severe manifestation of the disease. In addition, protection from malarial anaemia seems to be afforded by the class II alleles HLA-DRB1*1302/DQB1*0501. HLA alleles are ethnically and consequently geographically distributed, and similar effects can be found with different alleles in different populations. Indeed, in other parts of sub-Saharan Africa, the protective class I and II alleles for malaria are different from those in the Gambia *(Klein and Sato 2000)*.

Also in the Gambia, the class II molecule HLA-DRB1*1302 is more frequently seen in patients who clear acute hepatitis B infection. This protection from chronic disease is seen in both children and adults *(Thursz 1995)*.

Several studies have investigated association between HLA alleles and resolved acute versus chronic hepatitis C infection. Tibbs *et al.* (1996) reported the results from a UK Caucasian population and found under-representation of HLA-DQA1*03 and DQB1*0302 in patients with anti-HCV antibodies (99 HCV RNA positive, 5 HCV RNA negative) *(Tibbs 1996)*. In 1997, Alric *et al.* reported a white French population of HCV antibody positive patients (103 RNA positive, 25 RNA negative)

compared with 800 healthy controls. This study suggested that HLA-DB1*1101 and DQB1*0301 were over-represented in patients with resolved acute HCV infection, whilst in the CHC patients these alleles occurred at the same frequency as the control population *(Alric 1997)*. Thursz *et al.* confirmed this finding in a large multi-centre study across Europe including 8 hepatology units in the UK, France, Spain, Greece, Italy and Sweden *(Thursz 1999)*. Both of the latter 2 studies failed to show a correlation between liver fibrosis and the frequency of HLA class II alleles, although the Alric study did demonstrate a lower Knodell score in HLA-DQB1*0301 positive CHC patients. No other significant class I or II associations were reported.
It has been well documented that HLA-DQB1*0301 and DRB1*1101 (which are in strong linkage disequilibrium) appear protective from chronic infection, but only one study to date has been specifically designed to investigate the correlation between HLA class II alleles and severity of liver disease in CHC. The study of Tillmann *et al.* looked at the frequency of these 2 alleles in end-stage HCV related liver disease requiring liver transplantation. They found that both alleles occurred at lower frequency in end stage liver disease when compared with the background frequency in the local healthy population. The conclusion drawn from this observation was that these 2 alleles are protective from severe liver disease in chronic hepatitis C infection *(Tillmann 2001)*.

3.2 Aims

The aim of this study was to investigate the frequency of HLA-DQB1*0301 in chronic hepatitis C and correlate this with the severity of liver damage, categorised as either mild or severe.

3.3 Methods

5ml of blood was collected in EDTA or sodium citrate from each patient. DNA was extracted using a rapid salting out technique. The DNA was then used in PCR reactions using sequence specific primer pairs for the allele of interest. (*See materials and methods)*. Results were analysed using a Chi squared and Fisher's exact test to generate a p value and odds ratios for presence of the allele in different clinical situations. A power calculation was used to calculate the number of extra specimens required to achieve statistical significance of the results.

3.4 Results

3.4.1 Patient Demography

Patients were included in the study if they had had a liver biopsy to confirm the severity of their liver disease, or if they had a clinical diagnosis of cirrhosis (based on the Child-Pugh classification of cirrhosis, with Child-Pugh B or C).
Using the IPA score from the liver biopsy, the patients were divided into 2 groups. Those with an IPA score which totaled >4 were designated as severe liver disease, whilst a total score of <4 was considered mild disease. Patients with a total IPA score equaling 4 were excluded from this part of the analysis (Table 3.01). Further analysis was undertaken using the combined I and P components of the score alone to allow categorisation on inflammatory change. Finally, the A score was used in isolation for an investigation on the effect of certain alleles on severity of liver fibrosis. (Patients with a total IPA score of 4 were included in these sections of the study)
Patients with a clinical diagnosis of cirrhosis were included in the severe liver disease and severe fibrosis groups.

	Mild Liver Disease (Total IPA <4) N=86	**Severe Liver Disease (Total IPA>4) N=41**
Mean Age (Range)	42.6 (22-66)	48.6 (37-75)

Table 3.01 Age of CHC patients by severity of liver disease
Statistically different p=0.003 by students T test

The control group consisted of 220 solid organ donors from the local population.

3.4.2 Confounding Factors

Several factors are generally accepted to increase the rate of progression of liver damage in chronic hepatitis C infection. These include, increasing age, excess alcohol consumption and male sex. Concurrent liver disease, such as hepatitis B infection (HBV) or hereditary haemochromatosis (HHC), also accelerates this progressive liver damage. *(Poynard 2000)*

Information regarding these prognostic influences was gathered on each of the patients (Table 3.02). Patients with severe disease were significantly older than those with mild disease. Patients with concurrent liver disease due to hepatitis B infection and haemochromatosis were excluded from the analysis. The study is prospective and the patients were not age and sex matched as the samples were collected. This was undertaken during analysis.

Prognostic Indicator	**Mild Liver Disease N=86**		**Severe Liver Disease N=38**	
	No.	**%**	**No.**	**%**
Age ≥40	47*	54.7	34*	89.4
Male	52	60.5	26	68.4
Excess Alcohol Consumption	32/68**	47	14/16**	87.4
Concurrent Liver Disease (HBV or HHC)	0	0	0	0

Table 3.02 Frequency of known prognostic indicators of liver disease in CHC patients. Alcohol history was not available on all of the patients
*p<0.0002 by Chi Square. Relative Risk 1.64 (1.31-2.04)
**p<0.004 by Chi Square. Relative Risk 1.86 (1.36-2.54)

3.4.3 Frequency of HLA DQB1*0301 by Severity of Liver Disease

All patients eligible for the study were typed for HLA DQB1*0301. The results were then correlated with severity of liver disease (Table 3.03).

There was no association between DQB1*0301 and severity of liver disease. At this level of difference between the control group and severe liver disease group, a power calculation (95% CI, 80% Power) reveals that 1339 patients would be required to achieve statistical significance. If the frequency of the allele was only 20% in severe liver disease, then 232 patients would be required.

As there was no age and sex matching of the 2 populations, a subset analysis was undertaken following matching for confounding factors of age, sex and alcohol intake. No significant difference was demonstrated between these two matched groups.

HCV Status	Severity of Liver Disease	Sex	Mean Age	No. Positive (%)	Statistical Significance
Ab - / RNA -	Normal Controls (n=220)	**All**		**70 (31.8)**	**N/S**
Ab + / RNA +	All CHC (n=141) (with or without liver biopsy findings)	**All**		**38 (27)**	**N/S**
Ab + / RNA +	Mild Liver Disease (Total IPA <4)(n=86)	M	41.9	15 (28.8)	**N/S**
		F	43.7	9 (26.5)	**N/S**
		All	**42.6**	**24 (28)**	**N/S**
Ab + / RNA +	Severe Liver Disease (Total IPA >4)(n=38)	M	40.6	6 (23.1)	**N/S**
		F	52.5	4 (33.3)	**N/S**
		All	**48.2**	**11 (26.8)**	**N/S**
Age, Sex and Alcohol Intake Matched CHC Patients					
Ab + / RNA +	Mild Liver Disease (Total IPA <4)(n=19)	**All**	**47.5**	**6 (31.6)**	**N/S**
Ab + / RNA +	Severe Liver Disease (Total IPA >4)(n=19)	**All**	**48.1**	**8 (42.1)**	**N/S**

Table 3.03 Frequency of HLA-DQB1*0301 in HCV positive patients and controls

3.4.4 Frequency of HLA DQB1*0301 by Inflammatory and Fibrotic Index

Further analysis was undertaken in patients with chronic infection. Patients with an IPA score were grouped according to inflammatory and fibrotic index. Inflammatory index was dictated by combination of the I and P components of the score, and fibrosis by the A component (Table 3.04).

There was no significant difference in frequency of HLA DQB1*0301 between the groups discordant for severity of inflammation or fibrosis as individual markers of severity of disease. However, there was a significant under-representation of the allele in the small sub-group with mild inflammation but severe fibrosis.

Histological Index	Sex	No. Positive (%)	Statistical Significance
Mild Inflammation – IP Score <4 (n=46)	M	8 (23.5)	N/S
	F	4 (33)	**N/S**
	All	**12 (26.1)**	**N/S**
Severe Inflammation – IP Score ≥ 4 (n=32)	M	7 (31.8)	**N/S**
	F	3 (30)	**N/S**
	All	**10 (31)**	**N/S**
Mild Fibrosis – A Score 0 or 1 (n=109)	M	19 (27.5)	**N/S**
	F	11 (27.5)	**N/S**
	All	**30 (27.5)**	**N/S**
Severe Fibrosis – A Score 2 or 3 (n=32)	M	5 (20)	**N/S**
	F	2 (28.6)	**N/S**
	All	**7 (21.9)**	**N/S**
Severe Inflammation and Mild Fibrosis (n=11)	**All**	**3 (27.7)**	**N/S**
Mild Inflammation and Severe Fibrosis (n=9)	**All**	**0 (0)**	**p<0.05** by Fisher Exact Test

Table 3.04 Frequency of HLA DQB1*0301 in CHC by severity of fibrosis and inflammation

3.4.5 Confirmation of Validity of Results using Regression Analysis

In total, data was collected on 142 patients with CHC who were then HLA typed for the presence of HLA DQB1*0301. However, not all of the data was complete on all of the patients. To ensure that all of the data collected was utilised, regression analysis was applied to look for correlations between the frequency of HLA DQB1*0301 and the severity of the resultant liver disease in CHC. Correlations were also sought between severity of disease and duration of infection, sex, age and alcohol to confirm that our cohort was representative of other populations used in similar studies. Finally, correlation between severity of fibrosis and severity of inflammation were investigated to confirm that the inflammatory response in CHC is associated with progressive fibrosis.

For the regression analysis fibrosis, inflammation and excess alcohol history were ascribed a bi-modal score whilst age and duration of infection were used as integers.

There were no significant correlations between the frequency of HLA DQB1*0301 and severity of fibrosis, inflammation or total IPA score, age, sex, alcohol intake or duration of infection. (Table 3.05)

		Mild Fibrosis	**Age**	**Sex F**	**High Alcohol**	**Duration**	**IPA Mild**	**IP Mild**
DQB1 *0301 Positive	Pearson Correlation	0.051	0.076	0.018	0.057	0.184	0.01 1	-0.05
	Signif. (2 tailed)	0.544	0.365	0.832	0.575	0.066	0.9	0.666
	No.of Pts	142	144	144	98	101	127	77

Table 3.05 Correlation between HLA DQB1*0301 positivity and recognised confounding factors for severity of liver disease in CHC

Regression analysis of the data controlling for sex, alcohol intake, duration of infection, and age failed to show a significant correlation between frequency of HLA DQB1*0301 and severity of liver disease.

Pearson Correlations between the well recognised confounding factors for severity of liver disease in CHC revealed relationships consistent with those found by other groups. There were positive correlations between severe fibrosis and severe total IPA and high alcohol intake, and severe total IPA and duration of infection and age (as evidenced by negative correlations with mild disease in the bimodal analyses). In addition, there was a positive correlation between mild total IPA and mild fibrosis. In contrast to other groups, there was no correlation demonstrated between sex and severity of liver disease. (Table 3.06)

		Age	**Sex Female**	**High Alcohol**	**Duration**	**Total IPA Mild**
Mild Fibrosis	Pearson Correlation	-0.198	0.151	-0.372	-0.219	0.82
	Sig (2 tail)	0.018	0.074	0.00	0.29	0.00
	No.of Pts	142	142	97	100	126
Age	Pearson Correlation	1	0.085	-0.75	0.6	-0.294
	Sig (2 tail)		0.313	0.463	0.00	0.001
	No.of Pts		144	98	101	127
Sex Female	Pearson Correlation	0.85	1	-0.291	-0.088	0.1
	Sig (2 tail)	0.313		0.004	0.381	0.264
	No.of Pts	144		98	101	127
High Alcohol	Pearson Correlation	-0.075	-0.291	1	-0.034	-0.343
	Sig (2 tail)	0.463	0.004		0.766	0.001
	No.of Pts	98	98		80	86
Dura-tion	Pearson Correlation	0.6	-0.88	-0.34	1	-0.29
	Sig (2 tail)	0.00	0.381	0.766		0.006
	No.of Pts	101	101	80		90

Table 3.06 Correlations between confounding factors and severity of fibrosis and total liver disease in CHC. (Positive correlations are shown in blue and negative correlations in red)

When the partial correlation coefficient for the correlation between severity of fibrosis and high alcohol intake was calculated, using regression analysis controlling for female sex, age, DQB1*0301 positivity and duration of infection, there was a highly significant relationship. There was a negative correlation between the presence of mild fibrosis and high alcohol intake signifying a positive correlation between high alcohol intake and fibrosis; partial correlation coefficient -0.3023 (p=0.008, n=73). Finally, the relationship between severity of inflammation and severity of fibrosis as independent indices of severity of liver disease was examined. This was to see if there was a link between inflammation and fibrosis in CHC, which could support the hypothesis that it is the inflammatory response which drives the fibrosis. (Table 3.07)

		Mild Fibrosis	**Mild IP Score**	**Mild Total IPA**
Mild Fibrosis	Pearson Correlation	1	0.450	0.82
	Signif. (2 tailed)		0.00	0.00
	No.of Pts		76	126
Mild IP Score	Pearson Correlation	0.45	1	0.788
	Signif. (2 tailed)	0.00		0.00
	No.of Pts	76		60

Table 3.07 Correlations between Severity of Fibrosis, Inflammation and Total IPA score

3.5 Discussion

The rationale for examining the relationship between frequency of HLA DQB1*0301 and severity of disease in chronic hepatitis C infection is founded in observations from other viral infections *(Thursz 1995)*. In addition, it is well described that this allele is protective from the development of chronic infection *(Tibbs 1996; Alric 1997; Thursz 1999)*. This may suggest that antigen presentation through this allele is critical to the nature and magnitude of the immune response in HCV infection. Another possible explanation for this phenomenon is that the HLA DQB1*0301 allele is in linkage disequilibrium with another gene that influences the outcome of HCV infection, such as a member of the tumour necrosis factor α (TNF-α) genetic locus or the transporters associated with antigen processing (TAP) genes. TNF-α gene loci are closely related to the DQB class II alleles and certain polymorphisms may associated with over- or under-expression of the cytokines, leading to possible skewing of the immune response. Polymorphisms appear frequently in the TAP1 and 2 genes which are also coded in the MHC region, and these can affect antigen processing and the ability to load antigenic fragments into class I MHC molecules. If this were the case, then the DQB1*0301 could be acting as a marker for polymorphism in another gene.

Numbers in this study are small at present, and the frequency of HLA-DQB1*0301 in the CHC patients is 27%, directly comparable with the healthy controls (31.8%). This finding is in agreement with the study of Tibbs *et al.* which was based on a UK white Caucasian population similar to ours, but that study also included antibody positive RNA negative individuals *(Tibbs 1996)*. The finding of no statistically significant differences between HLA DQB1*0301 frequency in a CHC population and healthy controls is in contrast to the study of Thursz, but in agreement with Alric. The population studied in the Alric study is Northern European and therefore likely to have more ethnic similarity to the present population than that in the Thursz study, which includes Mediterranean patients *(Alric 1997; Thursz 1999)*. Both of these studies excluded resolved acute infections, as we did in the present study.

This study was designed specifically to investigate the correlation between HLA-DQB1*0301 and severity of liver disease in CHC, resulting in more power than

the Thursz and Alric studies which examined multiple alleles and were primarily investigating at their effect on viral clearance *(Alric 1997; Thursz 1999)*. Only the Tillmann study has demonstrated a correlation between DQB1*0301 and severity of liver disease *(Tillmann 2001)*. The Tillmann study found under-representation of this allele in patients with HCV related cirrhosis when compared with the local population. The patient population in Tillmann's study were from Hannover in Germany, and therefore likely to be more ethnically similar to our UK population. However, although Tillmann concluded that HLA DQB1*0301 is protective from severe liver disease in HCV infection, there were no data recording the frequency of this allele in mild HCV related liver disease in their local population. The present study aimed to HLA type a large group of patients with mild CHC liver disease and see if there was relative over-representation of the allele HLA DQB1*0301 when compared with patients with more severe liver disease. The results showed no significant differences in allele frequency between the 2 groups discordant for severity of liver disease. However, there was a clear relationship demonstrated between increasing age and alcohol consumption leading to increased severity of liver disease in CHC. This finding is in keeping with the current literature *(Poynard 2000)*.

When analyzing the data further by sub-group analysis, it was found that there was a statistically significant under-representation of the HLA DQB1*0301 allele in the group of patients with severe liver fibrosis but only mild inflammation. This under-representation was not seen in the groups where mild inflammation or severe fibrosis was considered in isolation, although there was a trend towards under-representation in the group with severe fibrosis which did not reach statistical significance. However, all but 2 of the patients with severe fibrosis but only mild inflammation had a history of excess alcohol abuse. Therefore, this extreme phenotype could represent alcoholic liver damage, and may not represent a solely hepatitis C induces pattern of liver injury. Numbers were too small to match the patients in this sub-group analysis for alcohol intake, which must be considered as a possible confounding factor to the apparently statistically significant under-representation of the allele.

Regression analysis of our cohort did confirm that the expected correlations between severity of liver disease and alcohol intake, duration of infection and age were present

in our population. We did not however, demonstrate the commonly seen relationship between more severe liver disease and male sex which has often been demonstrated in other populations. This more detailed analysis still failed to show any relationship between HLA DQB1*0301 and severity of liver disease.

If HLA-DQB1*0301 confers protection from severe fibrosis but susceptibility to more severe inflammatory changes in the liver in chronic hepatitis C infection this raises interesting questions about the mechanisms which underlie this phenomenon. It may be that the inflammatory response evoked in individuals with the DQB1*0301 allele is phenotypically anti-fibrotic. This may be due to the manner of antigen presentation in the DQB1*0301 positive antigen presenting cells, skewing towards a Th1 type pro-inflammatory anti-fibrotic phenotype in the $CD4^+$ cells. Another possibility is that this disease pattern may be related to other factors, such as TNF-α polymorphisms, leading to differential expression levels of the cytokine which are involved in inflammatory and fibrotic processes in the liver.
However, this hypothesis must be considered in the light of the regression analysis which showed a strong positive relationship between fibrosis and inflammation, in the absence of a relationship between either factor and HLA DQB1*0301 positivity. Targeting the small group discordant for severity of inflammation and fibrosis with HLA DQB1*0301 allele testing may help to clarify this apparent anomaly.

To further investigate the current hypothesis, consideration of other genes which may be in linkage disequilibrium with HLA-DQB1*0301 will be important. These would include other HLA alleles such as DRB1*1101, TNF-α polymorphisms and TAP genes. In addition, the effects of hetero- and homo-zygosity of these polymorphisms will give insight into their individual relevance and assist in determining the dominant factor. Twin studies would give also invaluable information about the importance of host genetic factors in the course of hepatitis C infection.

Chapter 4

Chapter 4

The $CD4^+$ Immune Response by Severity of Disease

4.1 Introduction

The $CD4^+$ T lymphocyte response in hepatitis C virus infection has been studied in patients with acute disease, chronic disease, and in patients with evidence of spontaneously resolved infection. These responses have been investigated using freshly isolated peripheral blood mononuclear cells *(Diepolder 1995; Hoffmann 1995; Diepolder 1997; Cramp 1999; Gerlach 1999; Rehermann 1999; Rosen 1999; Takaki 2000)*.

Previous studies have shown that resolution of infection is associated with a strong, $CD4^+$ proliferative response to at least one HCV antigen, and secretion of IFN-γ in response to multiple HCV epitopes. This suggests that the Th1 $CD4^+$ response correlates with viral clearance. In at least 40% of patients with evidence of previously resolved infection, these strong $CD4^+$ responses are maintained for many years *(Cramp 1999)*.

In addition, studies of acute HCV infection reveal that clearance of HCV RNA from the blood follows the development of strong $CD4^+$ proliferative responses. However, if these responses are not maintained then viraemia recurs, suggesting that for sustained clearance of HCV infection, the $CD4^+$ responses must be maintained *(Hoffmann 1995; Diepolder 1997)*.

In chronic hepatitis C infection, the $CD4^+$ proliferative responses are weak and not always associated with demonstrable HCV antigen specific cytokine secretion. If there are significant responses, these are usually directed against at most one HCV antigen. However, early studies grouped all the outcomes of CHC as "chronic hepatitis", without regard to severity of liver damage *(Cramp 1999)*. Some attempt has been made more recently to correlate HCV specific $CD4^+$ T cell responses with severity of liver disease, and has found that class II restricted HCV specific proliferative and Th1 cytokine responses are found more frequently in CHC patients with more severe liver disease *(Rico 2002)*.

If HCV infection is not cleared in the acute phase, there is an infiltration of the

liver with T and B lymphocytes, suggesting an on-going local immune response to the virus in the liver *(Bedossa and Poynard 1996)*. It is generally accepted that the liver damage in CHC is a direct result of this ongoing immune responsiveness *(Koziel 1997)*. It may be that the differences in severity of liver damage in CHC between individuals are the result of, and reflected in, variations in the virus specific $CD4^+$ response. To date, there has not been a thorough investigation of the $CD4^+$ response in patients with established chronic hepatitis C infection discordant for severity of liver disease.

4.2 Aims

We aimed to investigate the HCV antigen specific $CD4^+$ immune response in patients with chronic hepatitis C infection, and correlate the magnitude and phenotype of this response with the severity of the resultant liver disease.

4.3 Methods

Fresh peripheral blood mononuclear cells were isolated from heparinised whole blood by density gradient centrifugation. The cells were established in culture in the presence or absence of recombinant HCV proteins. The cells were then used in lymphocyte proliferation assays or FACS analysis for surface markers of activation and intra-cellular cytokines. Parallel cultures were established to allow harvest of supernatants for ELISA based estimation of cytokine production. Positive control cultures used either mitogen stimulation, or antigens derived from influenza and tetanus toxoid. For a full description of the methods, see *Materials and Methods* chapter.

4.4 Results

4.4.1 $CD4^+$ Lymphocyte Proliferation in Resolved Acute HCV Infection versus Chronic Hepatitis C Infection

To confirm the differences between the $CD4^+$ lymphocyte proliferation responses in resolved acute and chronic hepatitis C infection in our cohorts, HCV specific lymphocyte proliferation was measured in patients representing the 2 groups, together with a group of healthy controls (Figure 4.01).

Significant HCV antigen specific lymphocyte proliferation (proliferation index >2.5) was found in 50% (3/6) of the patients with resolved acute infection. Of all of the HCV antigen specific assays performed in these HCV antibody positive/RNA negative patients, 29% (10/25) were significantly positive.

In contrast, only 6.5% (2/31) patients with chronic hepatitis C demonstrated HCV antigen specific lymphocyte proliferative responses. This represents 2.33% (3/130) of all of the HCV antigen specific assays in the HCV antibody positive / HCV RNA positive individuals. The difference between the assays in resolved acute patients and CHC patients was statistically significant (p=0.001 by Fishers exact test, with respect to numbers of antigens recognised). (Table 4.01)

HCV Status	No. of Patients	No. of Patients with Significant Proliferative Responses (%)	No. of Assays	No. of Assays with significant proliferative Responses (%)
Resolved Acute HCV Infection	6	3 (50)	25	10 (29)*
Chronic Hepatitis C	31	2 (6.5)	130	3 (2.3)*

Table 4.01 Frequency of significant HCV Specific lymphocyte proliferative responses in resolved acute versus chronic hepatitis C infection
**p=0.001 Fishers exact test*

To ensure that the low magnitude of the HCV specific $CD4^+$ proliferation response in chronic hepatitis C did not represent a global immunosuppression, the patients were tested against non-HCV recall antigens (Figure 4.02). When compared with HCV specific proliferative responses, the magnitude of the responses to non-HCV recall antigens is strikingly high. In addition, the frequency at which significant responses occur is much greater than with the HCV antigens. (Table 4.02)

These responses were comparable with a group of healthy control individuals who are HCV antibody and RNA negative (fig 4.01 and 4.02).

Recall Antigen	No. of CHC Patients	No. of Patients with Significant Proliferative Responses (%)	No. of Healthy Controls	No. of Controls with Significant Proliferative Responses (%)
HCV Antigens	31	2 (6.6)	6	0 (0)
Influenza	10	8 (80)	6	5 (83.3)
Tetanus Toxoid	9	8 (88.9)	6	6 (100)

Table 4.02 Frequency of significant antigen specific lymphocyte proliferative responses to HCV and other recall antigens in chronic hepatitis C patients

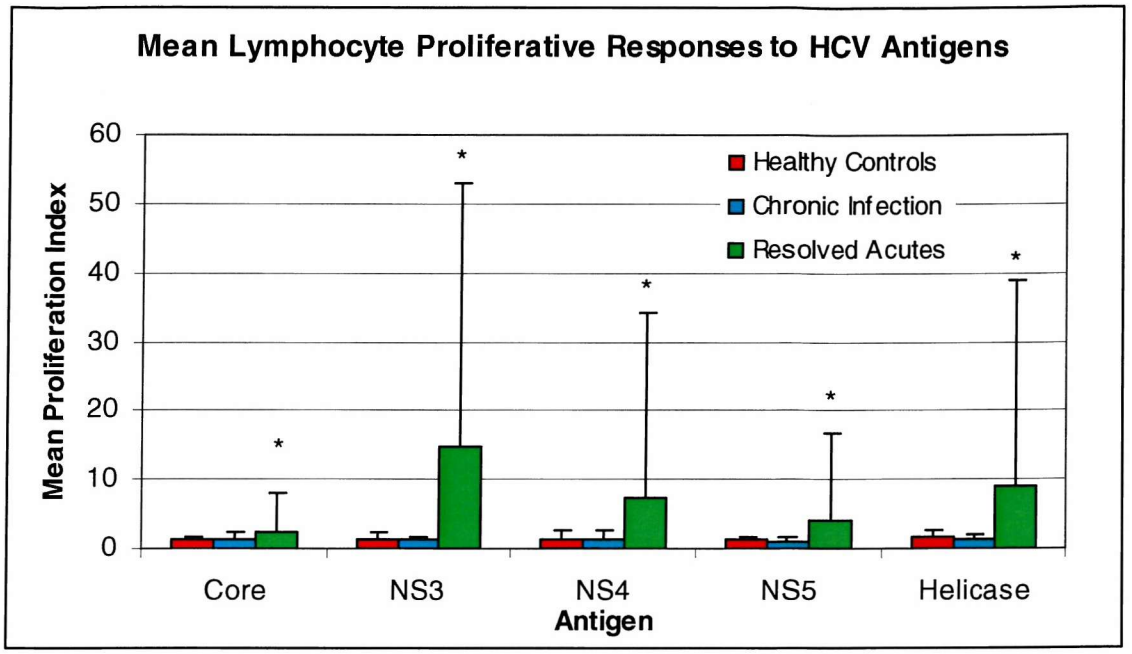

Figure 4.01 HCV Antigen Specific Lymphocyte Proliferation Indices in Resolved Normal healthy controls (n=6), resolved acute hepatitis C (n=6) and chronic hepatitis C infection (n=31)
*$p \leq 0.02$ *when comparing Resolved acute with Chronic Hepatitis C*

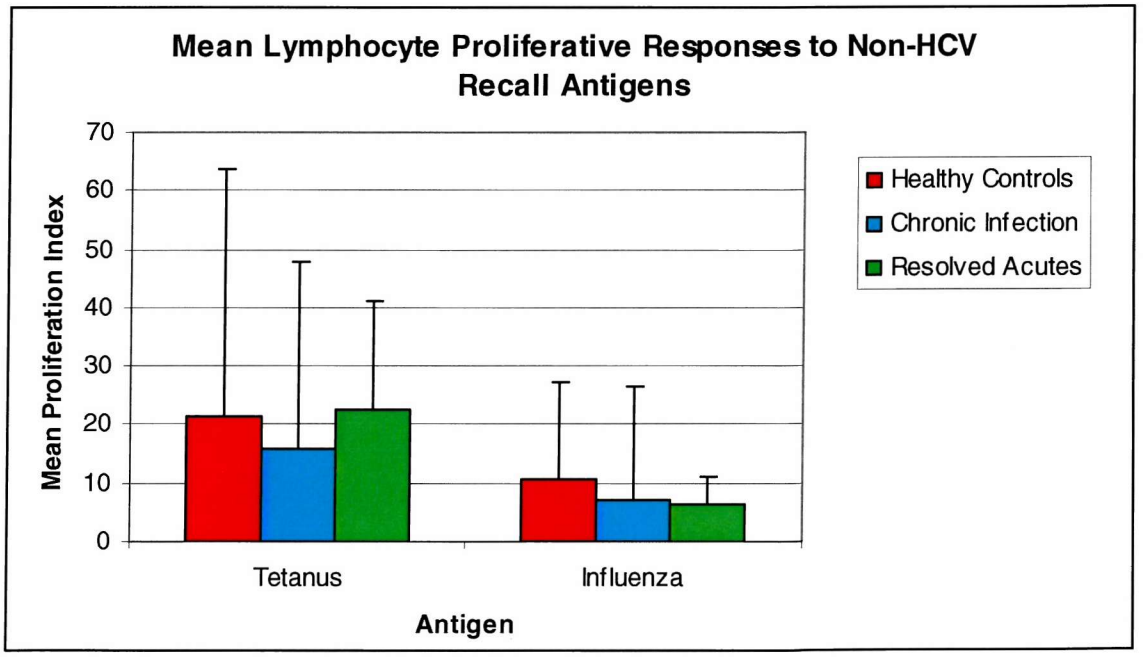

Figure 4.02 Antigen Specific Lymphocyte Proliferation Indices to non-HCV Recall antigens in Patients Normal Controls (n=6), chronic infection (n=10) and resolved acute infection (n=3). There are no statistical differences between the groups.

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4.4.2 $CD4^+$ Lymphocyte Proliferation and Cytokine Production in Chronic Hepatitis C Infection, Analysed by Severity of Liver Disease

There are striking differences in the HCV specific lymphocyte proliferative responses between patients with chronic hepatitis C infection and resolved acute infection. In order to test the hypothesis that the magnitude of the $CD4^+$ proliferative response in CHC correlates with the severity of the resultant liver disease, patients were stratified according to their liver histology score. The components of the IPA score (*see Materials and Methods)* were added together to provide a total IPA score. (Table 4.03). Sub-group analysis using the inflammatory elements (I and P scores) and the architectural elements (A score) as separate stratifying components did not produce significantly different results from using the IPA score as a whole.

Total IPA Score	Categorisation of Liver Disease
<4	Mild Liver Disease
4	Moderate Liver Disease
>4	Severe Liver Disease

Table 4.03 Classification of Severity of Liver Disease using IPA Liver Biopsy Score

4.4.3 HCV Peptide Specific Lymphocyte Proliferation

Initially, $CD4^+$ proliferation experiments were undertaken using HLA class II restricted peptides. The HCV core and NS3 peptides were chosen because they are well studied in the literature, and display promiscuous class II binding specificity. Furthermore, the NS3 peptide is believed to be immunodominant. *(Hoffmann 1995; Diepolder 1997)*

HCV Core peptide: Amino acid 23-42 KFPGGGQIVGGVYLLPRRGP

HCV NS3 peptide: Amino acid 1248-1261 GYKVLVLNPSVAAT

There were no differences in the mean HCV peptide specific $CD4^+$ proliferative responses between the patient groups discordant for disease severity (figure 4.03). There was considerable inter-individual variation between patients in each of the groups (figure 4.04). This heterogeneity may represent variability in HLA class II binding, or may represent differences in exposure to wild –type virus of variable genotype. Although the peptides were chosen for their good conservation across various HCV genotypes, and their promiscuous class II binding, there will be differences in both of these factors between individuals. Such heterogeneity in the test population confers difficulties and limitations to studies of large groups using peptides. However, there was no correlation between the presence of the allele HLA DQB1*0301 and magnitude of HCV peptide induced $CD4^+$ responses.

The level of significance of the proliferation index was determined for each peptide according to convention by testing the proliferative responses to this peptide in HCV negative controls. The mean proliferation index + 2 standard deviations was calculated and used to define the upper limit of normal. This gave the threshold of significance as 1.32 for the NS3 peptide.

There were no significant differences between the results of the HCV core or immunodominant NS3 peptide specific lymphocyte proliferation between patients discordant for severity of liver disease.

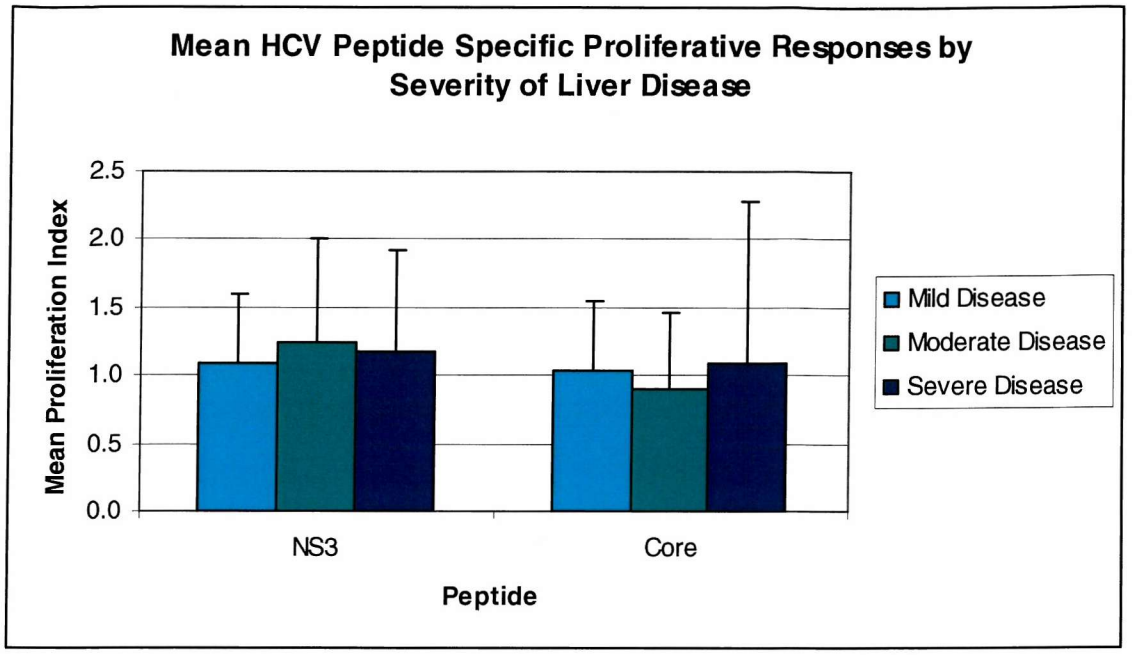


Figure 4.03 Mean Antigen Specific Lymphocyte Proliferation Indices to HCV Peptides in Patients with Chronic HCV Infection Discordant for Severity of Liver Disease
(Mild disease N=22, Moderate disease N=8, Severe Disease N=18)

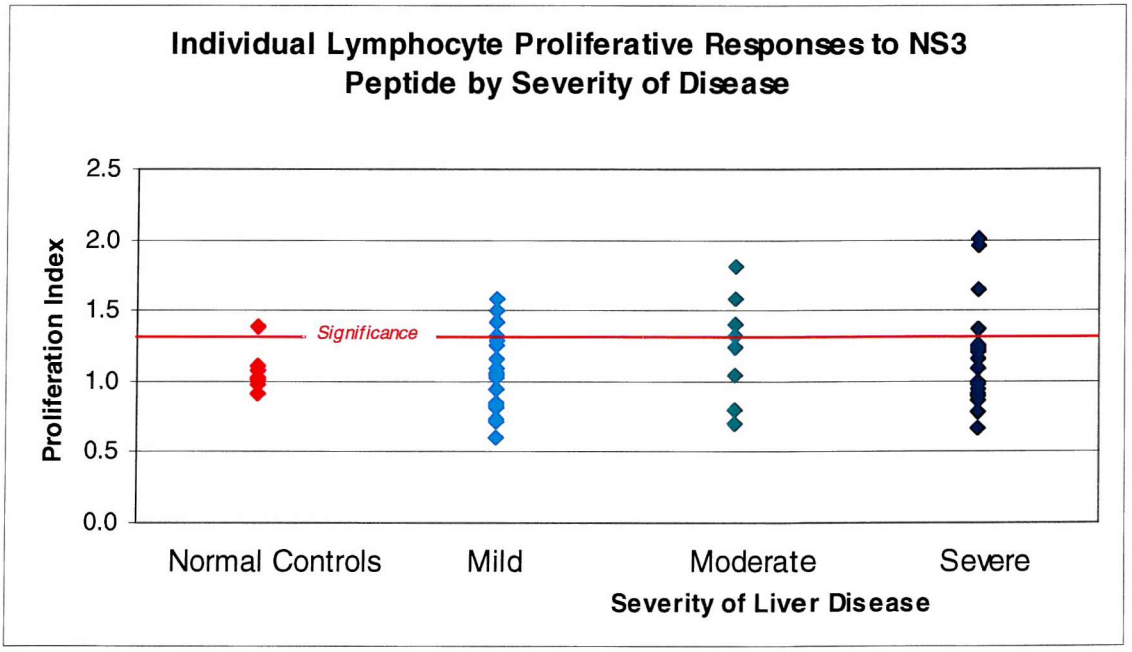


Figure 4.04 Antigen Specific Lymphocyte Proliferation Indices to HCV NS3 Peptide in Patients with Chronic HCV Infection Discordant for Severity of Liver Disease
(Mild disease N=22, Moderate disease N=8, Severe Disease N=18)

4.4.4 HCV Protein Specific Lymphocyte Proliferation

As explained above, the HCV peptides were chosen for their promiscuous MHC class II binding properties, but there are limitations to working with peptides in this system. Despite promiscuity in binding, there will inevitably be individuals that do not have appropriate class II molecules to present a specific peptide. In addition, the peptides each represent a single epitope, and as there is not a universally immuno-dominant epitope, it is not possible to predict how many patients might be expected to react to any single epitope.

To address these problems, experiments were undertaken using recombinant HCV proteins (figure 4.05). This allows processing of antigen by antigen presenting cells (APC), that generate epitopes appropriate to the HLA restriction of the APC. In addition, many epitopes will be generated from each protein, increasing the chance of the presence of an immunoactive peptide in the assay. The details of the patient groups are shown in table 4.04.

Severity of Liver Disease	Mean Age (Range)	M/F (%Male)	Total No. of Patients
Mild	38.36 (32-46)	9/5 (64.3)	14
Moderate	46.67 (40-51)	4/2 (66.7)	6
Severe	46.64 (41-53)	9/2 (81.8)	11

Table 4.04 Demographics of CHC patients used in HCV protein specific lymphocyte proliferation assays

There were no differences in the HCV protein specific $CD4^+$ proliferative response between the patient groups discordant for disease severity (figure 4.06).

To eliminate bias from age and sex, where possible the results were further analysed with patients matched across the groups for these variables. There were no differences in the HV proteins specific $CD4^+$ proliferative response between the patient groups discordant for disease severity when the groups were age and sex matched (figure 4.07).

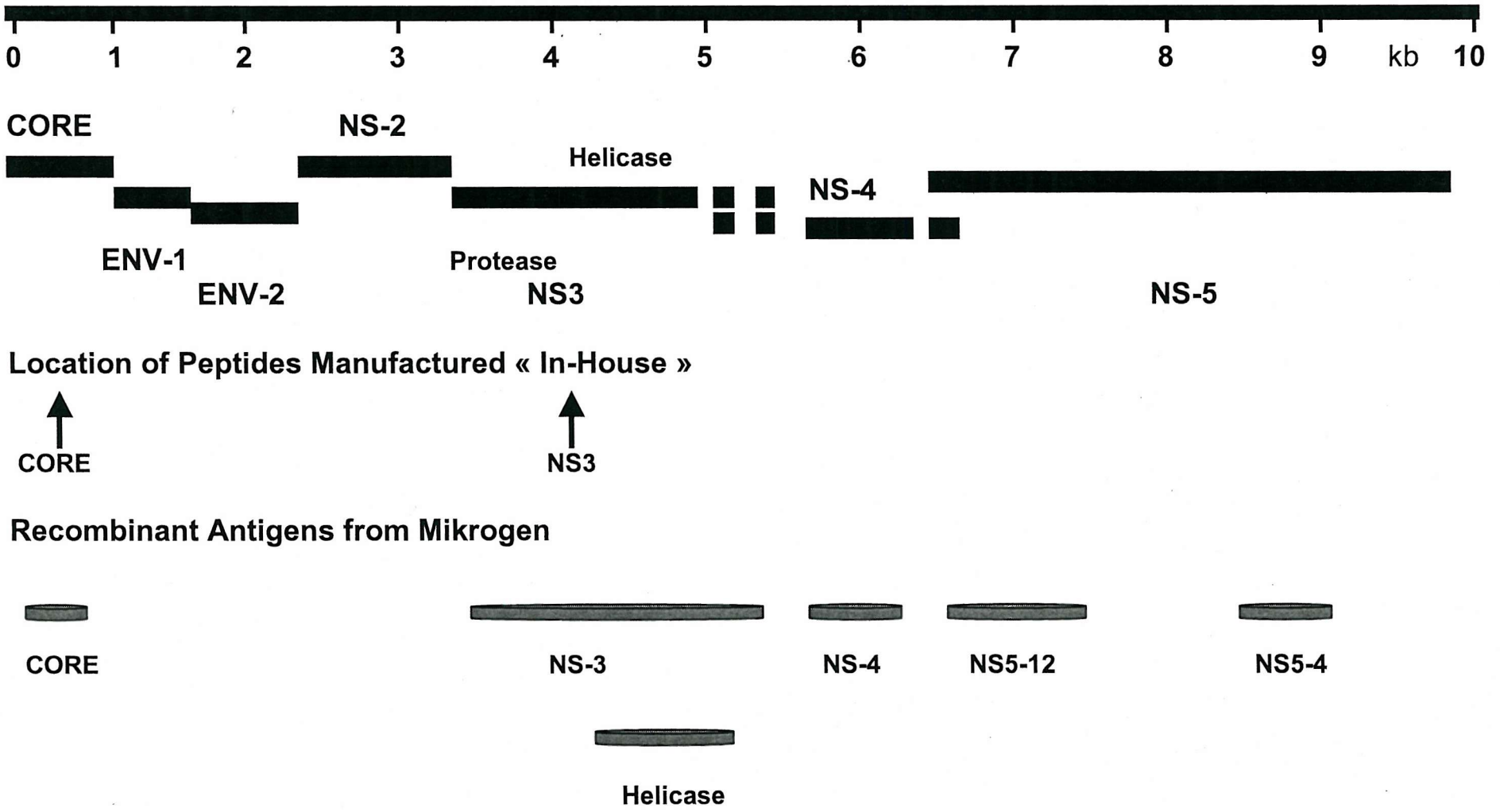


Figure 4.05 Hepatitis C Virus: Localisation of viral proteins and recombinant proteins supplied by Mikrogen

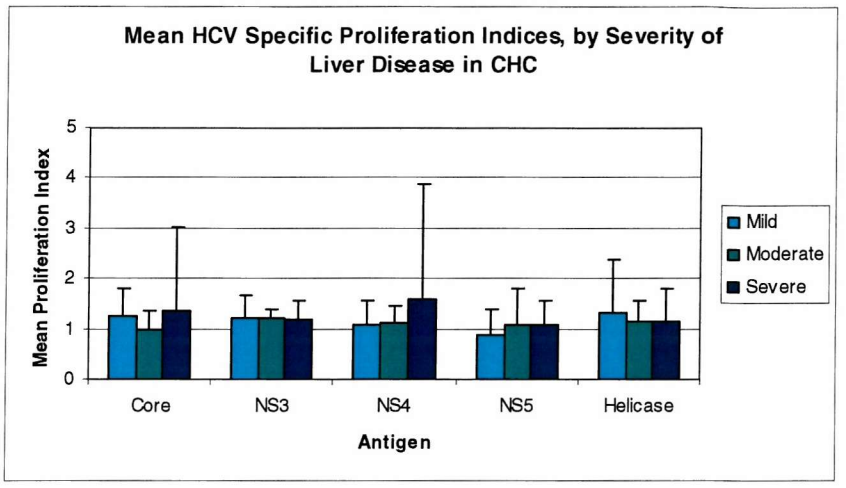
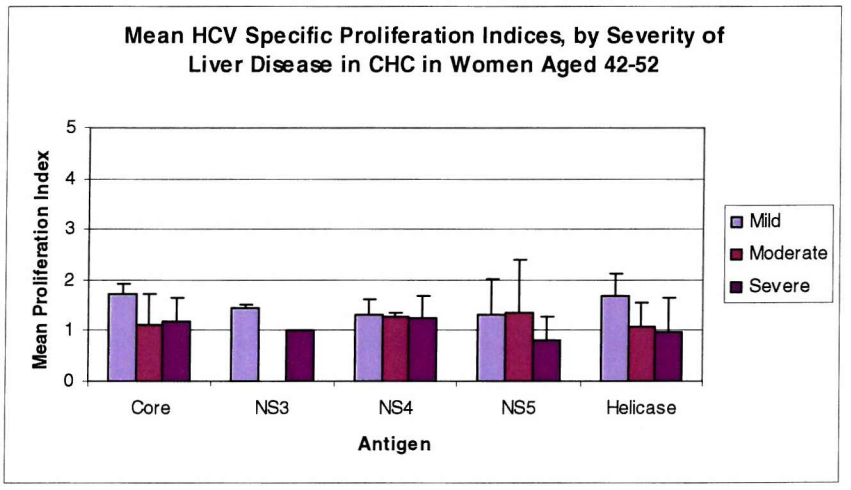
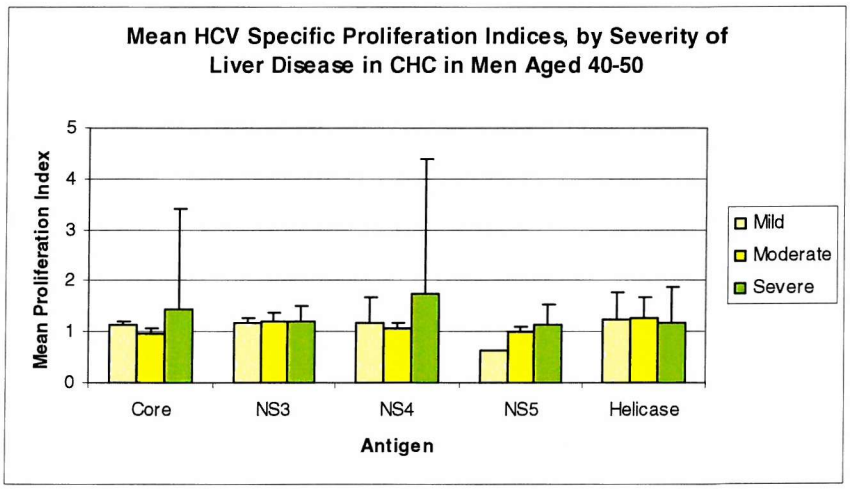


Figure 4.06 Antigen Specific Lymphocyte Proliferation Indices to HCV Proteins in Patients with Chronic HCV Infection Discordant for Severity of Liver Disease



A



B

Figure 4.07 **A** Antigen specific lymphocyte proliferation to HCV proteins in women aged 42-52 with chronic HCV infection by severity of liver disease
B Antigen specific lymphocyte proliferation to HCV proteins in men aged 40-50 with chronic HCV infection by severity of liver disease

4.4.5 HCV Protein Specific Lymphocyte Cytokine Production

In chronic hepatitis C infection, $CD4^+$ lymphocyte proliferative responses do not correlate with the severity of the liver damage which occurs. $CD4^+$ activation in response to antigen stimulation is characterised not only by proliferation, but also cytokine production. Furthermore, fully activated T cells may not proliferate further *in vitro* in response to the same antigen. There is also emerging evidence that some HCV proteins, NS4A and B, may inhibit protein synthesis and lymphocyte proliferation. *(Kato 2002)*

The pattern of cytokine production defines the Th1 / Th2 polarity of the response, but the amount of cytokine production may vary between individuals.

Cytokines have well-defined effects on the inflammatory and fibrotic response, which may profoundly influence the nature of the resultant liver disease in chronic hepatitis C infection.

To investigate the phenotype and magnitude of the cytokine response in CHC patients with known severity of liver disease, Enzyme Linked Immuno-Sorbant Assay (ELISA) was used to quantitate Interferon-gamma (IFN-γ) production by freshly isolated peripheral blood mononuclear cells stimulated with hepatitis C antigens. The cells were cultured in the presence of HCV antigen or appropriate positive (PHA) or negative controls for 3 days, and the supernatant was harvested for use in the sandwich ELISA. The numbers of patients in each group, and the frequency of positive findings is shown in table 4.05. The individual results are represented graphically in Figure 4.08, by nature and severity of liver disease (all of the patients in the moderate disease group had an total IPA score of 4, but a fibrosis score of only 1). All the patients had high levels of IFN-γ production with PHA stimulation. Of the mild disease patients, only one had an IFN-γ response to any of the HCV antigens, and this was solely against the NS3 protein. None of the moderate disease patients had any IFN-γ responses to any of the HCV antigens.

2 out of 8 severe disease patients had antigen specific IFN-γ production to HCV antigens; 1 patient to NS3 and NS4, and the other to core, NS4, NS5 and helicase. The severe disease patient with IFN-γ production to 4 antigens also had a high background level of IFN-γ production in the unstimulated cells. However, IFN-γ levels in the HCV antigen stimulated cells were at least 45% higher than the baseline level in this patient.

3 out of 4 resolved acute patients had antigen specific IFN-γ production to the HCV antigens; the first reacted only to helicase, the second to NS3, NS4 and helicase, and the third to core, NS3, NS4 and helicase.

The difference in the number of patients with responses was significant when the CHC group as whole were compared with the resolved acute group.

The number of positive assays in each of the groups as a whole were significantly different when resolved acute were compared with all CHC patients, or with the severe disease patients (stratified by total IPA score). On this occasion there was a significant difference when considering the architectural element of the IPA score as a sole component. There was a statistically significant difference when comparing the mild fibrosis sub-group (fibrosis score 0 or 1) with the severe fibrosis sub-group (fibrosis score 2 or 3).

Severity of Liver Disease	No. Patients with a response (%)	Total No. of Patients Tested	No. of Antigen Specific Responses (%)	Total No. of Responses measured
Resolved Acute Infection versus All CHC and Stratified by Severity of Disease				
Resolved acute Infection	3 (75)*	4	7 (44.4)	18**‡
All CHC (Mild, Mod and Severe)	3 (14.3)*	21	7 (7.2)	97‡
Mild Disease	1 (11)	9	1(2.6)	39
Moderate Disease	0 (0)	4	0 (0)	18
Severe Disease	2 (25)	8	6 (15)	40**
Comparison of Mild versus Severe Fibrosis Sub-groups				
Mild Fibrosis (A score 0 or 1)	1 (7.7)	13	1 (1.75)	57‡‡
Severe Fibrosis (A score 2 or 3)	2 (25)	8	6 (15)	40‡‡

Table 4.05 Frequency of HCV antigen specific interferon-γ production in freshly isolated peripheral blood mononuclear cells, by severity of liver disease
*p=0.031, by Chi Square. Relative risk=0.19 (Taylor series 95% Confidence Intervals 0.19<RR<0.63)
**p=0.22 by Chi square. OR 0.22 (0.05-0.92) RR 0.34 (0.14-0.83)
‡p<0.001 by Chi square. OR 0.09 (0.02-0.34) RR 0.15 (0.06-0.36)
‡‡p<0.02 by Chi Square RR Severe Fibrosis 8.55 (1.07-68.31)

Transforming Growth factor-beta (TGF-β) has an important role in activation of stellate cells, promotion of a fibrotic response and the mediation of immune tolerance. Therefore, the production of TGF-β in the supernatants from the HCV stimulated PBMC was also assessed by ELISA. (Table 4.06)

There was no demonstrable antigen specific TGF-β production in any of the patients. However, the matched antibody pairs used for this ELISA were specific for active TGF-β and do not detect latent TGF-β. TGF-β is secreted with a latency

associated propeptide from which it must be cleaved to become biologically active. *(Gleizes 1997)*The lower limit of detection of this assay for active TGF-β was 10 pg/ml, and although prior activation of the TGF-β would undoubtedly have yielded measurable quantities of active TGF-β, it was felt that this was not physiologically relevant. A sample standard curve for the TGF-β ELISA, confirming that the assay was a technical success, can be found in the materials and methods. (figure 2.04)

Severity of Liver Disease	**No. Patients with a response**	**Total No. of Patients Tested**	**No. of Antigen Specific Responses**	**Total No. of Response measured**
Resolved acute Infection	0	3	0	14
Mild Disease	0	6	0	27
Moderate Disease	0	3	0	14
Severe Disease	0	9	0	44

Table 4.06 Frequency of HCV antigen specific TGF–β production in freshly isolated peripheral blood mononuclear cells, by severity of liver disease

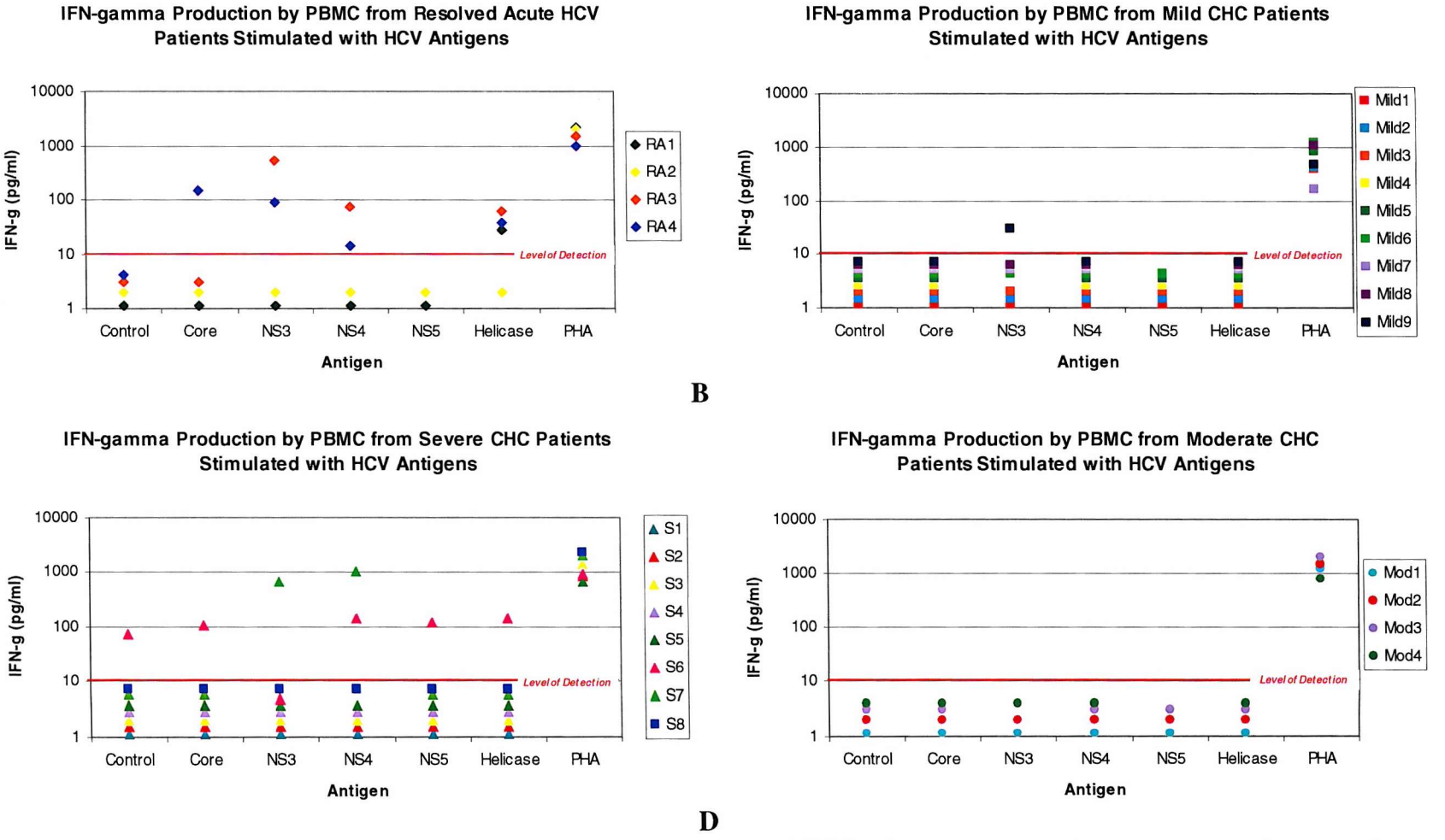


Figure 4.08 Interferon–γ levels detected in the supernatants of freshly isolated PBMC cultured for 3 days in the presence of HCV antigens or PHA (as a positive control): **A** Patients with resolved acute HCV infection **B** Patients with mild CHC liver disease **C** Patients with moderate CHC liver disease (and fibrosis score 0 or 1) **D** Patients with severe CHC liver disease (and fibrosis score 2 or 3).

4.4.6 Fluorescence Activated Cell Scanning Analysis of Intracellular Cytokine Production and Markers of Activation in HCV Antigen Stimulated Peripheral Blood Mononuclear Cells

As already demonstrated, lymphocyte proliferation by standard methods were not discriminatory between groups of chronic hepatitis C patients discordant for disease severity. This is possibly because the small subset of responsive cells was lost by dilution amongst the majority of non-responsive cells, as other groups have demonstrated the presence of HCV specific T cells in low numbers in peripheral blood. *(Minutello 1993; Cramp 1999)*In this experimental technique, peripheral blood mononuclear cells (PBMC) were isolated and maintained in culture for several days before cells were assayed.

IFN-γ secretion was discriminatory between groups of patients discordant for severity of CHC disease, with more antigen specific IFN-γ production seen in patients with more severe liver disease. However, although this technique is useful when looking at groups of patients, it still does not discriminate individual patients. In addition, this technique uses responses from cells in culture for several days prior to testing.

In order to study early responses of freshly isolated PBMC, cells were stimulated with recombinant HCV proteins and then assayed within 24 hours using the fluorescence activated cell scanner (FACS). Using this technique, it was possible to study cytokine production patterns and markers of activation (CD69) in a specific population of cells ($CD4^+$).

Cells were stimulated with antigen for 6 hours (HCV core or helicase, influenza), and treated with brefeldin-A for the last 5 hours of culture to paralyse export of cytokines. The cells were stained with antibodies to the surface markers CD4 and CD69, then fixed and permeabilised before staining for either IL-2, -4, -10 or IFN-γ. FACS used 3 colour fluorescence with anti-CD4 labeled with APC, anti-CD69 with FITC and anti-cytokine with PE, measured through FL4, FL1 and FL2 respectively.

The details of the patients used in the study are shown in table 4.07.

The results of the assays are shown in figure 4.09.

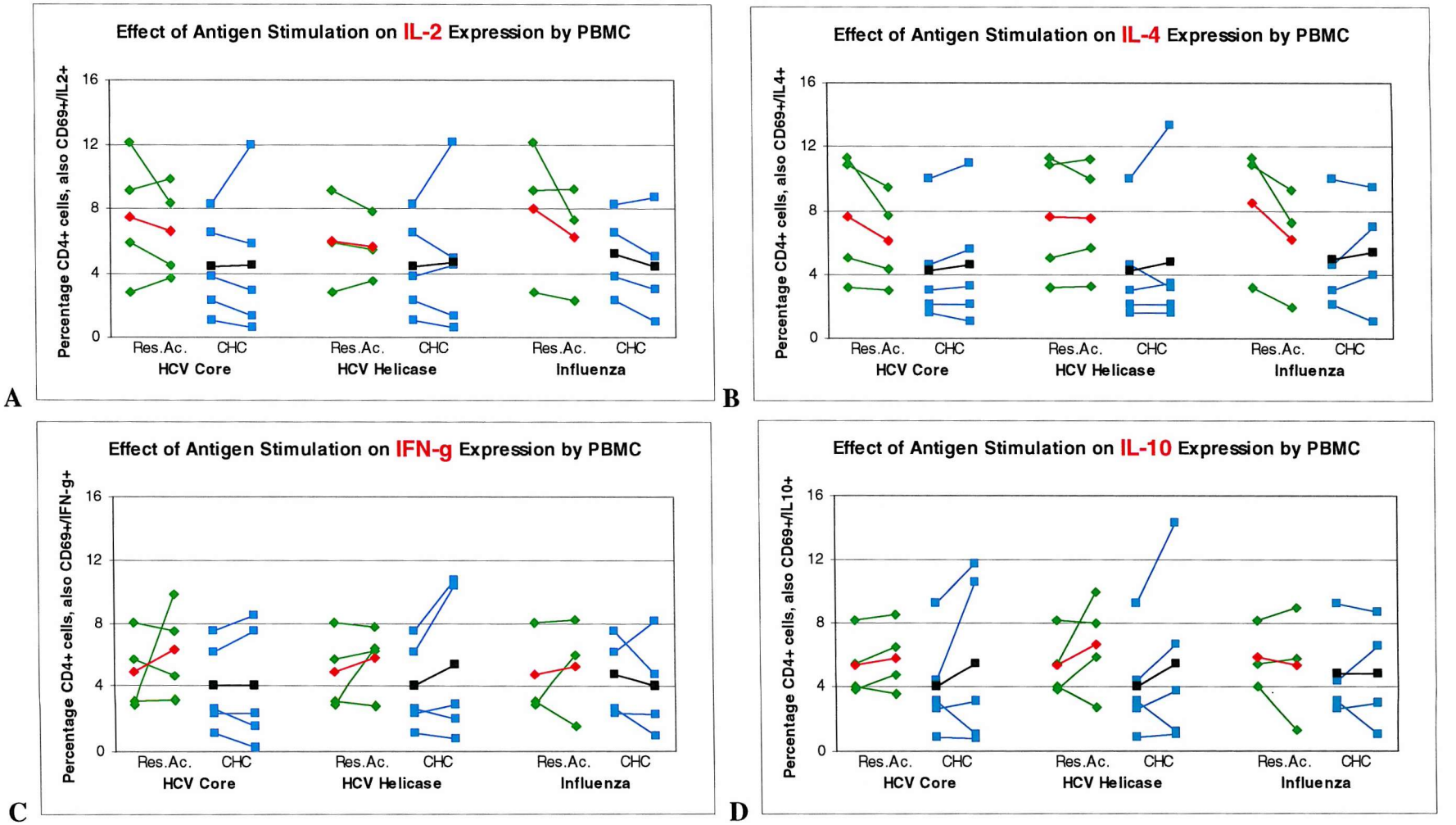


Figure 4.09 **Graphs** showing the change in percentage of $CD69^+$ cytokine expressing $CD4^+$ cells with antigen stimulation. Resolved Acute HCV patients are shown in green, with the mean in Red. CHC patients are shown in blue with the mean in black.
A IL-2 **B** IL-4 **C** IFN-γ **D** IL-10

HCV Status	Pt Number	Sex	IPA Score	Severity of Liver Disease
Resolved Acute	RA1	M	Not Applicable	
	RA2	M		
	RA3	F		
	RA4	M		
Chronic Hepatitis C	CHC1	M	1,0,0	Mild
	CHC2	M	2,1,1	Moderate
	CHC3	M	2,1,1	Moderate
	CHC4	M	3,1,2	Severe
	CHC5	F	3,2,2	Severe

Table 4.07 Details of patients used in the FACS study of responses in HCV

There were changes in the percentage of cells with cytokine expression following antigen stimulation. Although there were no significant differences between the groups, either between the resolved acute patients and the CHC patients, or between the groups of CHC patients discordant for disease severity there were differences in the trends.

Resolved acute patients had a trend towards an increase in IFN-γ and IL-10 expression on stimulation with HCV antigens, whilst there was a trend towards a decrease in IL-2 and IL-4 expression.

CHC patients had a trend towards an increase in all cytokine expression, most marked in IL-10 and IL-4.

These results are not statistically significant, but the trends support the hypothesis that resolved acute infection is associated with a Th1 response, whilst chronic hepatitis C is associated with a predominantly Th2 response.

4.4.7 Further Investigation and Confirmation of the Brisk $CD4^+$ Responses in Resolved Acute HCV Infection

The purpose of the investigation of the immune response in CHC was to identify differences between groups of CHC patients discordant for severity of liver disease. This would test the hypothesis that differences in the magnitude and phenotype of the $CD4^+$ response in CHC dictate the severity of the resultant immune mediated liver damage. I have demonstrated differences between discordant groups in terms of HCV antigen specific IFN-γ secretion, but there were no differences in proliferative response. However, there were marked differences in all assays between the CHC patient group and those with previously resolved acute disease.

The patients with resolved acute disease and brisk responses to the recombinant antigens did not respond to all of the antigens against which they were tested. This may reflect differences in sequence between the test antigens, and those to which the patient was exposed through natural infection. An alternative hypothesis is that the same mechanisms which suppress the detectable immune response in CHC are acting in these low magnitude response assays. TGF-β levels were low in all patients, which suggests that this cytokine is unlikely to be the key factor in any immuno-modulatory mechanism, but there are other cytokines, such as IL-10 which warrant consideration. To further investigate this possibility, 2 of the resolved acute patients who had previously shown brisk proliferative and IFN-γ responses were studied in more depth.

Initial experiments were designed to confirm that the proliferative responses were reflective of $CD4^+$ activation. In order to confirm this, freshly isolated PBMC were incubated with anti-CD4 coated magnetic beads, and then passed through a magnetic column to create 2 populations; the eluate which was deplete of $CD4^+$ cells and the captured fraction which was $CD4^+$ enriched. Standard proliferation assays and supernatant collections were then performed on the individual fractions. The proportion of CD4+ cells in the different fractions was confirmed by FACS analysis after staining with anti-CD4 antibodies (Figure 4.10, table 4.08). The results of these proliferation experiments are shown in figure 4.11.

The results of the cytokine assays on the supernatants are shown in figure 4.12. TGF-β was low in the supernatants from all fractions, and the results are not shown.

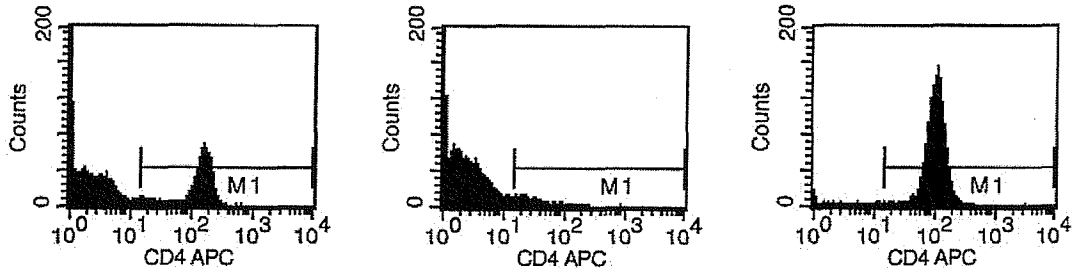
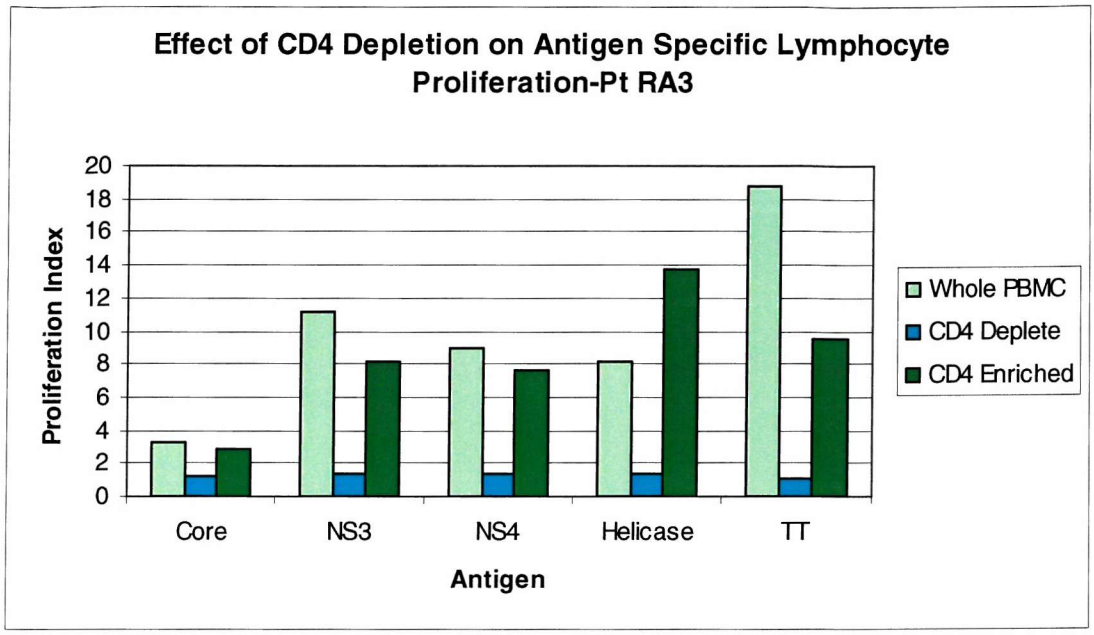


Figure 4.10 FACS analysis from CD4 depletion / enrichment experiment, results shown are from patient RA3
A Whole PBMC fraction
B $CD4^+$ Depleted
C $CD4^+$ Enriched

Patient	Percentage $CD4^+$ Cells		
	Whole PBMC	**CD4 Depleted**	**CD4 Enriched**
RA3	40.35	7.42	97.67
RA4	42.63	2.73	90.43

Table 4.08 Table showing the percentage $CD4^+$ cells in the different fractions of cells from patients RA3 and RA4; Whole PBMC and the 2 fraction after anti-CD4 Magnetic bead separation.



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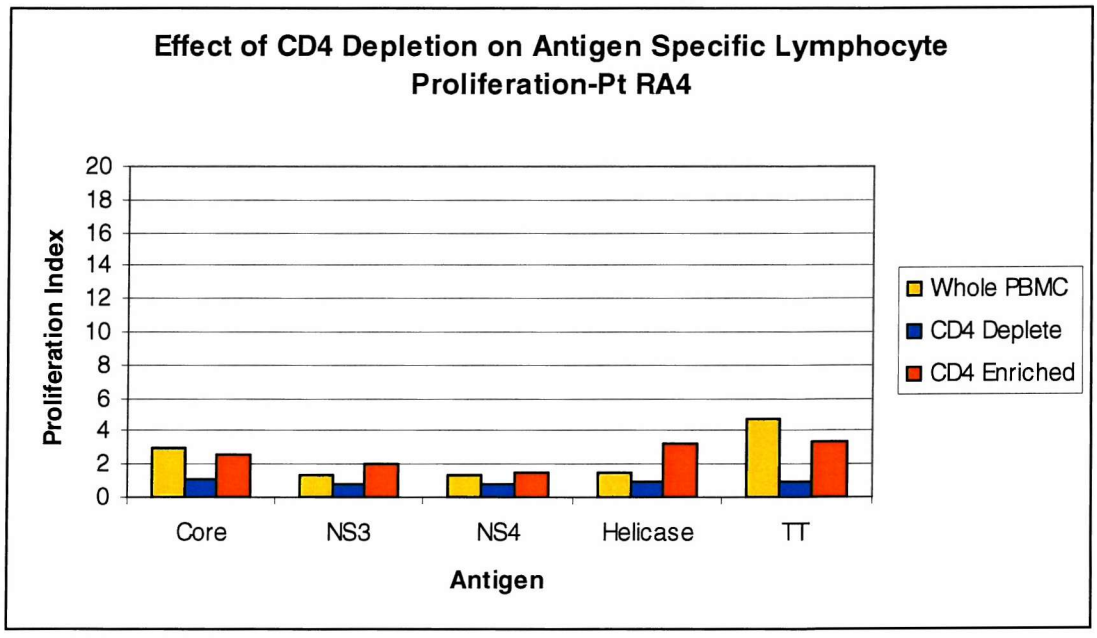


B

Figure 4.11 The effect of CD4 depletion on antigen specific lymphocyte proliferation in each of 2 resolved acute HCV infection patients. Each graph shows the antigen specific proliferation with whole PBMCs without magnetic bead separation, and each of the 2 fractions produced by the anti-CD4 magnetic bead positive selection process.

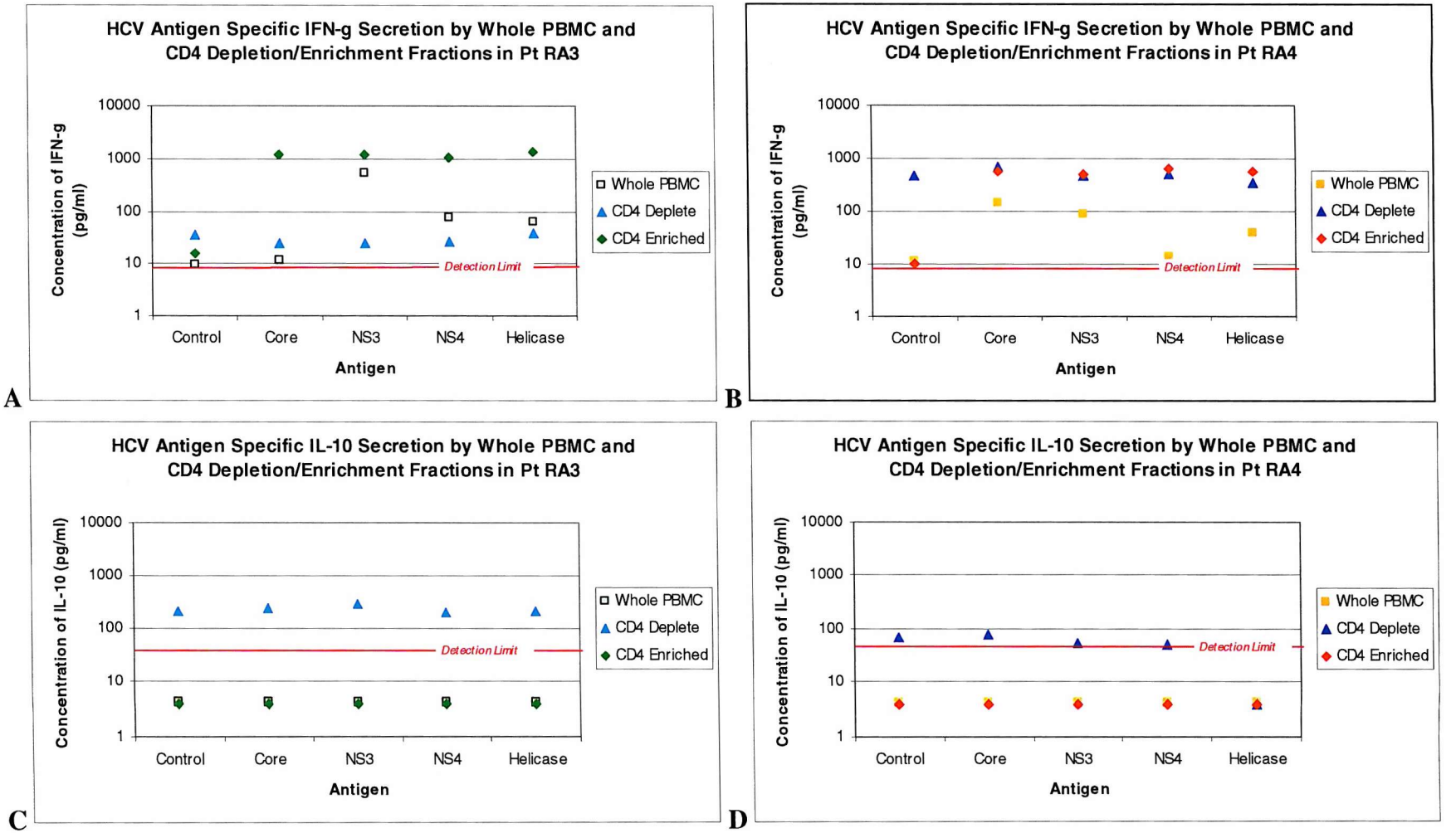


Figure 4.12 Graphs showing IFN–γ (A and B) and IL-10 (C and D) secretion into the supernatants of different fractions of HCV antigen stimulated PBMC. **A** and **C** from patient RA3 and **B** and **D** from patient RA4.

The anti-CD4 magnetic beads are designed to positively select a $CD4^+$ population. By using some of the freshly isolated PBMC as a whole sample, and some separated using the magnetic beads, it is possible to obtain a $CD4^+$ enriched population. Collecting the fraction of the bead treated PBMC not captured in the magnetic column, it is possible to also have a $CD4^+$ depleted population, the cells which make up the non-$CD4^+$ portion of the whole PBMC population. The FACS data confirms that there is at least 90% purity in the $CD4^+$ enriched population in the 2 donors used, and that there were no more than 7.5% $CD4^+$ cells in the depleted population.

Antigen specific proliferation varies with time amongst individuals, as the 2 subjects chosen for the experiments had much higher proliferation indices to some of the HCV antigens when they were previously tested (particularly RA4), and lower proliferation indices with others. However, cytokine responses were much more consistent.

There was no antigen specific proliferation to either HCV or non-HCV antigens in either patient with the $CD4^+$ depleted fraction. In all assays, the proliferation index was higher in both the whole PBMC sample and the $CD4^+$ enriched fraction, even if it did not reach significance. In patient RA3, all of the antigens produced a significant proliferative response with the whole PBMC sample and the $CD4^+$ enriched fraction. Patient RA4, had significant proliferation with core and tetanus toxoid using the PBMC sample; these responses were maintained in the $CD4^+$ enriched sample, but there was also a significant proliferative response to helicase with this fraction.

IFN-γ secretion showed a similar pattern in both patients. There was low background IFN-γ production in the whole PBMC portion, and each had antigen specific IFN-γ production to 3 HCV antigens (RA3; NS3, NS4 and Helicase: RA4; Core, NS3 and Helicase).

Both patients had high IFN-γ background production in the $CD4^+$ deplete fraction, which was maintained with all of the HCV antigens with no antigen specificity.

Both patients showed low background and high and homogeneous antigen specific IFN-γ production to all of the HCV antigens in the $CD4^+$ enriched fraction.

Background IL-10 production was low in both patients in the whole PBMC samples, there was no antigen specific production.

In the $CD4^+$ deplete fractions of both patients there was high background production of IL-10 which did not change with antigen stimulation. $CD4^+$ enriched fractions did not produce detectable background or antigen specific IL-10 in either patient.

TGF-β production was low in all supernatants collected.

4.5 Discussion

There are clear differences in the proliferative responses between patients who have resolved acute HCV infection and patients with chronic disease, both in percentage of patients with significant responses and the magnitude of those responses. This must be considered in light of the fact that patients with chronic infection have ongoing exposure to viral antigens whilst individuals with resolved acute infection (HCV antibody positive and HCV RNA negative) have often been aviraemic for many years. The lack of proliferative responses in chronic hepatitis C infection does not represent a global immuno-suppression. Testing against other recall antigens, such as influenza virus and tetanus toxoid, reveals that the responses of CHC patients mirror those of the resolved acute HCV individuals and normal controls. It is possible that the lack of reactivity to the recombinant HCV antigens used in this investigation is due to genotype and quasi-species difference in the virus strains infecting the experimental subjects but this is unlikely, as the same differences would affect the responses in the resolved acute HCV patients. In addition proteins were used rather than genotype specific peptides, this may allow generation of epitopes which are preserved across genotypes, although this cannot be guaranteed in all individuals.
A possible explanation for the difference in proliferative responses to HCV antigens and other recall antigens in CHC, is that hepatitis C virus can specifically suppress immune responses to itself in some individuals. If this occurred, chronic infection would be established. These experiments were not designed to investigate the mechanisms for such a phenomenon which are hampered by the lack of a small animal model of HCV infection, or an in vitro HCV culture system.

The liver damage in chronic hepatitis C is believed to be immune mediated, leading to the hypothesis that the discordance seen in the severity of the liver damage is due to differences in the immune response to the virus. The $CD4^+$ proliferation responses of the CHC patients have been correlated with the severity of the liver disease in each patient. There are no statistically significant differences in the HCV antigen specific

$CD4^+$ proliferative responses across the groups with mild, moderate or severe liver disease, even when controlled for age and sex.

The HCV specific cytokine responses from the same $CD4^+$ cells do differentiate between the groups. Using ELISA techniques to examine the supernatants of HCV antigen stimulated PBMC cultures for the pro-inflammatory cytokine IFN-γ and the pro-fibrotic cytokine TGF-β has revealed a significantly antigen-specific IFN-γ production in severe liver disease. The IFN-γ response in severe CHC liver disease is similar to that in resolved acute HCV infection.

Increased HCV specific IFN-γ production in severe CHC supports the hypothesis that the brisk and effective immune response seen in resolved acute infection can become harmful if viraemia is not cleared and chronicity is established. The proliferative and cytokine responses that are beneficial for virus eradication may contribute to liver damage if maintained over prolonged periods.

It might be predicted that because TGF-β is thought to be pro-fibrotic and IFN-γ is regarded as anti-fibrotic, that severe CHC liver disease would be associated with high TGF-β and low IFN-γ. The pattern of cytokine secretion found using ELISA techniques is contrary to this prediction. A possible explanation for this phenomenon is that although cytokines in isolation may have a predictable effect on liver fibrogenesis, in CHC they are part of a cytokine mêlée. I have only measured 2 cytokines using these methods, and have not looked for other cytokines which could have antagonistic effects. In CHC, it is possible that the predominant effect of TGF-β is in promoting immune tolerance rather than fibrosis, and that low levels in severe liver disease allow an unchecked immune response to damage the liver. Although IFN–γ is regarded as anti-fibrotic, it is also involved in inflammatory cell recruitment, and it may be that in severe CHC, this is the predominant effect. In this case it could be other cytokines produced by recruited inflammatory cells that promote fibrogenesis. Alternatively, although the cells are secreting an anti-fibrotic cytokine, they may signal directly to promote cell damage and fibrosis by direct cell to cell contact with hepatocytes and hepatic stellate cells.

The FACS data suggests that in CHC liver disease, HCV induces IFN–γ production in $CD4^+$ cells at a lower level than in resolved acute disease. In addition, IL-4 expression is increased in chronic disease and reduced in resolved acute disease. These data support the hypothesis that the $CD4^+$ response in CHC is predominantly Th2 and inadequate to cause viral clearance. There is a measurable IFN-γ response by $CD4^+$ cells in CHC, although it is less marked than the response in resolved acute disease which is associated with viral clearance. This IFN-γ response, whilst not representing a brisk Th1 response allowing viral clearance, may contribute to liver disease by inflammatory cell recruitment.

The $CD4^+$ depletion / enrichment experiments confirmed that the antigen specific responses that are seen using the techniques employed in the present study are generated by $CD4^+$ cells. However, there is significant IFN-γ and IL-10 production in the CD4 depleted fraction, which is not antigen specific, and not seen in the whole PBMC samples. This suggests that there are complex interactions in the whole PBMC samples; background IL-10 and IFN-γ from the non-$CD4^+$ fraction are suppressed to undetectable levels in the current assays, and some of the antigen specific IFN-γ production form the $CD4^+$ cells is also suppressed. Cytokine production in the $CD4^+$ deplete fraction is not associated with proliferation. In the whole PBMC samples and the $CD4^+$ enriched samples, there is sometimes significant proliferation with low levels of IFN-γ production (patient RA3, core, whole PBMC), but more often IFN-γ production without proliferation (patient RA4, NS3 and helicase, whole PBMC, and NS3 and NS4 with the $CD4^+$ enriched fraction).
TGF-β levels were low in all of the assays, which suggests that this is not important in any immunosuppressive mechanisms that may be involved in HCV induced $CD4^+$ responses, however, the current study suggests that IL-10 may be a more important immuno-modulatory cytokine in this system.
Future work should investigate the role of IL-10 in CHC immune responses.

One of the most clinically significant effects of chronic hepatitis C infection is liver fibrosis. Some patients will not develop fibrosis despite decades of infection, whilst others reach end stage fibrosis or cirrhosis within 10-20 years. The effector cells in the liver believed to be responsible for abnormal matrix deposition are the hepatic

stellate cells. These usually quiescent lipid storing cells can activate following a variety of insults to become myofibroblast-like cells responsible for type I collagen production and the disruption of normal basement membrane matrix. Future experiments to elucidate the effects of the $CD4^+$ response in CHC should explore any differential effects of the Th response on hepatic stellate cell activation. This would have to involve the effects of conditioned supernatants, and if HLA matching could be achieved, then experiments involving direct cell to cell contact.

Chapter 5

Chapter 5

The $CD4^+$ Response during Treatment Interferon-α and Ribavirin

5.1 Introduction

There is not yet an effective vaccination for hepatitis C virus. Instead, in the face of chronic infection, pharmacological strategies are employed to boost host immunity and act as anti-viral agents *(Hultgren 1998; Cramp 2000)*.

The major clinical problems in chronic hepatitis C infection are caused by liver fibrosis. Many complications of the disease can be prevented if this progressive fibrosis can be arrested or reversed *(Poynard 2000)*. Current treatment development strategies are aimed at achieving sustained virological clearance through direct anti-viral action and manipulation of the immune response. If these treatments could also reverse the fibrotic process, or be used in combination with anti-fibrotic agents, then mortality and morbidity could potentially be significantly reduced.

Until recently, the most effective treatment for CHC was Interferon-α injected thrice weekly in combination with daily oral Ribavirin. In patients with moderate to severe HCV related liver damage the rate of sustained virological response to this therapy was around 40% *(McHutchison 1998; Poynard 1998)*. It is generally believed that the predominant action of interferon-α is anti-viral, whilst the ribavirin serves to augment the host's immune response, possibly through the induction of interleukin 1 *(Hultgren 1998; Ning 1998)*. Cramp *et al.* published data showing that during such combination treatment, $CD4^+$ proliferative responses increase in magnitude. If these responses become significant and are maintained after the cessation of treatment, a sustained virological clearance is much more likely *(Cramp 2000)*.

Pharmacological engineering if Interferon-α has lead to the development of PEGylated interferon-α, so called because the interferon molecule is bound to a poly ethylene glycol (PEG) moeity. This slows the rate of absorption of drug from the injection site, allowing more sustained delivery and constant plasma levels. These

PEGylated-interferons can be administered once weekly, and trial data suggests that they are approximately twice as effective as standard interferon-α when used as monotherapy for CHC *(Heathcote 2000; Zeuzem 2000)*. PEGylated interferons are just undergoing licensing for use in the UK, but whilst waiting for approval, trials using the PEGylated molecules in combination with ribavirin have just been completed. Sustained virological response rates in CHC for combination PEGylated Interferon-α and ribavirin are in excess of 55% *(Fried 2001; Manns 2001)*. The effect of PEGylated interferon-α on T cell responses in CHC is undetermined at present. Other immunomodulatory strategies which have been considered in the treatment of chronic disease include; the use of cytokines to bias the immune response towards either a Th1 or Th2 phenotype; the use of cytokines to act as anti-fibrotic agents; specific blocking of the actions of pro-inflammatory or pro-fibrotic cytokines; the development of vaccines which target specific antigens (host or foreign) with an immune system "alert signal", such as tetanus toxoid, packaged alongside. Some of these mechanisms could potentially be used in the treatment of CHC *(McHutchison 1999; Rosen and Gretch 1999; Nelson 2000)*.

5.2 Aims

The aim of this study was to investigate the effects of treatment for CHC on antigen specific $CD4^+$ lymphocyte responses to HCV antigens.

5.3 Methods

A pilot study examined the effect of combination treatment with standard interferon-α and ribavirin on HCV specific lymphocyte proliferation in PBMC. 3 patients due to start treatment were identified through the routine Southampton HCV service; they were consented for regular donation of blood for research purposes. Standard lymphocyte proliferation assays were undertaken on freshly isolated PBMC at baseline and then at 4 monthly intervals from start of treatment. Cramp *et al.* published their study of $CD4^+$ responses on anti-viral therapy as the pilot finished.

Patients enrolled into a clinical trial of PEGylated Interferon-α in combination with Ribavirin for treatment of chronic hepatitis C were assayed for HCV specific $CD4^+$ lymphocyte proliferation at baseline (before starting treatment) and then at various points during treatment. In addition, FACS analysis for surface markers of activation and cytokine secretion after HCV protein stimulation was undertaken at regular intervals. The study followed patients for 6 months after cessation of treatment and correlated the results with virological response.

Patients were treated with 180μg PEGylated Interferon-α (with a PEG moiety of 40kDa) weekly and randomised to receive either the standard dose of ribavirin (1000mg or 1200mg daily, depending on weight) or 800mg daily. Doses were reduced or withdrawn after starting therapy if toxicity caused significant anaemia (ribavirin), neutropaenia or thrombocytopaenia (interferon). Patients were also randomised to receive either 24 or 48 weeks of treatment.

Viral loads were measured on serum separated from fresh whole, clotted blood. The assays used TAQman based technology for quantitation and were undertaken by Dr Steve Hadfield as part of his PhD work.

For the cellular assays, fresh peripheral blood mononuclear cells were isolated from heparinised whole blood by density gradient centrifugation. The cells were established in culture in the presence or absence of recombinant HCV proteins. These freshly isolated PBMCs were then used in lymphocyte proliferation assays or FACS analysis for surface markers of activation and intra-cellular cytokines. In some cases, parallel cultures were established to allow harvest of supernatants for ELISA based estimation of cytokine production. Positive control cultures used either mitogen stimulation, or antigens derived from influenza and tetanus toxoid.

For a full description of the methods, see *Materials and Methods* chapter.

5.4 Results

5.4.1 Pilot Study Examining Serial HCV Specific Lymphocyte Proliferation Whilst on Standard Therapy for HCV Infection

Three patients were recruited into the study. The details of the patients are shown in table 5.01. Patients were all treated with Interferon-α 3million units thrice weekly, and ribavirin 1000mg or 1200mg daily depending on body weight (>75kg 1200mg).

Patient	Age	Sex	Route of Infection	IPA Score on Liver Biopsy
X	46	F	Unknown	1,1,1
Y	32	M	IVDA	1,1,0
Z	47	M	IVDA	3,2,3

Table 5.01 Details of patients recruited into pilot HCV treatment study

Patient X was treated for 48 weeks, and patients Y and Z for 24 weeks each.

The PBMC from each patient were tested against 4 recombinant HCV antigens at each time point. Figure 5.01 shows the proliferation of the greatest magnitude for each patient at each time point.

Patient X achieved transient viral clearance only, despite continuation of therapy for 48 weeks, and was therefore a relapsed responder. Patients Y and Z both achieved viral clearance by 16 weeks treatment, and stopped treatment at 24 weeks. Patients Y and Z were both sustained responders to anti-viral therapy.

Patient Z had developed significant proliferative responses to 2 antigens (NS3 and Helicase) by 32 weeks (8 weeks after stopping treatment). These high magnitude responses were maintained for at least 24 weeks post cessation of treatment, and were associated with a sustained virological response. These responses were statistically significantly higher than responses at baseline ($p<0.05$ by students t test).
Patients X and Y did not develop significant proliferative responses to any of the HCV antigens at any time point. Patient X was a relapsing responder, and patient Y a sustained responder.

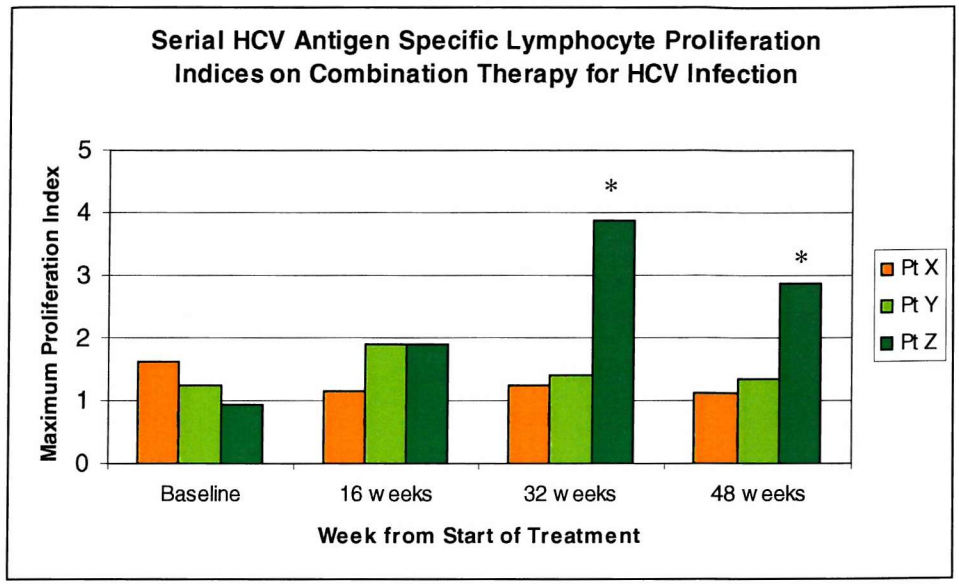


Figure 5.01 Serial lymphocyte proliferation indices for patients on combination treatment for CHC infection. For each patient, the proliferation index from the antigen which gave the greatest magnitude response is shown at each time point to demonstrate the maximal HCV specific $CD4^+$ response. *$p<0.05$

These results were entirely consistent with those published by Cramp *et al.* in their study of 28 patients undergoing treatment for CHC. The results from this pilot study were complete just as the published study came into the public domain. Therefore, to avoid needless repetition of a carefully conducted study, it was decided to focus the investigation of $CD4^+$ responses during treatment for CHC on patients being treated with the newly available PEGylated Interferon-α. It would then be possible to compare these results with the data of Cramp *et al.* from standard treatment regimes.

5.4.2 The Effect of PEGylated Interferon-α in Combination with Ribavirin on HCV Specific $CD4^+$ Responses

5.4.2.1 Patient Details

Eight patients were recruited into the study, but only 6 patients were studied at baseline due to problems with sample collection. The details of the patients are shown in table 5.02.

Changes in dosing are shown in table 5.03.

Pt A withdrew from the treatment trial at week 29, stopping all treatment and refusing further blood donation for several weeks. Pt G was withdrawn from the treatment trial at week 28, as he was still PCR positive for HCV at week 24 (he is included in the 24 week treatment arm of the study for analysis purposes).
The dose of ribavirin was reduced in patients B and H due to symptoms of breathlessness exacerbated by the induced haemolytic anaemia. Patients D and E both developed hypersensitivity to ribavirin, causing severe skin rashes and necessitating withdrawal of this drug.

Patient	Age	Sex	Route of Infection	IPA Score on Liver Biopsy
A	40	M	IVDA	2,0,2
B	50	M	IVDA	2,1,1
C	45	M	IVDA	2,2,2
D	36	M	IVDA	2,2,2
E	44	F	IVDA	2,1,1
F	56	F	Unknown	3,1,2
G	44	M	Unknown	2,1,2
H	49	M	IVDA	2,1,1

Table 5.02 Details of patients recruited into PEG-IFN / Ribavirin treatment study

Patient		Week of Treatment																		
		Base-line	1	2	3	4	5	6	7	8	9	10	11	12	13-14	15-16	17-24	25-29	30-36	36-42	42-48
A	I	180	180	0	180	0	90	90	90	0	0	45	45	45	45	45	45	45	NIL		
	R	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl			
B	I	180	180	180	180	180	180	180	180	180	180	135	135	135	135	135	135	135	135	135	135
	R	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	0	600	600	600	600	600
C	I	180	180	180	180	135	135	135	135	135	135	90	90	90	90	90	90	90	90	90	90
	R	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl
D	I	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180	NIL			
	R	Bl*	NIL																		
E	I	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180
	R	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	0	200	NIL				
F	I	180	180	135	90	90	90	0	45	45	90	90	0	45	45	45	45	45	45	45	45
	R	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl
G	I	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180	NIL (from week 28)		
	R	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl			
H	I	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180	180	NIL			
	R	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	0	600	600				

Table 5.03 Dose changes in PEG-IFN and Ribavirin study. I=PEG-Interferon dose (mcg/week), R=Ribavirin dose (mg/day), Bl=Blinded dose. *Blinded dose for 4 days, then 2 days no treatment, then 1 further dose before Ribavirin treatment was suspended

5.4.2.2 Viral Load Measurements

Viral load measurements were taken at weeks 0, 1, 2, 4, 6, 8, 12, 18, 24 and 48. Measurements were also taken at week 72 where appropriate (in the 48 week treatment patients).

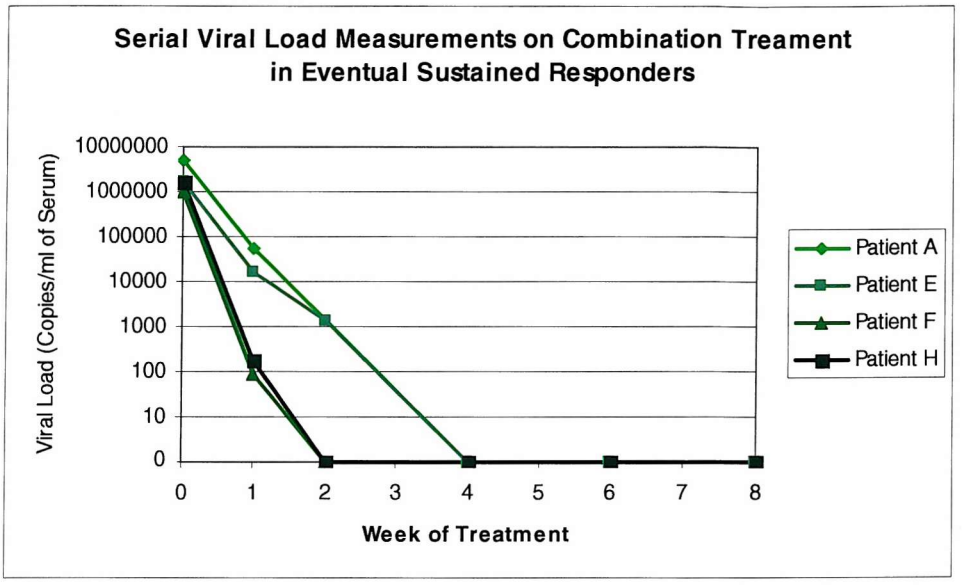
Sustained responders (SR) are defined as those patients who remained sero-negative for the virus 24 weeks after cessation of treatment. Relapsed responders (RR) were negative for HCV RNA during treatment but relapsed in the 24 week post-treatment follow-up phase of the trial. Non-responders (NR) did not clear the virus at any stage.

Of the 8 patients; 4 were sustained responders, 3 relapsed responders and 1 a non-responder. Serial viral load measurements are shown in table 5.04, and there is a graphical representation of the early change in viral load by eventual outcome in figure 5.02.

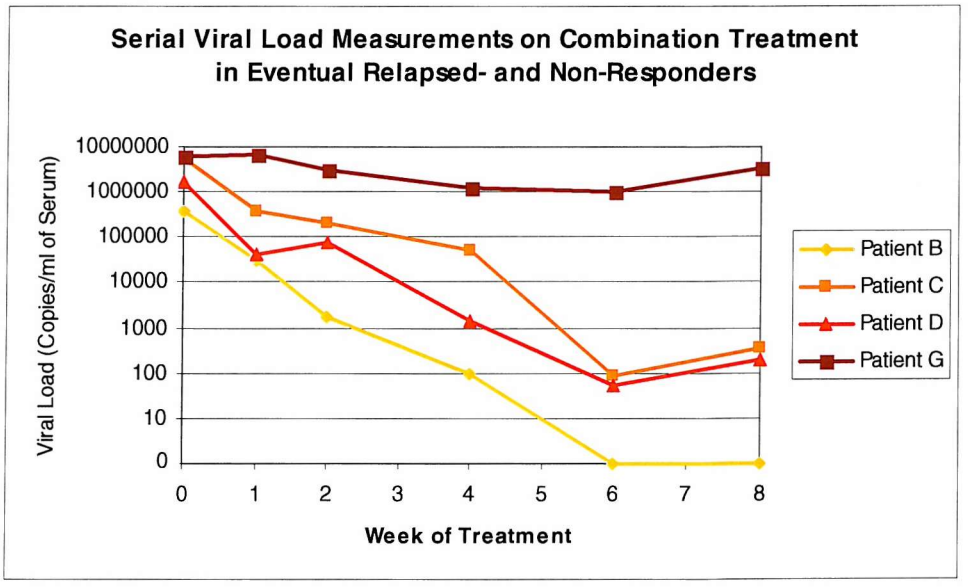
Pt.	**Week of Treatment**										
	0	**1**	**2**	**4**	**6**	**8**	**12**	**18**	**24**	**48**	**72**
A	5.2 x10^6	5.6 x10^4	N/T	0	0	0	0	0	0	0	
B	3.9 x10^5	3.2 x10^4	1.7 x10^3	1.0 x10^2	0	0	0	0	0	0	1.3 x10^6
C	5.7 x10^6	3.7 x10^5	2.0 x10^5	5.0 x10^4	8.4 x10^1	3.6 x10^2	5.0 x10^1	0	0	0	7.0 x10^5
D	1.7 x10^6	4.1 x10^4	7.6 x10^4	1.4 x10^3	5.1 x10^1	1.9 x10^2	0	0	0	2.4 x10^5	
E	1.6 x10^6	1.6 x10^4	1.4 x10^3	0	0	0	0	0	0	0	0
F	9.7 x10^5	9.4 x10^1	0	0	0	0	0	0	0	0	0
G	6.1 x10^6	6.7 x10^6	2.9 x10^6	1.3 x10^6	1.0 x10^6	3.4 x10^6	N/T	1.3 x10^6	4.4 x10^6	6.0 x10^6	
H	1.6 x10^6	1.9 x10^2	0	0	0	0	0	0	0	0	

Table 5.04 Serial viral load measurements throughout treatment with combination therapy with PEG-IFN and Ribavirin. Results are shown in copies of virus per ml of freshly isolated serum.
Patients A, E,F and H are Sustained Responders
Patients B, C and D are Relapsed Responders
Patient G is a Non-Responder

The graphs showing changes in viral load throughout the early time points show a clear difference between those who have an eventual sustained response, and those who do not. The rate of fall in viral load is much quicker in those who achieve a SR than RR or NR, and all of the SR patients were negative for HCV RNA by 4 weeks (figure 5.02).
Only one patient appeared to be truly resistant to therapy, as his level of viraemia dropped by a maximum of 6 fold throughout the entire treatment course. This maximum reduction in viral load (to $1x10^6$ copies/ml) occurred at 6 weeks, but by the end of treatment the level had risen again (to $4.4x10^6$ copies/ml).



A



B

Figure 5.02 Serial viral loads by eventual virological outcome whilst on treatment with combination PEG-Interferon and Ribavirin. These graphs show serial viral loads over the first 8 weeks of therapy.
A Eventual Sustained Responders
B Eventual Relapsed and Non-Responders

5.4.2.3 Serial Lymphocyte Proliferation Assays

Serial lymphocyte proliferation assays were undertaken using the recombinant HCV antigens; core, helicase, NS3 and NS4.

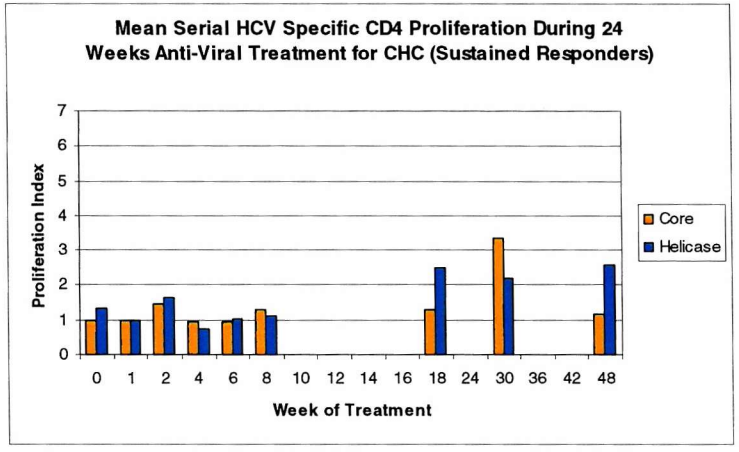
The results were analysed by length of treatment and response to therapy, giving 4 groups; 24 weeks treatment sustained responders and non-responders (comprising relapsed and true non- responders), and 48 weeks treatment sustained and relapsed responders. There were 2 patients in each group.

For greater clarity the results from just core and helicase antigens are shown in figure 5.03.

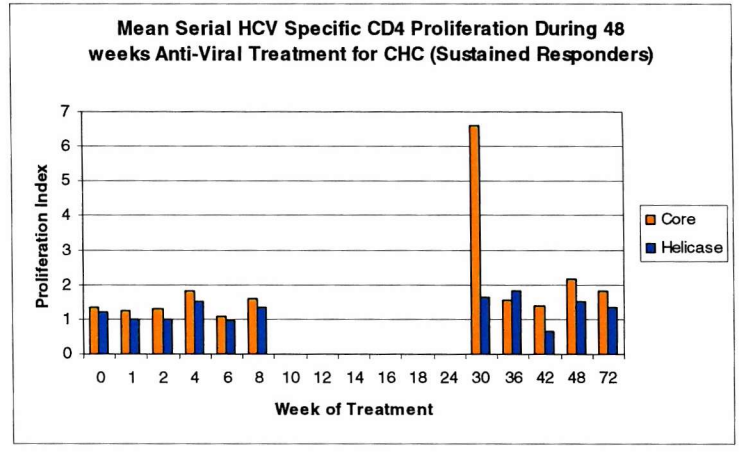
In figure 5.04, the serial maximum mean proliferation index value for any HCV antigen is shown. This allows direct comparison of the maximal proliferative response to HCV antigens during treatment between sustained and non-responders.

There are no significant differences between HCV specific $CD4^+$ proliferative responses in the sustained responders and the non- or relapsed responders during the course of treatment, although numbers were small in each group. Proliferative responses did increase in magnitude during the course of therapy in all groups, but returned to near baseline within 6 months of cessation of treatment. The sustained responders were not left with the higher magnitude proliferative responses seen in many patients after spontaneously resolved acute disease.

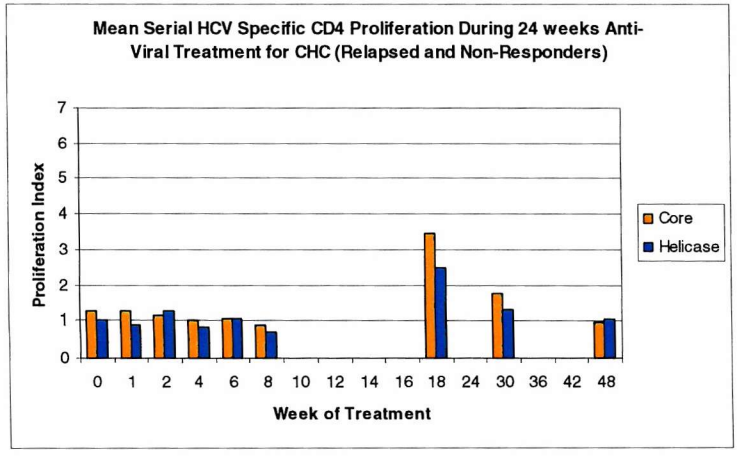
At week 30 in the sustained responder group, there seems to be a high magnitude proliferative response to core protein, but this mainly reflects the very brisk response in one individual.



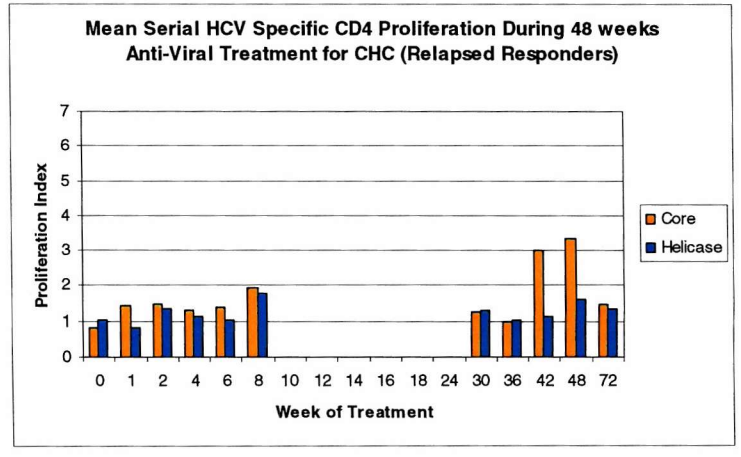
A



B

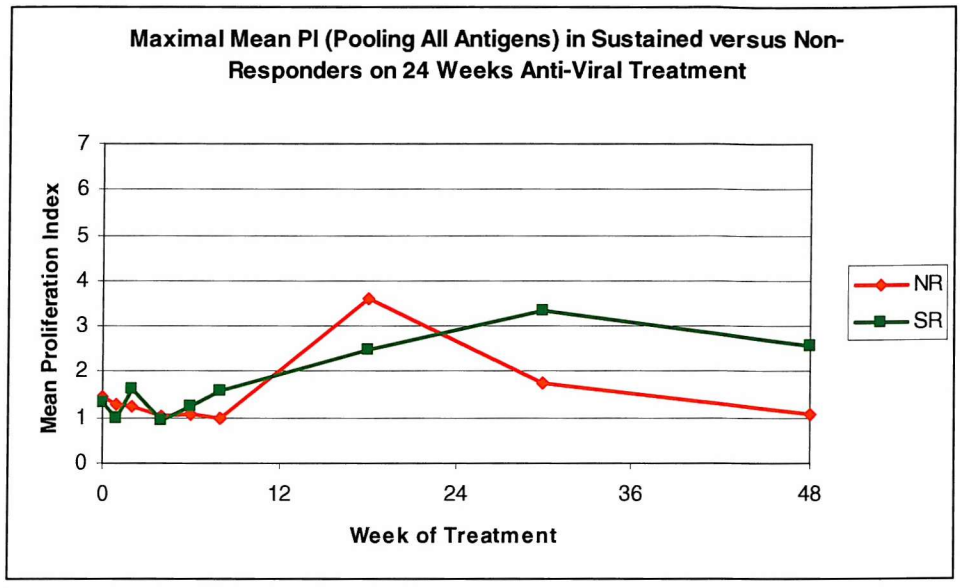


C

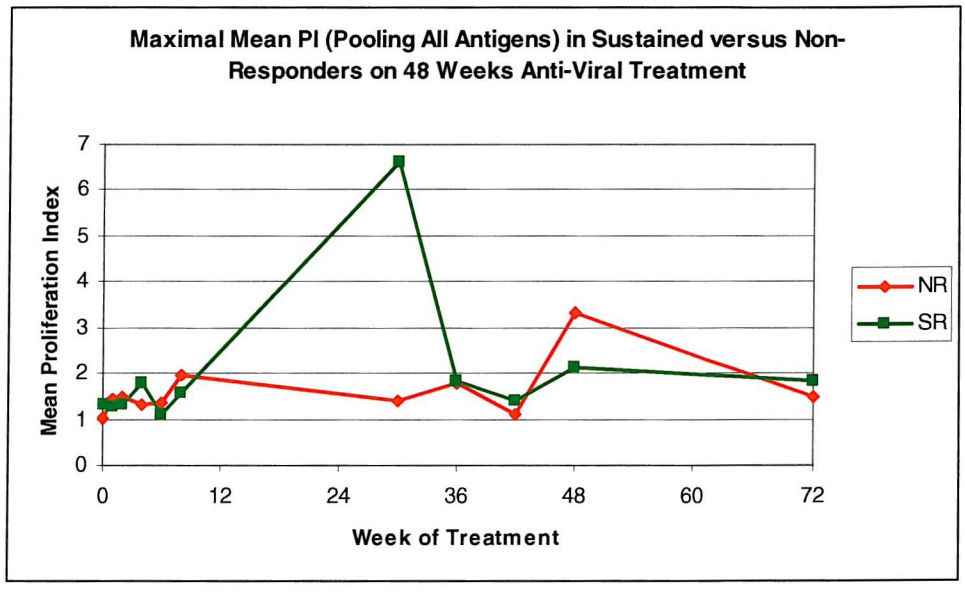


D

Figure 5.03 Serial lymphocyte proliferation indices by final outcome of treatment. **A** and **B** are eventual sustained responders, and **C** and **D** are relapsed and non-responders. N=2 in each group (**A** and **C** 24 weeks therapy, **B** and **D** 48 week)



A



B

Figure 5.04 Serial maximum proliferation indices to HCV antigens, by outcome of therapy. **A** During 24 weeks of treatment **B** During 48 weeks of treatment

5.4.2.4 Serial FACS Analysis for Markers of Activation and Intracellular Cytokines

FACS analysis for CD4, CD69 and intracellular cytokines (IL-2, -4, -10, IFN-γ) was undertaken at weeks 0, 12, 24, 48 and where appropriate, week 72. Freshly isolated PBMCs were assayed after 6 hours culture with either recombinant HCV core or helicase. The results of the IL-4 and IFN-γ FACS analysis are shown in table 5.05. The pooled results of IL-4, -10 and IFN-γ for the first 24 weeks of treatment (both 24 and 48 week treatment groups) and the post-treatment time points are graphically represented in figure 5.05. The other results are not shown. Some of the data sets are not complete due to difficulties with isolating sufficient PBMCs for study at every time point, and also several missed appointments by patients.

There was no statistical difference between the groups, and there were no discriminating factors which could predict prior to the initiation of therapy or during therapy, what would be the final virological outcome. However, this may be because this is a pilot study looking at small numbers treated in a clinical trial, as PEGylated interferon-α was not yet licensed for use in combination with Ribavirin in the UK. The responses at 12 weeks were examined in greater detail as clinical trials have suggested this time point as critical for achieving virological clearance that will be sustained after cessation of treatment.

All groups did show a rise in the proportion of antigen specific $CD4^+/CD69^+$ cells secreting cytokines during treatment, and this proportionate increase compared to non-antigen stimulated controls, declined after cessation of therapy.

There was a trend towards a reduction in the proportion of antigen specific IL-4 producing cells maintained at 24 weeks post treatment in the sustained responders compared with the proportions at baseline. This trend was not seen in the non-responders to treatment. In addition there was a trend towards an increase in the proportion of antigen specific IFN-γ producing cells maintained at 24 weeks post cessation of therapy in the sustained responder group. This trend was not seen in the non-responder group. In

contrast, there was a trend towards increased proportion of IL-10 producing cells in the non-responder group maintained at 24 weeks post cessation of therapy. Taken together, this relative reduction in IL-4 production and increase in IFN-γ production could represent a switch from a Th2 predominant $CD4^+$ response to Th1 predominance.

The data examining the first 12 weeks of therapy, to investigate the clinical trial data suggestive of 12 week predictability of treatment does seem to suggest trends in IL-4 production and IFN-γ production which can differentiate the sustained and non-responders. There is a trend towards "switching on" and IFN-γ response in the eventual sustained responders and "switching on" an IL-4 response in the eventual non-responders. As with the data for the end of study cytokine profiles, this suggests that development of a Th1 predominant $CD4^+$ response is more likely to be associated with viral clearance.

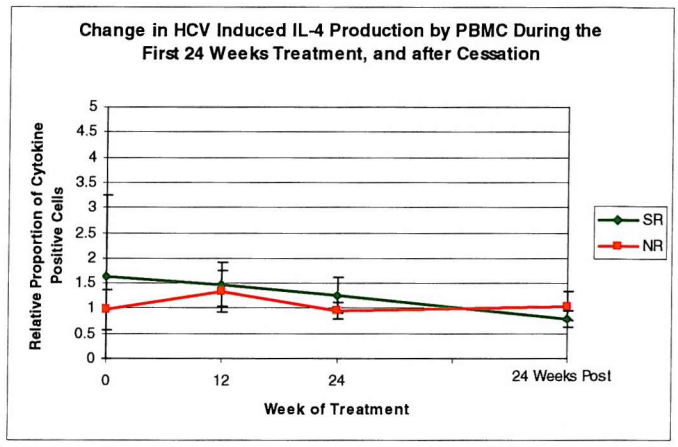
		Week of Treatment				
24 Week Groups		**0**	**12**	**24**	**48**	**72**
Core	SR	2.77 (2.21)	1.85 (5.81)	1.27 (5.2)	0.69 (5.04)	
	NR	0.69 (1.4)	1.69 (1.76)	0.94 (1.48)	0.86 (6.13)	
Helicase	SR	1.89 (0.34)	1.21(3.08)	1.22 (5.01)	0.63 (4.66)	
	NR	1.25 (2.54)	1.31 (1.40)	0.99 (1.56)	0.83 (5.92)	
48 Week Groups						
Core	SR	0.69 (0.48)	1.47 (4.0)	1.4 (3.91)	1.11 (6.47)	0.89(4.46)
	NR	No Result	1.05 (4.64)	No Result	No Result	1.37 (9.0)
Helicase	SR	1.29 (0.83)	1.22 (3.45)	1.57 (3.26)	0.94 (5.48)	0.95(4.92)
	NR	No Result	1.34 (5.93)	No Result	No Result	0.88 (5.9)

A IL-4

		Week of Treatment				
24 Week Groups		**0**	**12**	**24**	**48**	**72**
Core	SR	0.29 (0.99)	1.0 (3.99)	1.35 (4.98)	1.09 (7.3)	
	NR	1.27 (3.69)	1.09 (1.86)	1.07 (1.29)	0.45 (4.03)	
Helicase	SR	No Result	0.82 (3.25)	1.21 (4.47)	1.02 (6.84)	
	NR	0.43 (1.24)	0.84 (0.89)	0.79 (0.96)	0.57 (5.06)	
48 Week Groups						
Core	SR	0.38 (0.21)	1.9 (3.46)	1.12 (3.75)	1.15 (5.46)	0.59(4.09)
	NR	No Result	1.64 (5.38)	No Result	No Result	1.01(6.08)
Helicase	SR	0.55 (0.3)	1.84 (3.1)	1.63 (3.07)	0.72 (3.41)	0.74(4.94)
	NR	No Result	1.39 (4.41)	No Result	No Result	0.98(5.97)

B IFN-γ

Table 5.05 Tables showing the mean proportion of CD4+ cells expressing IL-4 (A) and IFN-γ (B) following antigen stimulation with recombinant HCV core or helicase protein. The first number is the ratio compared to non-antigen stimulated control $CD4^+$ cells, and the number in brackets represents the absolute percentage of cytokine expressing cells.
SR=Sustained Responders NR=Non and Relapsed responders



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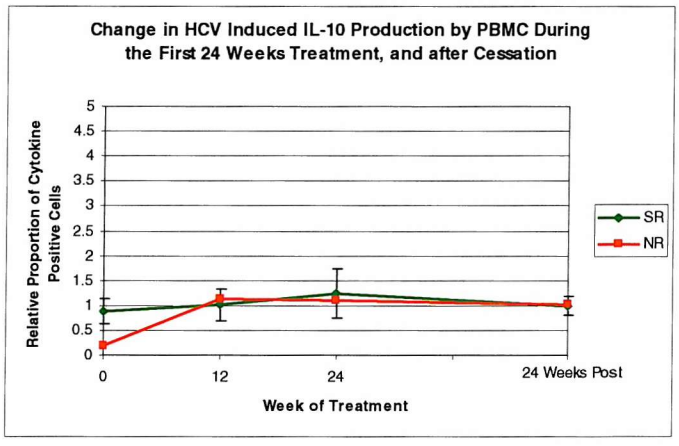

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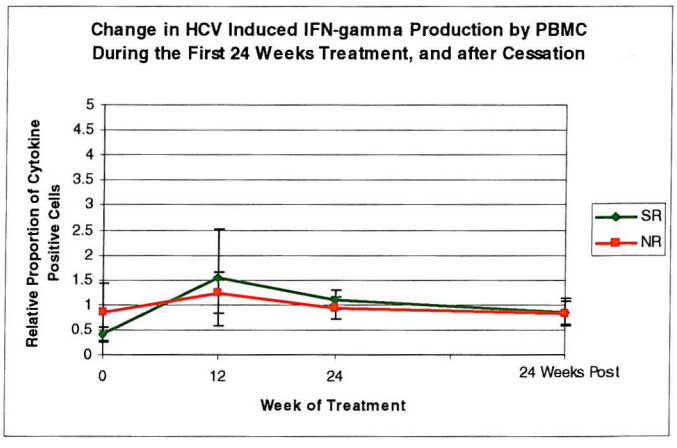
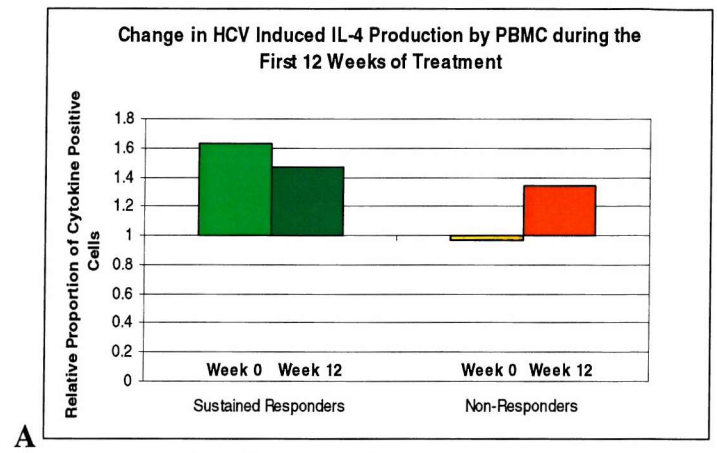


C

Figure 5.05 Graphs showing the mean proportion of antigen specific cytokine secreting cells relative to non-antigen stimulated controls during the course of 24 weeks combination therapy, by eventual virological outcome. The results of the core and helicase proteins have been pooled.
A IL-4 secreting cells **B** IL-10 secreting cells
C IFN-γ secreting cells



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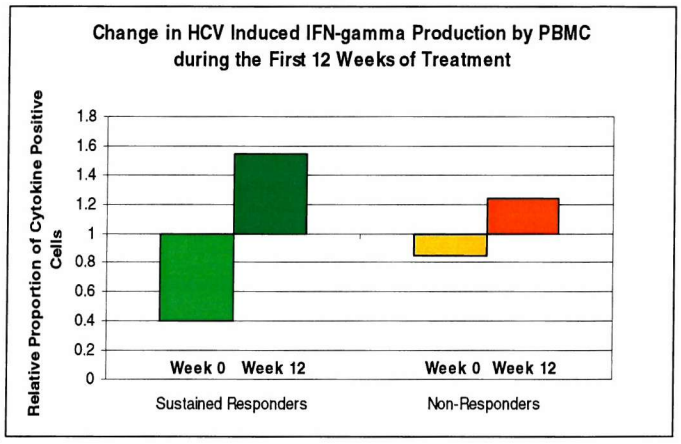

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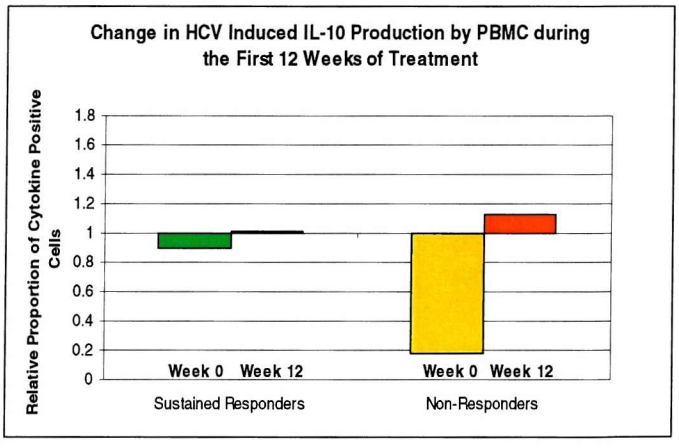


C

Figure 5.06 Graphs showing the mean proportion of antigen specific cytokine secreting cells relative to non-antigen stimulated controls during the first 12 weeks of combination therapy, by eventual virological outcome. The results of the core and helicase proteins have been pooled.
A IL-4 secreting cells **C** IL-10 secreting cells
B IFN-γ secreting cells

5.5 Discussion

Chronic infection is associated with an immune response that appears to be incapable of clearing the infection. These inadequate immune responses are difficult to detect by classical T cell assays. However, it is the immune response in chronic hepatitis C infection that mediates the liver damage. It is my hypothesis that differences in the nature and magnitude of the immune response between individuals dictate the severity of the resultant liver disease. Effective therapy for HCV infection must aim to augment the immune responses to allow viral clearance whilst not exacerbating the liver damage.

The mainstay of treatment for CHC is interferon-α. This fulfills the requirements for a successful treatment for HCV infection, as it allows viral clearance by augmenting the immune response, has anti-viral properties, and also has an anti-fibrotic action in the liver. As a sole agent, the viral clearance rate is low, and the treatment is not well tolerated by patients. Future treatment options are likely to continue with interferon-α as a component, but to improve the chances of patient compliance through unpleasant and debilitating side-effects, the potential viral clearance rate must be much higher than it is currently.

Cramp *et al.* showed that $CD4^+$ responses become more vigorous during treatment with standard interferon-α and ribavirin, and that these magnified responses are maintained if virological clearance is sustained in some patients *(Cramp 2000)*. A pilot study in our population revealed results consistent with the published data, although numbers were small (0/1 Non-responder developed significant HCV specific proliferative responses, 1/2 sustained responders developed significant HCV specific proliferative responses which were maintained beyond cessation of treatment).

The use of PEGylated- interferon-α has improved both viral clearance rates and patient adherence. In combination with ribavirin, preliminary results show that this treatment achieves sustained viral clearance in 56% of patients *(Fried 2001; Manns 2001)*.

The present study shows that PEG-IFN treatment is also associated with an increase in the magnitude of $CD4^+$ responses as measured by classical means (proliferation assays), although these increased responses occur at a later time point than Cramp *et al.*, and are of a lower magnitude. The maintenance of the increased magnitude of $CD4^+$ responses associated with sustained virological clearance described in Cramps cohort was not seen in the present group. Although there is a significant rise in the magnitude of proliferative responses in some of the sustained virological responders whilst on treatment, this does not occur in all patients and is maintained after cessation of therapy in very few. This suggests that the sustained virological responders may clear the virus, but are not left with the same responses as patients who have previously spontaneously resolved acute HCV infection (see chapter 4). Thus the treatment of CHC does not permanently reverse any immunological deficit which allows chronic hepatitis C to become established, if such a defect exists.

In addition to the changes in proliferative responses, there seems to be a change in the cytokine profiles of the antigen specific $CD4^+$ cells in the PBMC. Although numbers are small, there is a trend towards an increase in the proportion of IFN-γ secreting cells and a decrease in IL-4 secreting cells during treatment amongst those who ultimately become sustained virological responders. This change in proportions is maintained for 24 weeks post cessation of therapy, and is seen as early as at 12 weeks of therapy. These trends are not seen amongst virological non-responders. Instead, there is a trend for an increase in proportion of IL-10 secreting cells in the non responder group. It is interesting to note that in this cohort of patients the eventual sustained responder group had a higher proportion of IL-10 and IL-4 secreting cells, and a lower proportion of IFN-γ secreting cells than the non-responder group at baseline. This may suggest that the group of patients that eventually achieve a sustained virological response with treatment initially have a more polarized Th2 type $CD4^+$ T lymphocyte response than eventual non-responders. The subtle reduction in IL-4 secreting cells and increase in IFN-γ secreting cells suggests a switch towards a Th1 response in the responders, which is not seen in the non-responder group. This hypothesis may mimic what happens in acute infection as resolution of acute HCV infection is associated with a brisk Th1 response *(Diepolder 1995; Diepolder 1997; Gerlach 1999)*.

The most discriminating factor dictating successful viral clearance was the rate of fall in viral load. All of the eventual sustained responders were HCV RNA negative by 4 weeks, whereas none of the non-responder/relapsed responder group was negative at 4 weeks. This finding is entirely consistent with data from other groups looking at all forms of anti-viral therapy.

Numbers are small in these studies, and the data sets are not always complete. These problems are inevitable when using a study population such as those with a chronic viral illness. Attendance for appointments can be unreliable and it is not always possible to obtain sufficient PBMC for investigation from a patient with neutro- and lympho-paenia due to their anti-viral therapy. In addition, there is individual variation in HLA repertoire, and genotype of infecting virus, with many quasi-species also involved. This will reduce the chances in each of the patients of having the potential to mount a response to each of the recombinant assay antigens. A much larger study involving more patients and more antigens would improve the potential for demonstrating differences between responders and non-responders to anti-viral therapy, and allow characterisation of what those differences are.

It is possible that the immunological changes which reflect or dictate viral clearance occur in the very early stages of treatment, and effectively and efficiently achieve viral clearance within the first 4 weeks. To investigate this, it would be necessary to repeat the assays described in the current study at much earlier and more frequent time points to document any of these obviously effective changes.

Chapter 6

Chapter 6

The Effect of the $CD4^+$ Immune Response on Hepatic Stellate Cells

6.1 Introduction

In the normal human liver, hepatic stellate cells (HSC) are fat storing cells located in the Space of Disse of the hepatic sinusoid, representing 5-8% of the total cells in the liver.In their resting state these cells have a globular morphology, contain fat droplets, and have long cytoplasmic processes, containing filaments and microtubules, which wrap themselves around the sinusoids. There is considerable inter-species variation in quiescent HSC, both in terms of morphology and distinguishing markers used in immunohistochemistry (i.e. rat HSC stain positive for desmin and GFAP whilst human HSC are negative for both of these markers) *(Geerts 2001)*.

The fat droplets which characterise the HSC quiescent phenotype are rich in vitamin A and this can allow identification of quiescent cells by autofluorescence following excitation with light at 328nm. Surface expression of Low-Affinity Nerve Growth Factor receptors and N-CAM has been demonstrated on quiescent human HSC, and hepatocyte growth factor and nerve growth factor can be detected in these cells. It has also been possible to identify the ECM component laminin within quiescent HSC, although staining for other matrix components is hampered by the close proximity of these cells to the basement membrane *(Geerts 2001)*.

There is marked heterogeneity of quiescent HSC within the liver, and the exact phenotype depends on the microanatomical position i.e. peri-portal or intra-lobular. The functions of quiescent hepatic stellate cells are; involvement in vitamin A homeostasis, extracelllular matrix synthesis (collagen types III and IV, laminin and a small amount collagen I), extracellular matrix degradation (matrix metalloproteinases 2, 3, 10, 13, 14 and TIMPs 1 and 2), regulation of sinusoidal blood flow by vasomotor activity, and secretion of paracrine, juxtacrine, autocrine and chemoattractant mediators (growth factors and cytokines). The normal liver architecture is maintained by continual turnover of ECM components in a balance of matrix formation and degradation *(Geerts 2001)*.

Following activation, the phenotype of these cells changes dramatically; the vitamin A rich fat droplets disappear and the cells acquire a myofibroblast-like phenotype, express α-smooth muscle actin (α-SMA) and proliferate. The fate of the fat droplets is not understood. Activated HSC have been shown to produce matrix degrading metalloproteinases, inhibitors of these proteinases and fibrillar collagens *(Friedman 1996)*.

The phenotype of activated hepatic stellate cells (HSC) and their distribution throughout the liver, has lead to the belief that these are the cells primarily involved in liver matrix remodeling and the abnormal accumulation of fibrillar collagen in pathological fibrotic processes *(Friedman 1993; Friedman 2000; Bataller and Brenner 2001)*.

In liver injury, the activation of HSC can be initiated by oxidative stress from hepatocytes or inflammatory cells, or by fibronectin from sinusoidal endothelial cells. It has recently been shown that the transcription / translation cascade which results from activation to promote such a profound change in phenotype can be reversed or driven towards apoptosis, resulting in arrest and possible reversal of the fibrotic process *(Iredale 2001)*.

There are multiple transcription factors involved in HSC activation. One particular transcription factor family, nuclear factor κB (NF-κB), is involved in responses to cytokines. NF-κB produces activation changes in the HSC which protect it from apoptosis and promote expression of immune-active surface and secreted molecules. There is a background level of expression of NF-κB in quiescent HSC, but it is controlled by the inhibitory complex I-κB. Activated HSC have a higher level of expression of NF-κB, supported by autocrine factors, and this increased activity helps to maintain the cells in a profibrogenic and proliferative state, protecting them from apoptosis. However, further up-regulation of NF-κB induced by stimulation with cytokines such as IL-1β and TNF-α, results in suppression of collagen α_1(I) expression and increased expression of ICAM-1, IL-6, MIP-2 and COX-2, which render the cells less proliferative and less fibrogenic *(Gallois 1998; Elsharkawy 1999; Eng and Friedman 2001)*.

In addition to matrix-remodeling proteins, activated HSC have been shown to express major histocompatibility complex (MHC) class II molecules, CD40 and Inter-cellular Adhesion Molecule-1 (ICAM-1) *(Hellerbrand 1996; Hellerbrand 1998; Schwabe*

2001). Activated HSC also secrete cytokines and chemokines; MCP-1, MIG, IL-6, IL-8, IL-10, TGF-β and others *(Wang 1998; Friedman 1999; Sprenger 1999)*. Some of these molecules are regulated by the transcription family NF-κB (i.e. ICAM-1).

The cytokines and chemokines produced by HSC have the potential to act on leukocytes of all types to affect recruitment and trafficking, and have been shown to have an autocrine effect on HSC themselves *(Friedman 1999; Mahalingam and Karupiah 1999; Baggiolini 2001)*. HSC are sensitive to the effects of the cytokines produced by leukocytes, which can either augment or suppress HSC matrix remodeling processes *(Friedman 1999; Pinzani and Marra 2001)*. These findings suggest that HSC produce, and are sensitive to, the soluble growth factors and cytokines in the liver microenvironment, and also have the capability to interact directly with immune active cells *(Maher 2001)*.

Some liver injury processes have been explored in animal models i.e. Carbon Tetra-Chloride injury, bile duct ligation, conconavalin A injury. However, there are many human liver diseases for which there is no reliable animal model e.g. hepatitis C and alcohol induced liver disease. In these diseases, the deposition of abnormal collagen to cause fibrosis and cirrhosis is variable between human individuals, both in amount of collagen deposited and the rate of progression of the resultant fibrosis *(Morgan 1994; Poynard 2000)*. It is likely that differential responses to the same insult are due to a combination of environmental and genetic diversity.

There are multiple levels at which human diversity may potentially influence the fibrotic process; frequency and magnitude of exposure to the insult, exposure to protective or exacerbating factors, individual sensitivity to the insult, specific nature of HSC reactivity to the insult, interaction between HSC and other cell types, regulation of the magnitude and prolongation of the activation processes once initiated.

Chronic hepatitis C (CHC) infection is a good example of how environmental and genetic diversity can influence the outcome of the fibrotic process. Increasing age, male sex and high alcohol intake are all associated with a high rate of progression of CHC fibrosis *(Di Bisceglie 1998)*. In addition, there are certain MHC class II alleles which are associated with a less rapid rate of progression of liver disease, which may be due to interaction between HSC and immune active cells following specific

priming signals during the antigen presentation process to $CD4^+$ cells through the class II pathway *(Alric 1997; Thursz 1999).*

Despite increasing breadth of knowledge about the clinical course and immunological mechanisms of CHC infection, very little is known about the interactions between the virus, the immune system and HSC, which may explain the discordance seen in the severity and rate of progression of liver fibrosis between individuals.

6.2 Aims

We aimed to investigate the effect of the HCV specific $CD4^+$ response on hepatic stellate cells.

- By the development of a simple assay for testing conditioned supernatants from the peripheral blood mononuclear cell cultures of CHC patients for effects on hepatic stellate cell activation through the NF-κB pathway
- To use this assay to identify supernatants that induce reactivity
- To characterise the nature of the effects of these reactive supernatants on the potential pro-fibrotic functions and immune interactions of HSC.

6.3 Methods

Primary hepatic stellate cells were isolated from normal human liver by collagenase digestion and density centrifugation. The stellate cells were then activated by culture on plastic and grown to confluence in tissue culture flasks. For the RNA assays and FACS analysis, the hepatic stellate cells were used as primary cells direct from these culture flasks. For the cellular ELISAs, cells were passaged once or twice using trypsin to bulk their numbers, and then grown to confluence in 96 well plates. Conditioned media were generated using freshly isolated peripheral blood mononuclear cells from HCV positive and negative individuals. The cells were cultured with recombinant HCV proteins or appropriate positive and negative controls, and the supernatants collected at day 3 for use in the experiments.

The effect of individual cytokines was assessed by culture of HSC in complete medium containing known dilutions of recombinant human cytokines.

The cellular ELISA for ICAM-1 was undertaken using HSC in 96 well plates cultured for 16-20 hours in the presence of test media. The cells were then fixed, blocked and stained with a biotinylated anti-ICAM-1 antibody. Streptavidin horse-radish peroxidase and a chromogenic substrate reaction allowed quantitation of the amount of anti-ICAM-1 bound to the cell surface.

FACS analysis was undertaken following 16 hour culture with test medium. The cells were then washed, blocked and stained for ICAM-1, CD40, α-SMA, class II MHC with fluorescently labeled specific antibodies. They were then fixed, permeabilised and then stained for intra-cellular markers.

FACS was undertaken using a FACScalibur flow cytometer, and analysis using Cell Quest software.

Semi-quantitative RNA estimation was undertaken on primary HSC cultured for 16 hours in test medium. The cells were lysed and total RNA was converted into cDNA by reverse transcription, involving incorporation of a biotin-labeled dUTP. The cDNA was then hybridised to a custom-made membrane with cross-linked DNA probes for the specific genes of interest. Streptavidin alkaline phosphatase incubation then preceded a chemiluminescent reaction as a readout. Quantitation was by normalisation to β-actin expression.

Chemokine ELISAs were undertaken by standard sandwich ELISA using specific matched antibody pairs. The supernatants were removed from the primary HSC immediately prior to their harvest for RNA.

Western blotting was kindly undertaken by Dr Lindsay Murphy using a standard technique and specific antibodies.

For a full description of the methods see *Chapter 2, Materials and Methods.*

6.4 Results

6.4.1 Confirming Purity of HSC Cultures

Freshly isolated human hepatic stellate cells were cultured in sterile plastic tissue culture flasks. Culture on plastic leads to activation of the HSC, causing them to adhere to the plastic, acquire a myofibroblast-like phenotype and proliferate. After 14-21 days the primary cultures were confluent and ready for passage or use in an assay.

To assess the purity of the HSC cultures, and ensure that there were no contaminating Kupffer cells or macrophages, FACS analysis was undertaken with staining for α-SMA to detect HSC. (Figure 6.01**A**).

FACS analysis of the hepatic stellate cell primary culture revealed expression of α-SMA by over 99% of cells, confirming that the stellate cell population was pure with no contaminating Kupffer cells. This result was confirmed with 2 other primary cultures prepared from different donors on different dates (Figure 6.01**B,C**). Due to pressure on numbers of primary HSC, it was not possible to undertake confirmatory FACS on every population, but the isolation procedure was standard and constant and therefore it was assumed that all of the subsequent HSC primary cultures were pure.

6.4.2 Expression of Inter-Cellular Adhesion Molecule-1 (ICAM-1) by Activated Primary HSC, and Up-Regulation of that Expression by Interferon-γ (IFN-γ)

Current literature has reported that whilst quiescent hepatic stellate cells do not express ICAM-1, activated myofibroblast-like stellate cells do.

6.4.2.1 FACS Analysis

Using FACS analysis and surface staining for ICAM-1 we confirmed that 40-90% of activated primary HSC express ICAM-1. Both the number of positive cells and the intensity of staining, indicating the level of expression on the cell surface, can be increased by culture in the presence of IFN-γ. (Figure 6.02) The same effect can be achieved by culture with Tumour Necrosis Factor-α (TNF-α, data not shown).

6.4.2.1 Cellular ELISA Analysis

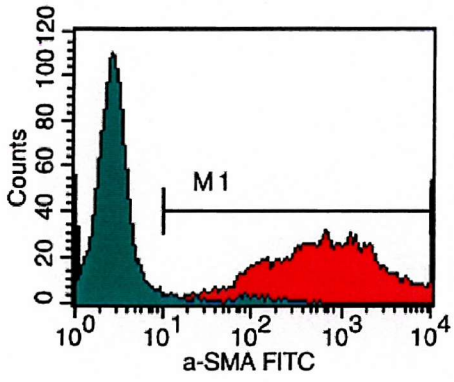
It was not possible to seed the primary HSC directly into 96 well plates, and so the ELISA assay was not technically possible using primary cells.

6.4.3 Expression of ICAM-1 by Passaged HSC, and Up-Regulation of that Expression by IFN-γ and TNF-α

In order to utilise changes in level of expression of ICAM-1 as a screening tool for change in activation of hepatic stellate cells, it was necessary to confirm the initial HSC findings in passaged HSC, as primary HSC are a valuable and limited resource.

6.4.3.1 FACS Analysis

It was possible to confirm that passaged HSC do express ICAM-1 and that this can be up-regulated by IFN-γ and TNF-α. (Figure 6.03).
Later passage HSC do not express as much ICAM-1 on their surface as early passage or primary HSC, but the level of expression can still be up-regulated by IFN-γ and TNF-α. (Figure 6.03).



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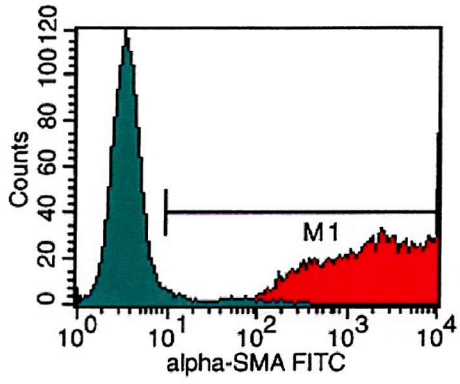

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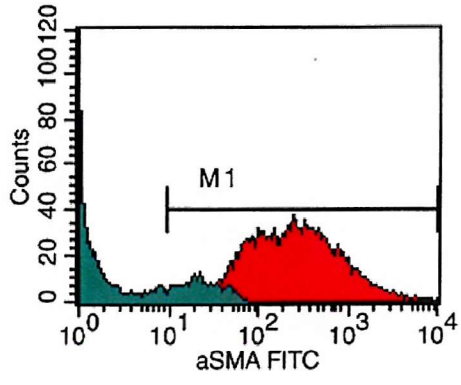
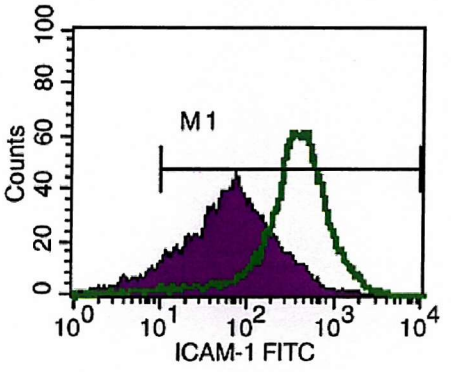


C

Figure 6.01 FACS analysis of 1^o hepatic stellate cells, permeabilised, fixed and stained with an FITC labeled anti-human α–SMA antibody (shown in red), or an isotype negative control (green).
A Donor 51 year old male **B** Donor 51 year old female
C Donor 28 year old male



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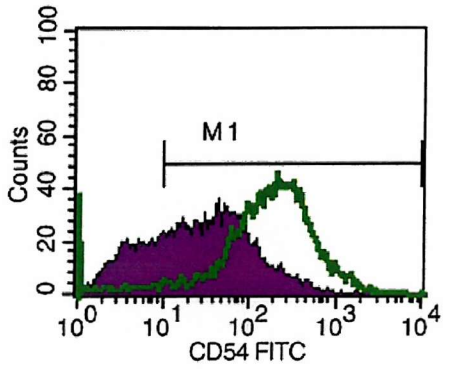
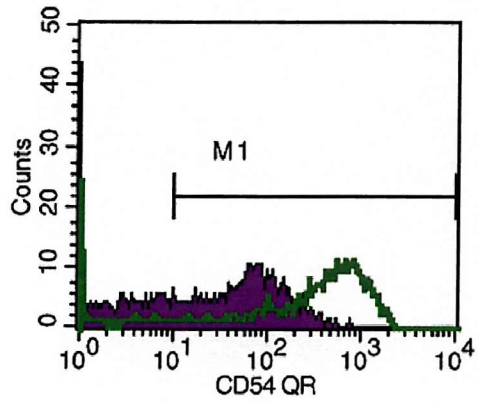
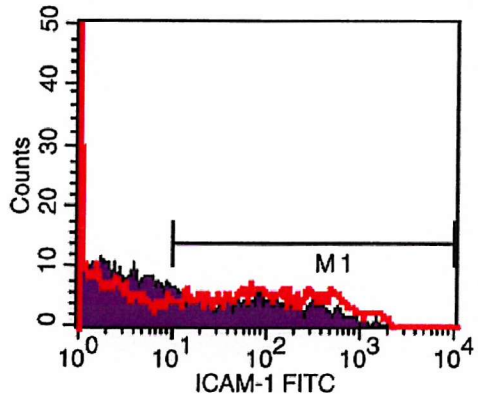


B

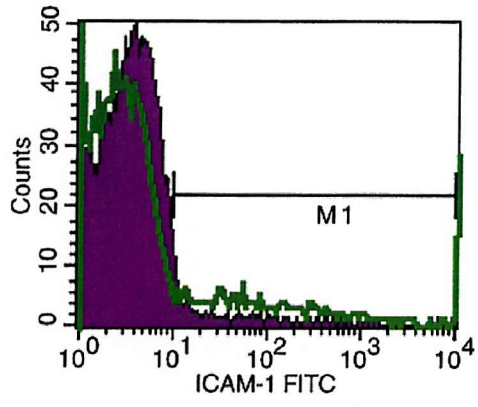
Figure 6.02 FACS analysis of 1^{o} hepatic stellate cells, stained with an FITC labeled anti-human ICAM-1 (CD54) antibody. Unstimulated cells shown in purple, with an overlay in green of cells stimulated overnight with IFN-γ 10ng/ml
A Donor 51 year old male
B Donor 51 year old female



A **Passage 2**



B **Passage 4**



C **Passage 6**

Figure 6.03 FACS analysis of passaged (p) hepatic stellate cells stained with either an FITC or quantum red (QR) labeled anti-human ICAM-1 (CD54) antibody. Unstimulated cells in purple, overlay in green of cells stimulated with IFN-γ 10ng/ml, or red for TNF-α 10ng/ml
A p2 cells **B** p4 cells **C** p6 cells

6.4.3.2 Cellular ELISA Analysis

The findings in the FACS analysis were reproduced in the cellular ELISA. There were significant increases in the OD readings from wells stimulated for 16-20 hours with IFN-γ or TNF-α. These findings were consistent across several populations of HSC and dose dependent. (Figure 6.04).

However, the fold changes seen with the later passage stellate cells were less marked than the earlier passage cells. This is consistent with the reduction in ICAM-1 expression seen with later passage cells using FACS analysis. (Figure 6.04) This suggests that the stellate cells begin to lose their characteristic phenotype with recurrent passage in cell culture.

Following this observation, stellate cells were used at passage 1 or 2 only for the cellular ELISAs.

6.4.4 The Effect of Single Cytokines on ICAM-1 Expression by Passaged Human Hepatic Stellate Cells

Using the established and reliable cellular ELISA for ICAM-1, other cytokines likely to be present in the sinusoidal microenvironment in CHC were screened for their effect on ICAM-1 expression. Each cytokine was applied to triplicates of confluent cells in a 96 well plate and incubated for 16-20 hours prior to staining for the assay. The cytokines tested were; Interleukins -4, -6, -10, -12 and -13 (IL-4,-6,-10,-12 and -13), Transforming Growth Factor-β (TGF-β), Platelet Derived Growth Factor (PDGF) and Interferon-α (IFN-α). Each of these cytokines has been implicated in the pathogenesis of CHC infection and/or the associated liver damage. In the case of IFN-α, this is the mainstay of treatment in CHC, and is reported to have anti-fibrotic effects in the liver.

Significant up-regulation of ICAM-1 was seen with IL-6, IL-10, TGF-β and IFN-α, each in a dose dependent manner. (Figure 6.05). The degree of up-regulation of ICAM-1 expression with these cytokines, was not of such great magnitude as with IFN-γ.

No significant up-regulation was seen with IL-4, -12 or -13 or PDGF. (Data not shown).

Of note there was no constant relationship of up-regulation of ICAM-1 with either pro- or anti-fibrotic cytokines. Current literature suggests that IL-4 and -13, TGF-β and PDGF are pro-fibrotic, whilst IL-10 and -12 and IFN-α are anti-fibrotic. *(Friedman 1999)*

6.4.5 The Effect of Recombinant Hepatitis C Proteins on ICAM-1 Expression by Passaged Human Hepatic Stellate Cells

In order to use the cellular ELISA assay to screen HCV protein stimulated PBMC supernatants from CHC individuals for their effect on HSC, it was necessary to ensure that the recombinant proteins themselves did not have an effect on ICAM-1 expression. This is to control for any effects that might be attributed to the recombinant proteins inevitably present in the conditioned supernatants when they are applied to the hepatic stellate cell cultures.

Each protein is in a protein carrier buffer, and so the ELISA was carried out using unstimulated cells as a first control, and the protein carrier buffer alone in the medium as a second.

There was no significant effect on ICAM-1 expression with any of the recombinant HCV proteins when compared with carrier buffer or unstimulated control. (Figure 6.06)

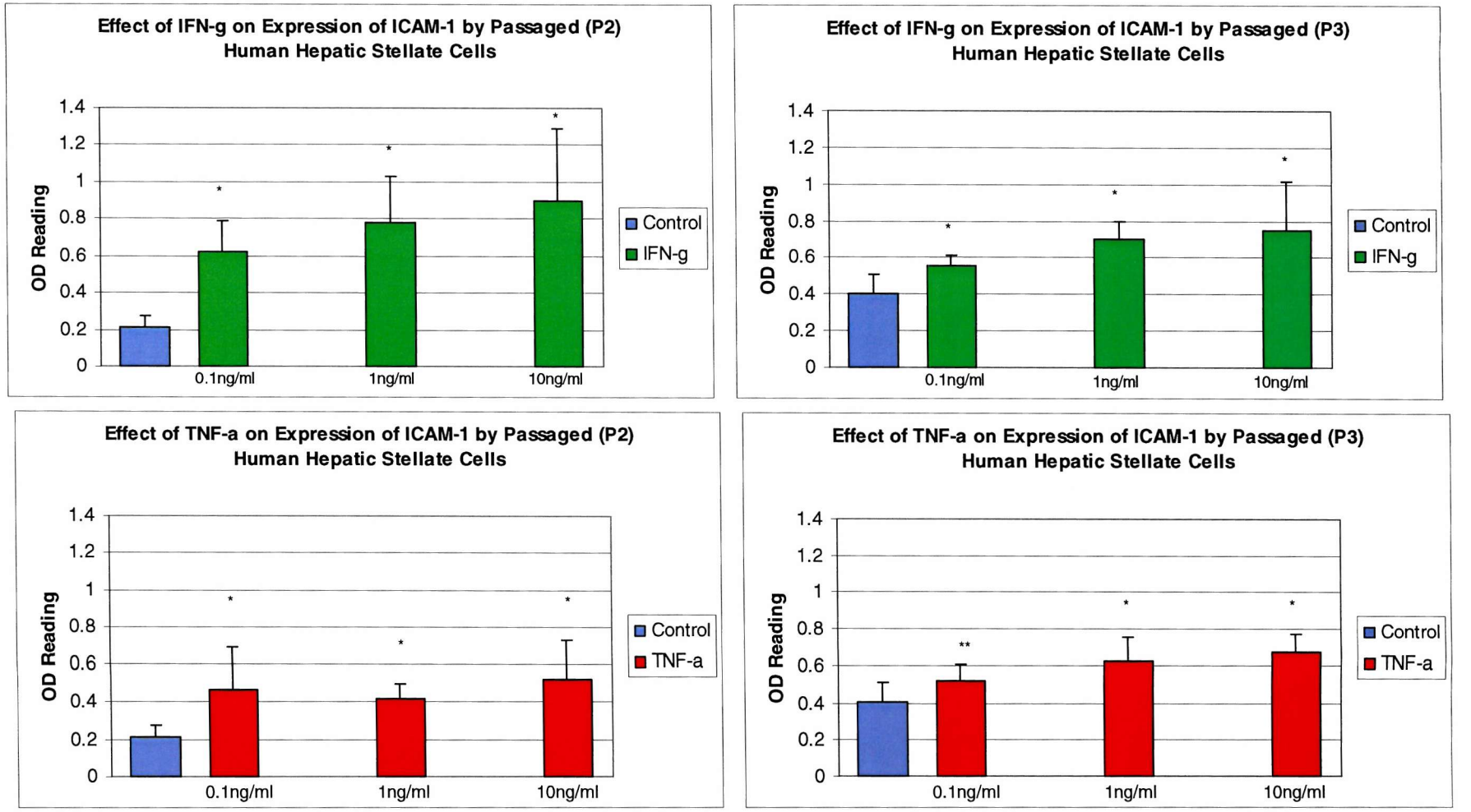


Figure 6.04 Cellular ELISAs for ICAM-1 using 2 stellate cell populations (one at p2 and the other at p3) from different donors. The OD readings represent the mean of OD readings from triplicates after 16 hour incubation with either control medium, or medium containing IFN-γ or TNF-α. ***p<0.02 ** p=0.05**

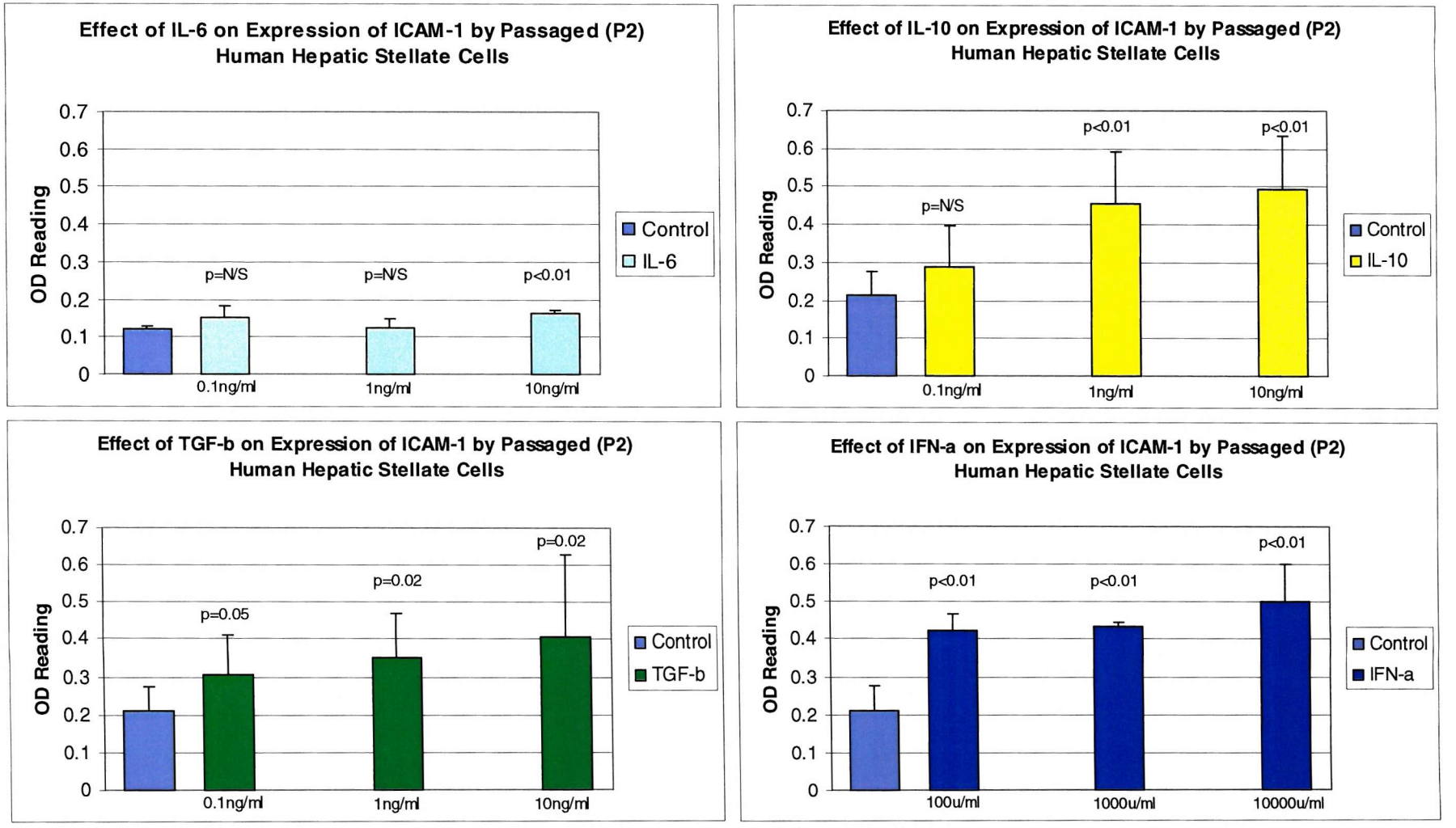


Figure 6.05 Cellular ELISAs for ICAM-1 using p2 stellate cell populations. The OD readings represent the mean of OD readings from triplicates in the presence or absence of cytokine. All of these cytokines reached significance.

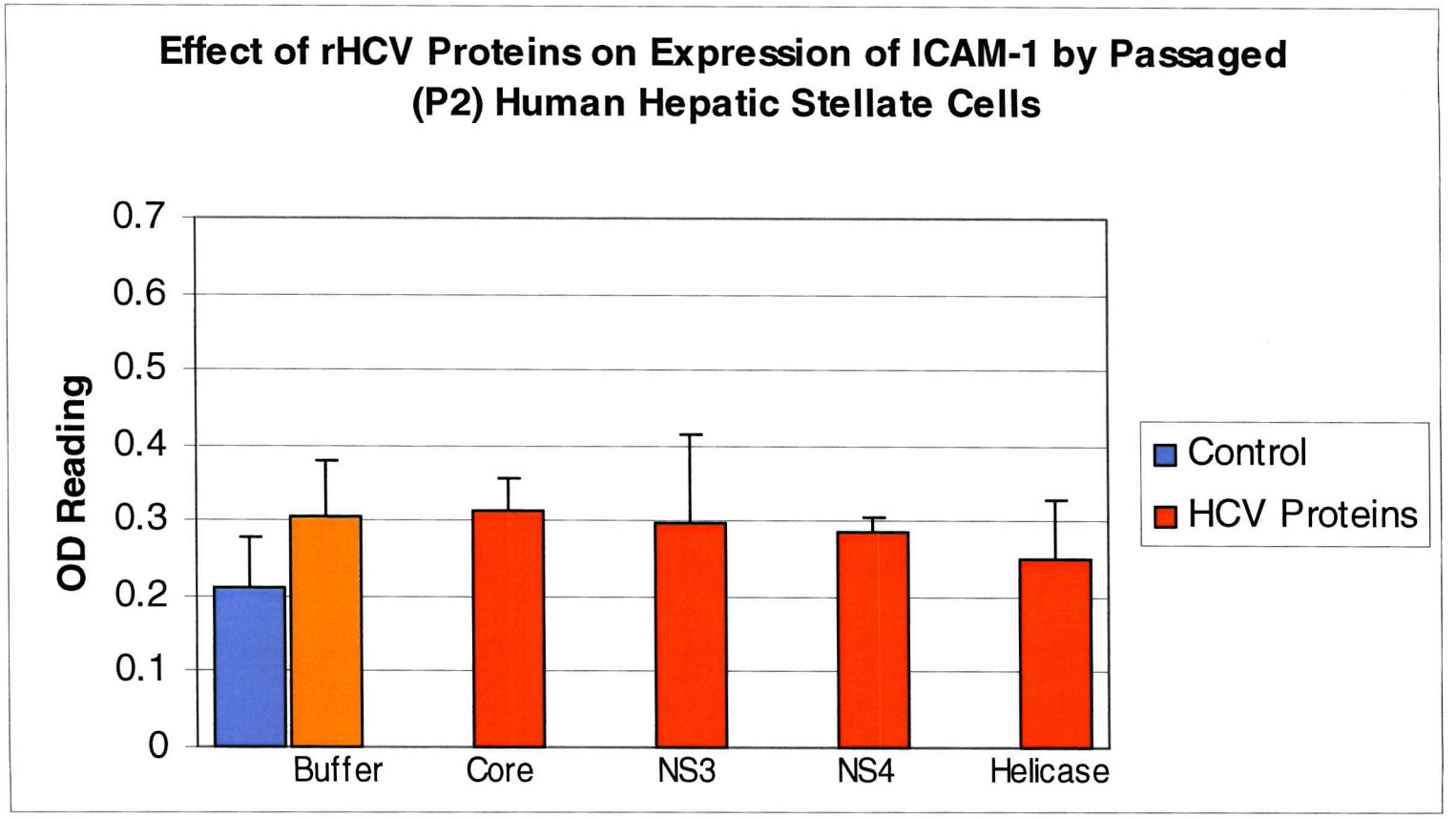


Figure 6.06 Cellular ELISA for ICAM-1 using a Passage 2 stellate cell population. The OD readings represent the mean of OD readings from triplicates in the presence of either medium alone (control), medium containing protein carrier buffer (Buffer) or each of 4 recombinant HCV proteins. None of the assays reached significance. NS5 protein was also screened for its effect on ICAM-1 expression, and did not cause significant up-regulation. *Data not shown.*

6.4.6 Screening Conditioned Supernatants for Effect on ICAM-1 Expression by Passaged Human Hepatic Stellate Cells

To assess the effect of the HCV specific $CD4^+$ response on ICAM-1 expression by hepatic stellate cells, conditioned supernatants were generated from the peripheral blood mononuclear cells (PBMCs) of 10 CHC patients. The PBMC cultures were stimulated with recombinant HCV proteins or appropriate positive and negative controls, and the supernatants were harvested at day 3. These supernatants were then incubated for 16-20 hours with confluent HSC monolayers in triplicate wells of 96 well plates.

The details of the 10 CHC patients are shown in table 6.01.

Patient	Sex	Age	Severity of Liver Disease	$CD4^+$ Proliferative Response
A	M	42	Severe (IPA Score 2,2,2)	No significant responses
B	M	48	Moderate (IPA 2,1,1)	No significant responses
C	M	50	Severe (IPA Score 2,0,3)	No significant responses
D	M	34	Mild (IPA Score 1,1,0)	No significant responses
E	M	45	Moderate (IPA Score 2,1,1)	No significant responses
F	F	45	Mild (IPA Score 2,1,0)	No significant responses
G	M	28	Mild (IPA Score 1,1,0)	No significant responses
H	M	37	Mild (IPA Score 1,0,0)	No significant responses
I	F	54	Severe (IPA Score 3,2,2)	No significant responses
J	M	47	Severe (IPA Score 2,1,3)	No significant responses

Table 6.01 Demographic details of the CHC patients used to generate supernatants for the HSC ICAM-1 cellular ELISAs

7 of the 10 supernatants screened using the ICAM-1 cellular ELISA assay, following stimulation with recombinant HCV proteins, had no effect on the level of ICAM-1 expression by HSC. (Figure 6.07). PHA stimulated supernatants, as the positive control, produced significant up-regulation of ICAM-1 expression in all these cases. These assays were repeated at least one further time using a different HSC population to confirm that there was no reactivity, and the results were reproducible.

Conditioned supernatants generated by the HCV protein stimulated PBMCs from 3 of the 10 CHC patients did produce up-regulation of ICAM-1 expression in HSCs. Patient H produced up-regulation with NS3 stimulated supernatant; patient I produced up-regulation with NS5 and Helicase; Patient J produced up-regulation with NS3 and NS4. All of these patients also produced up-regulation of ICAM-1 expression in HSCs with PHA stimulated supernatants as a positive control. (Figure 6.08) Assessed by standard lymphocyte proliferation assays on PBMC, none of the 10 patients had significant proliferative responses to any of the recombinant HCV proteins. Using the ICAM-1 cellular ELISA on HSC has revealed differences in the HCV specific PBMC responses of 3 patients that were not apparent using standard methods.

Patient	**Positive Assays with HCV Protein Stimulation**		**Positive Assays with PHA Stimulation**	
	LPA (Lymphocyte Proliferation Assay)	**HSC ICAM-1 Cellular ELISA**	**LPA**	**HSC ICAM-1 ELISA**
A	Nil Significant	Nil Significant	**Significant**	Not tested
B	Nil Significant	Nil Significant	**Significant**	**Significant**
C	Nil Significant	Nil Significant	**Significant**	Not tested
D	Nil Significant	Nil Significant	**Significant**	**Significant**
E	Nil Significant	Nil Significant	**Significant**	**Significant**
F	Nil Significant	Nil Significant	**Significant**	**Significant**
G	Nil Significant	Nil Significant	**Significant**	**Significant**
H	Nil Significant	**NS3 significant**	**Significant**	**Significant**
I	Nil Significant	**NS5, Helicase signif**	**Significant**	**Significant**
J	Nil Significant	**NS3, NS4 signif.**	**Significant**	**Significant**

Table 6.02 Table showing the results of the HSC ICAM-1 cellular ELISA using HCV protein conditioned supernatants in comparison with lymphocyte proliferation results from the same patients

The screening assay chosen identified 3 out of 10 CHC patients who generated a measurable response to recombinant HCV proteins. This is in contrast to the lymphocyte proliferation assays which identified 0 out of the 10 patients. Using an

IFN-γ ELISA, it was possible to identify 2 patients with reactivity in their supernatants, and these 2 patients are both included in our ICAM-1 ELISA positive group (group 2).

The 7 patients who did not test positive in our assay all have signs of immune mediated histological damage in response to HCV, indistinguishable from that seen in the test positive patients. Explanations for the lack of reactivity in the assays will be discussed.

For further investigation of the effect of the $CD4^+$ immune response on HSC, the supernatants which tested positive in our initial screening ICAM-1 ELISA assays were chosen for future experiments. Patient J underwent liver transplantation soon after his initial PBMC were harvested. Following liver transplantation, he was on an immunosuppressive drug regime, which rendered him unsuitable for further study.

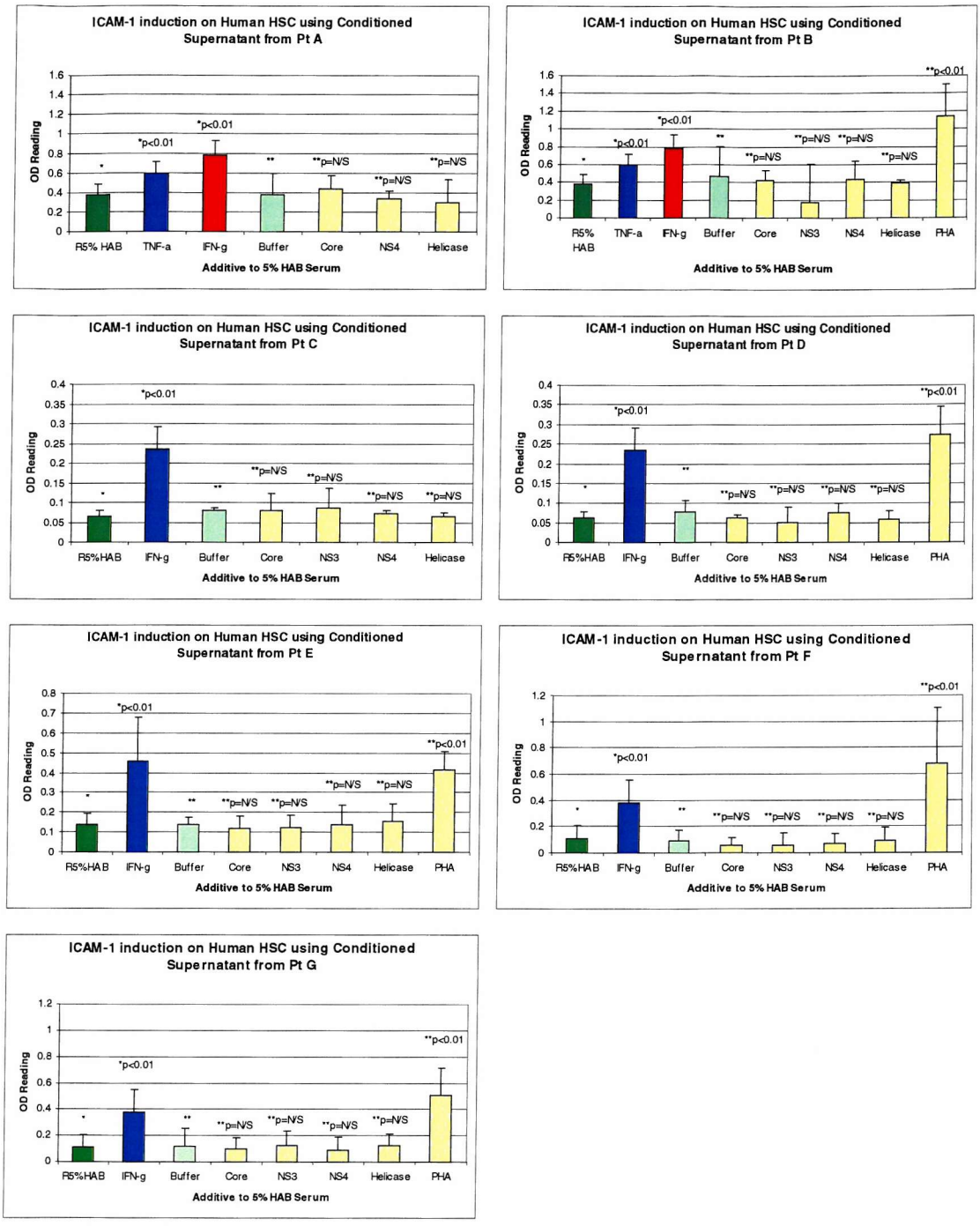
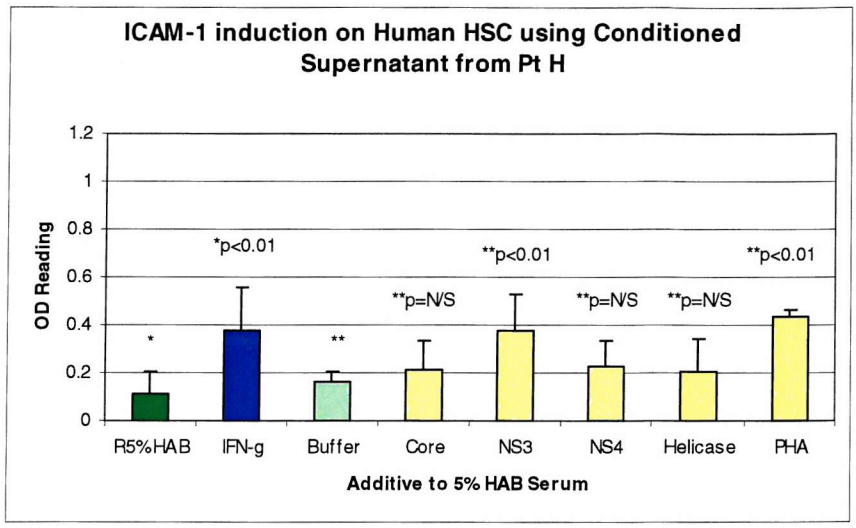
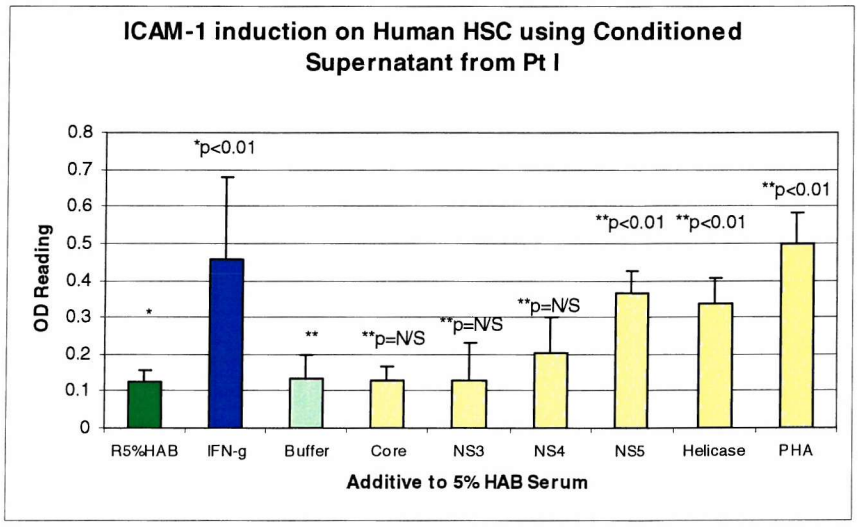


Figure 6.07 Cellular ELISA used to screen conditioned supernatants from the HCV protein stimulated PBMC of 7 CHC patients. In these patients, none of the HCV protein stimulated supernatants produced significant up-regulation of ICAM-1. PHA stimulated supernatants and IFN-γ positive control medium did produce significant up-regulation of ICAM-1 expression in all cases.($p<0.01$)





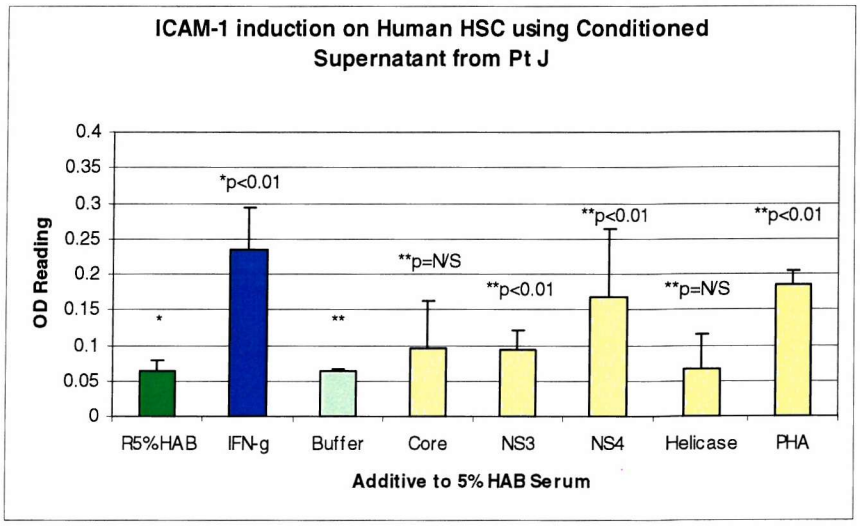


Figure 6.08 Cellular ELISA used to screen conditioned supernatants from the HCV protein stimulated PBMC of 3 CHC patients. The supernatants from PHA stimulated PBMC all produced up-regulation of ICAM-1. In addition, at least 1 HCV protein stimulated supernatant in each patient produced significant up-regulation of ICAM-1 expression by HSC.

6.4.7 Measuring Interferon-γ Levels in the Conditioned Supernatants

Screening individual cytokines revealed that IFN-γ was the most potent at producing up-regulation of ICAM-1 expression in hepatic stellate cells. To assess the likelihood that IFN-γ was the dominant cytokine causing ICAM-1 up-regulation in the supernatants generated from patients H,I and J, sandwich ELISA for IFN-γ was used to measure levels in each of the samples. The results are shown in table 6.02.

IFN-γ ELISA of Supernatants from PBMC used in HSC ELISA

Pt	Buffer	Core	NS3	NS4	NS5	Helicase	PHA
Patients with Non-Reactive HCV Stimulated Supernatants (Group 1)							
A	9.77	9.58		9.3	9.9	8.9	No test
B	15.2	17.1	12.9	14.5	14.2	17.3	447.5
C	6.4	6.1	6.1	7.3	8.4	5.9	No test
D	8.5	9.0	8.0	7.3	8.3	9.9	411.4
E	3.8	4.1	9.7	7.0		5.53	792.2
F	6.97	7.73	6.11	5.3		8.1	161.1
G	3.9	3.5	3.5	Low		6.7	1058.9
Patients with Reactive HCV Stimulated Supernatants (Group 2)							
H	6.6	8.8	29.9	10.3		8.4	471.3
I	73.3	106.3	Low	140.6	116.8	143.4	893
J	5.9	Low	5.9	3.7	Low	6.4	803

Table 6.03 IFN–γ levels in the PBMC conditioned supernatants used in ICAM-1 cellular ELISAs. Results are shown in pg/ml, as measured by sandwich ELISA. The supernatants which produced significant up-regulation of ICAM-1 are shown in red.

In group 1, the HCV protein stimulated supernatants all had low levels of IFN-γ, and there were no significant differences between levels in protein stimulated and buffer negative controls. Each of the PHA stimulated supernatants had high levels of IFN-γ. In group 2, patient H had low levels of IFN-γ in all of the supernatants except NS3 and PHA stimulated, which were significantly raised. Patient I had high levels in all of the supernatants except the NS3 stimulated. IFN-γ levels were significantly higher

in the PHA stimulated supernatant. Patient J had low levels of IFN-γ in all of the supernatants except PHA stimulated, which was high.

6.4.8 Assessing the Effect of Blocking Interferon-γ in Conditioned Supernatants in HSC Cellular ELISA for ICAM-1

To assess the contribution of IFN-γ in up-regulation of ICAM-1 expression by HSC, the supernatants from patient H were pre-incubated with an IFN-γ specific monoclonal antibody prior to incubation with HSC in the cellular ELISA assay. The high baseline level of IFN-γ in the patient I buffer control supernatant did not cause ICAM-1 up-regulation above the level of control medium. The IFN-γ levels in the 3 reactive supernatants (NS5, Helicase and PHA) were higher than buffer control. NS4 also had a high level of IFN-γ, which up-regulated ICAM-1 expression, but not quite to reach statistical significance. Blocking IFN-γ activity totally, would reveal whether the up-regulation in ICAM-1 expression seen with the patient I supernatants was due to the higher levels of this cytokine. Results are shown in figure 6.09.

The significant up-regulation of ICAM-1 in HSC seen with NS5 and Helicase stimulated supernatants is completely abrogated by pre-incubation with blocking anti-IFN-γ antibodies. This suggests that the increase in ICAM-1 expression above buffer-negative control in these samples was due to the higher levels of IFN-γ in these supernatants.

However, the PHA associated rise in ICAM-1 expression is only partially abrogated by blocking anti-IFN-γ antibodies. This is despite the fact that the level of IFN-γ in the supernatant was far lower than the 5000pg/ml recombinant cytokine used as a positive control in this experiment the total effect of which could be blocked by the antibody. This suggests that IFN-γ is not the only mechanism through which ICAM-1 is up-regulated in PHA stimulated supernatants.

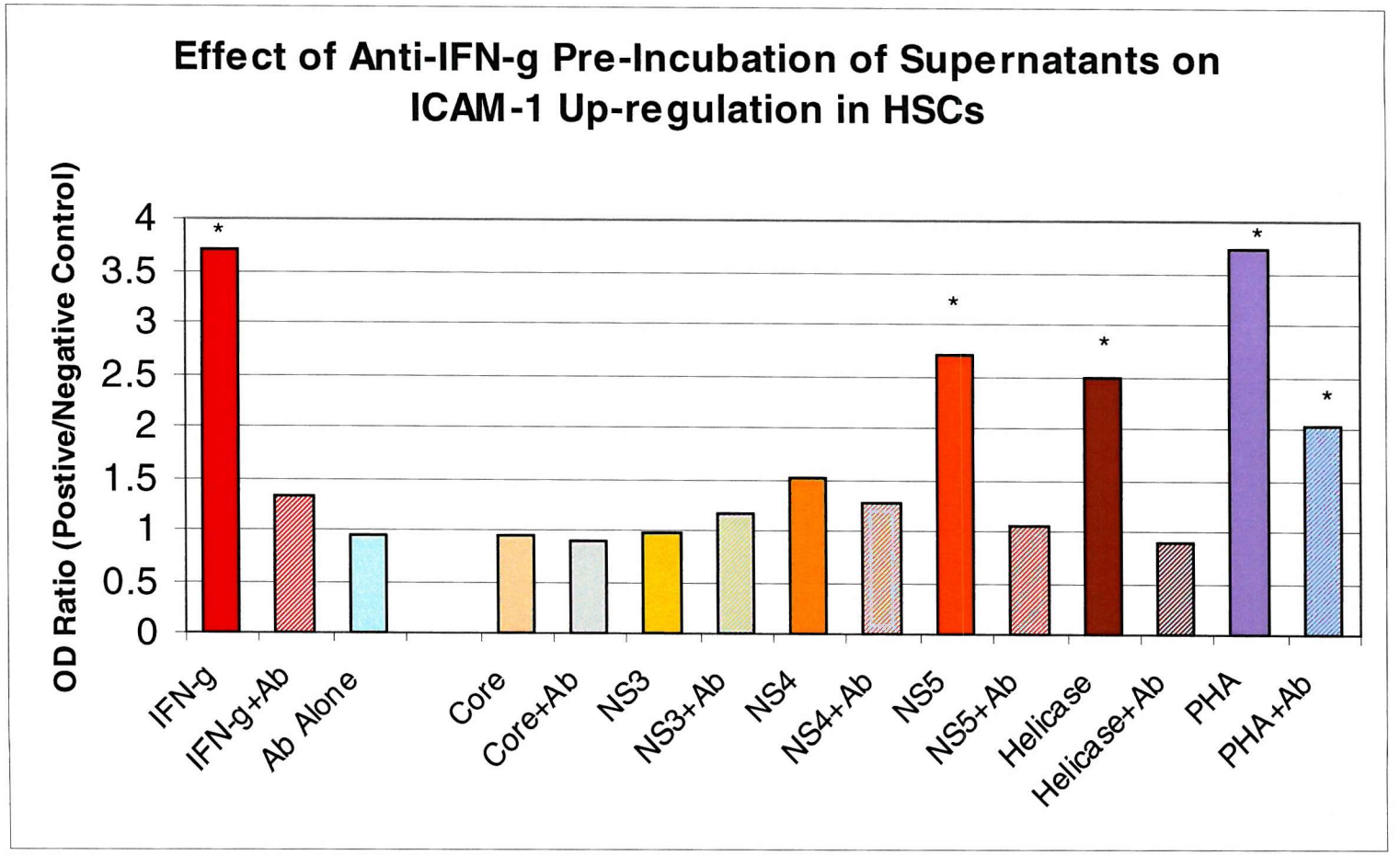


Figure 6.09 The effect of pre-incubation of conditioned supernatants with anti-IFN–γ monoclonal antibody. Control is with recombinant human IFN-γ at 5000pg/ml. The results are represented as a ratio of mean OD reading of test wells over mean OD reading of appropriate negative control, (where 1=no significant rise in OD reading, representing no up-regulation of ICAM-1).
***Denotes significantly raised OD readings above negative control p<0.01**

6.4.9 Investigation of Interferon-γ Regulated Immunological Pathways in HSC at a Molecular Level

IFN-γ causes up-regulation of ICAM-1 in HSC. This effect can be reproduced by the conditioned supernatants from the PBMC of patients with chronic hepatitis C. In order to investigate what other effects IFN-γ has on HSC, RNA was harvested from activated primary HSC after 16 hours incubation with either control medium or IFN-γ containing medium. Conversion of this RNA to cDNA allowed incubation with a membrane cross-linked with specific DNA probes to genes of interest. The amount of cDNA bound to the membrane could then give a semi-quantitative measure of mRNA expression, normalized to β-actin. In this way it is possible to assess the effect of IFN-γ on genes associated with fibrosis, immune active surface molecules, chemokines and cytokines.

Each assay produced 2 membranes with a chemiluminescent signal, which was then exposed to light sensitive film. (Figure 6.10) Densitometry was undertaken on the radiographs, and quantitative measurements were obtained by comparing the mean density of the two dots generated by the gene of interest to those of the β-actin gene. The array experiment was repeated 3 times, each time with primary hepatic stellate cells from a different donor. The cells were used at 14-21 days after isolation, depending on when they reached confluence in a tissue culture flask. All of the cells were activated due to their culture on plastic.

The mean results of 3 arrays are shown in figure 6.11. Each gene is represented as a percentage change with IFN-γ stimulation compared with control. Some genes were below the level of detection of the assay in either the control or IFN-g stimulated array. These genes are represented by a minimum percentage change, calculated by using the threshold level of detection of the assay as the value for the negative reading, and are marked with *.

IFN-γ stimulation has little effect on the expression of fibrosis associated genes, consistent with the established literature that IFN-γ is anti-fibrotic. There is a large increase in mRNA for ICAM-1, which is consistent with the FACS and ELISA data. In addition, HLA-DR is up-regulated and VCAM-1 and CD40 are switched on. In cytokine and chemokine genes, there is variability; IL-10, MCP-3, MIP1α, MIP-1β, TARC, TGF-β and TNF-α are all down-regulated; IL-6, IL-8, ITAC, MCP-1, MIG and IP-10 are all up-regulated. The relevance of this will be discussed later.

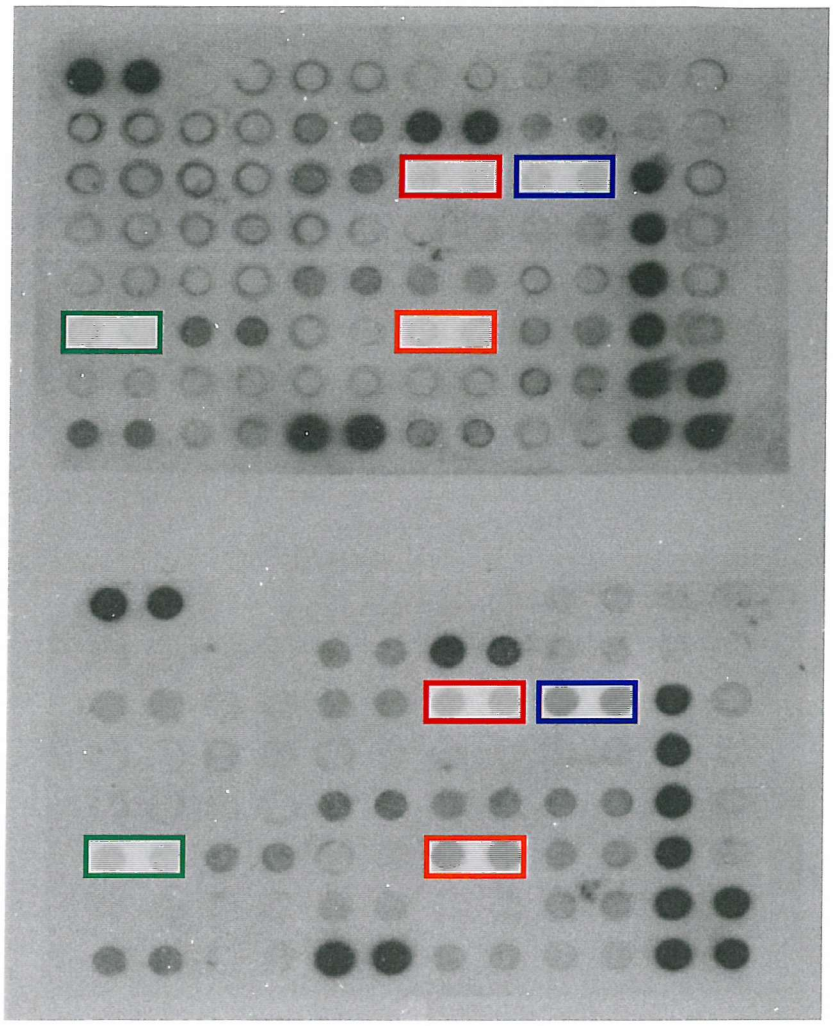
There is some biological variation between the responses of HSC from different donors, and the data from the 3 arrays is shown in figure 6.12.The inter-donor variability between HSC is most marked in the genes associated with fibrosis. HSC from one donor out of the three up-regulates α-SMA, collagen-1 and Gelatinase-A with IFN-γ, whilst cells from the other 2 donors down-regulate these genes. These differences are small, but may be important in helping to explain biological variation in rate of progression and severity of hepatic fibrosis in chronic liver disease.
To confirm that the mRNA values correlate with changes at a protein level, several genes were chosen for confirmatory testing. The results are represented in table 6.03.

Ref. Array	**Protein Tested**	**Method**	**% Change in Protein Expression IFN-γ Relative to Control** (Absolute No.)	**% Change in RNA Expression IFN-γ Relative to Control** (Absolute No.)	**Likely Level of Control**
1	a-SMA	Western	**102.7** .037/1.013)	**120** (1.11/0.92)	RNA
1	TIMP-1	Western	**102.3** (1.074/1.046)	**109** (1.09/1.00)	RNA
1	ICAM-1	FACS	**180.5** (83.6/46.3)	**148.4** (0.95/0.64)	RNA
1	CD40	FACS	**193.5** (3.62/1.87)	**105.1** (0.62/Neg)	Protein
1	HLA II	FACS	**147.9** (2.5/1.69)	**115.3** (0.68/Neg)	RNA
3	MIG	ELISA	**20131.8** (3221/16)	**137.3** (0.86/Neg)	Protein
3	MCP-1	ELISA	**138** 2432/1762)	**104.5** (0.46/0.44)	RNA
3	IL-8	ELISA	**80.2** 692.4/862.3)	**114.8** (0.44/Neg)	Protein

Table 6.04 Confirmation of RNA changes by using various protein assays to corroborate results from gene arrays. Western Blots used lysates from the same cells used in the gene array, and were analysed by densitometry (units=relative density to constant). FACS was carried out on freshly harvested cells from a parallel culture to that used in the array (units=percentage positive staining cells). ELISAs were undertaken on the supernatants from the cells used in the array (units=pg/ml)

All of the confirmatory tests produced protein assay results which correlated in direction of change with the RNA assays, except IL-8 in array 3. The percentage changes were not equivalent, but transcription does not always correlate exactly with translation.

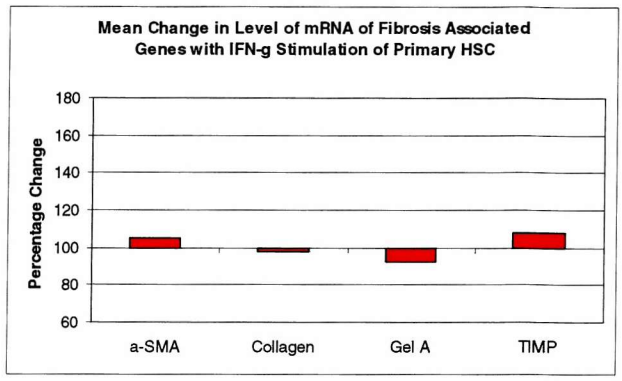
A



B

α-SMA	α-SMA	CCR3	CCR3	CCR4	CCR4	CCR5	CCR5	CD40	CD40	pUC18	
CD40L	CD40L	CD69	CD69	CD81	CD81	Coll-1	Coll-1	CXCR3	CXCR3	pUC18	
HLA-DP	HLA-DP	Eotaxin	Eotaxin	GEL-A	GEL-A	**HLA-DR**	**HLA-DR**	**ICAM-1**	**ICAM-1**	b-ACTIN	
IFN-γ	IFN-γ	IL-10	IL-10	IL-12A	IL-12A	IL-12B	IL-12B	IL-13	IL-13	b-ACTIN	
IL-18	IL-18	IL-4	IL-4	IL-6	IL-6	IL-8	IL-8	I-TAC	I-TAC	GAPDH	
MCP-1	**MCP-1**	MCP-3	MCP-3	MCP-4	MCP-4	**MIG**	**MIG**	MIP-1a	MIP-1a	GAPDH	
MIP-1b	MIP-1b	NCAM	NCAM	IP-10	IP-10	RANTES	RANTES	TARC	TARC	GAPDH	GAPDH
TGF-β1	TGF-β1	TGF-βR	TGF-βR	TIMP-1	TIMP-1	TNF-α	TNF-α	VCAM-1	VCAM-1	GAPDH	GAPDH

Figure 6.10 **A** Radiographs from a paired gene array used for assessment of cDNA from HSC RNA (Control cell RNA used for the upper membrane and IFN-γ stimulated cell RNA for the lower). The largest magnitude increases are highlighted and colour coded on the array and table. **B** Arrangement of the gene probes is shown in the table. Densitometric readings were taken from each dot for analysis.



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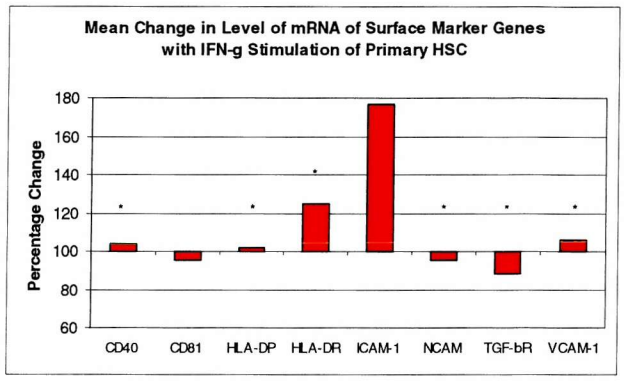

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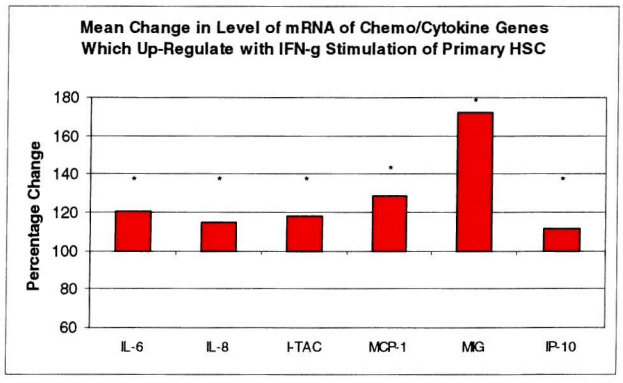

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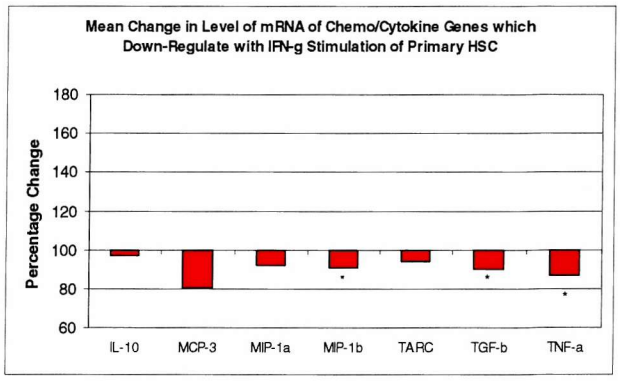
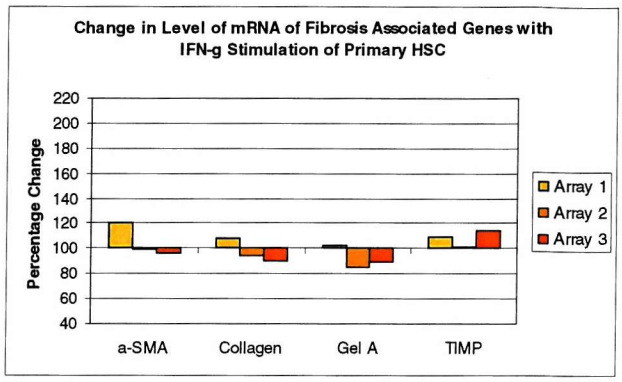


D

Figure 6.11 Mean results from 3 gene arrays, divided by nature of activity of the gene; **A** Fibrosis associated; **B** Surface molecules; **C/D** Chemo/ Cytokine molecules. Positive bars indicate up-regulation, and negative bars down-regulation of mRNA in relation to non-IFN-γ stimulated cells. *Indicates results representing minimum percentage change.



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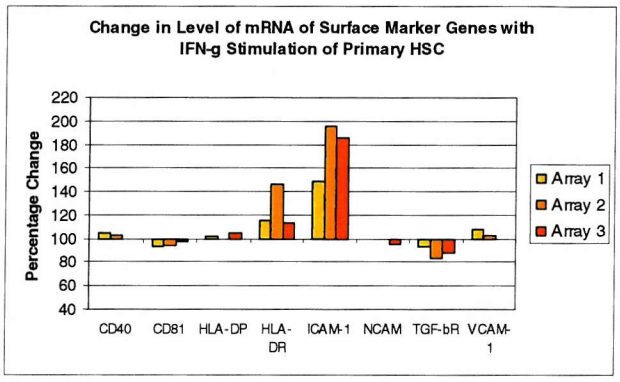

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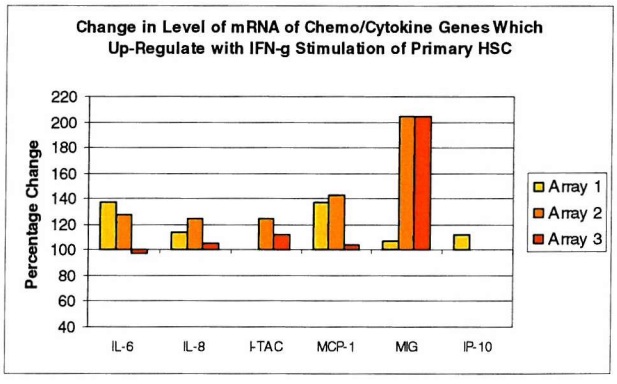

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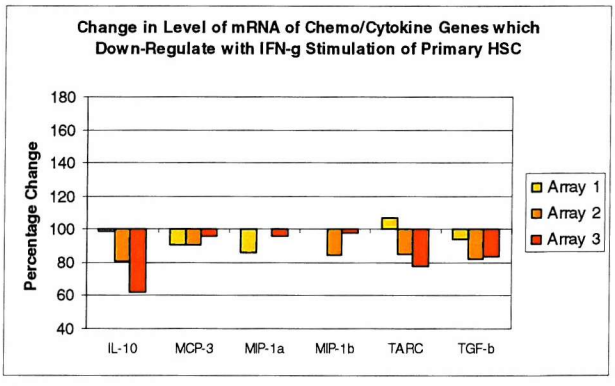

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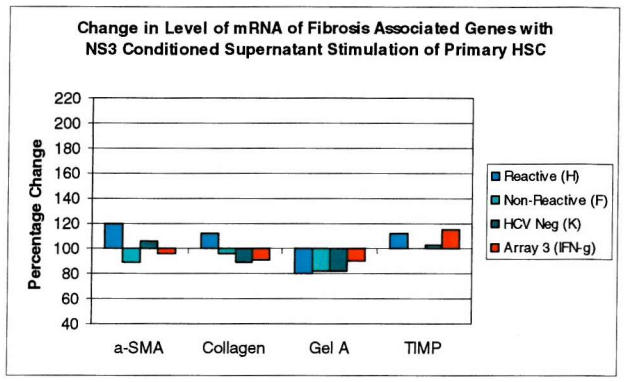
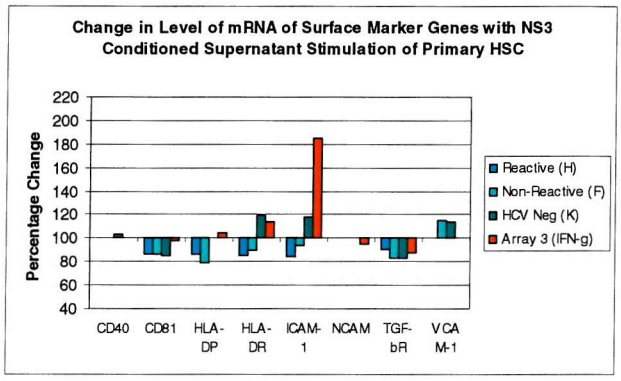
Figure 6.12 Results from 3 gene arrays, divided by nature of activity of the gene; **A** Fibrosis associated; **B** Surface molecules; **C/D** Chemo/ Cytokine molecules. Percentage change is with IFN-γ stimulation compared with control. If there is no bar, the gene was not detectable in that assay.

6.4.10 Investigation of the Effect of Conditioned Supernatant on Immunological Pathways in HSC at a Molecular Level

The conditioned supernatants from patient H produced up-regulation of ICAM-1 in the ELISA assays with NS3 stimulation, similar to that seen with recombinant IFN–γ. This was associated with a high level of IFN–γ in the relevant conditioned supernatant (29.9pg/ml in the NS3 stimulated supernatant compared with 6.6pg/ml in the buffer control). To further investigate the effects of conditioned supernatants on hepatic stellate cells, and assess whether the effect of IFN-γ containing supernatant is entirely due to IFN-γ, further experiments were devised using the gene arrays. Therefore supernatant from patient H buffer control and NS3 stimulated PBMCs was used to stimulate 2 flasks of primary HSC overnight, and the cells were then harvested for RNA extraction and use in a gene array. In parallel, as a positive control for this experiment, standard medium versus medium containing recombinant IFN-γ was used to stimulate HSC from the same donor. As a negative control, HSC from the same donor were also incubated over night with buffer and NS3 stimulated supernatants from patient F, (a chronic hepatitis C patient with no reactivity in the ICAM-1 assays and low levels of IFN–γ), and buffer and NS3 stimulated supernatants from a HCV negative control. The results of the positive control array pair and the patient H stimulated array pair are shown in figure 6.13. The results from the patient F and HCV negative control (patient K) array pairs are also shown in figure 6.13.



A



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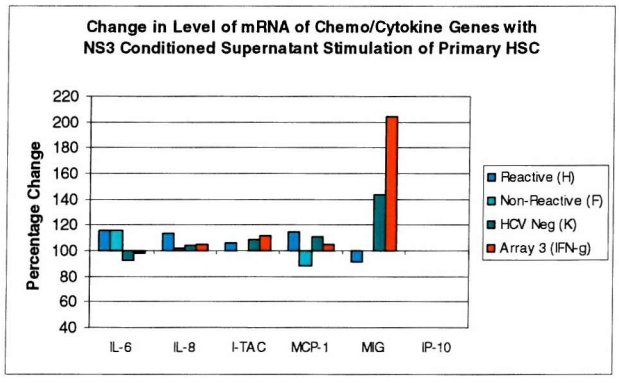

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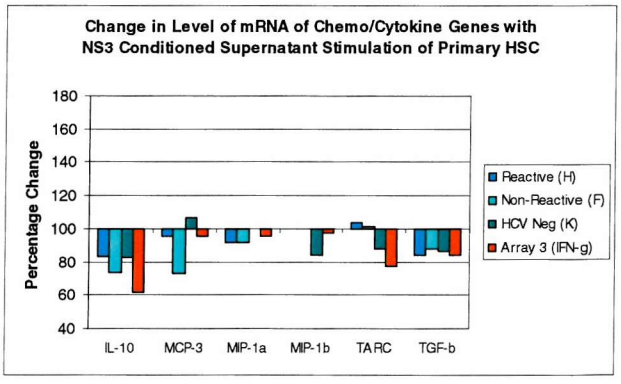

D

Figure 6.13 Gene arrays from HSC from one donor stimulated by conditioned supernatants from pts H,F and K, and a control array stimulated with IFN-γ (cf. fig 6.12).**A** Fibrosis associated; **B** Surface molecules; **C/D** Chemo/Cytokine molecule genes. Percentage change is between NS3 and control. If there is no bar, the gene was not detectable

6.5 Discussion

Hepatic stellate cells can be reliably and safely isolated from human liver resections. The quiescent HSC which are harvested from a liver preparation gradually activate and acquire a myofibroblast phenotype over the course of 7-10 days when cultured in serum rich media on plastic. The cells proliferate and reach confluence in a tissue culture flask within 14-21 days. These activated primary human HSC can be used as an *in vitro* model of HSC behaviour during an active fibrogenic process. They express typical markers of HSC activation, as seen *in vivo*, and have similar morphology to those cells seen in collagen bands of a fibrotic liver.

Stimulation with IFN-γ or TNF-α produces changes in the phenotype of the activated, cultured HSC which can be assessed by various experimental techniques i.e. FACS analysis, Western Blotting, supernatant ELISA. This suggests that these cultured primary cells may be a useful model to examine interactions between the immune system and HSC. This would be a particularly attractive prospect for diseases with immune mediated chronic fibrotic liver disease in which there is no reliable small animal model i.e. chronic hepatitis C.

Human hepatic stellate cells are a valuable and scarce commodity. However, passaged cells show many of the same characteristics as primary cells, particularly in their responses to IFN-γ and TNF-α. This maintenance of phenotype lasts for at least 2 passages of cells in culture, and IFN-γ is always the most potent stimulus. Passaged human stellate cells from different donors show the same ICAM-1 response to any given cytokine stimulus, and the results are reliable and reproducible. Thus, ICAM-1 expression in passaged human stellate cells can be used as a screening tool for identifying interesting interactions between the immune response and HSC which warrant further investigation using primary cells.

Conditioned supernatants were generated by stimulating freshly isolated PBMC from CHC patients with recombinant HCV proteins. After 3 days the supernatants were harvested and used in HSC experiments. Previous work in chapter 4 has confirmed that this method of stimulating PBMC will primarily identify a $CD4^+$ response, if there is one to detect. 10 CHC patients were used in these experiments. None of the

10 patients had a measurable $CD4^+$ proliferative response to the recombinant HCV proteins. 2 patients had significant antigen specific IFN-γ secretion to at least one HCV protein, although in one patient this was in the presence of high background IFN-γ production. Each set of supernatants was tested against passaged HSC from at least 2 different donors, using the ICAM-1 cellular ELISA. Significant up-regulation of ICAM-1 expression was produced by supernatants from 3 of the 10 patients.

These results demonstrate that standard methods for measuring antigen specific $CD4^+$ function may not detect all significant responses. Proliferation was negative in all 10 patients, whilst IFN-γ production was positive in 2 of the 10 patients. Using the HSC ICAM-1 assay, both of the IFN-γ producers were positive, and a further patient was identified whose supernatants produced significant up-regulation of ICAM-1. This suggests that the HSC ICAM-1 cellular ELISA is more sensitive than standard assays for detecting a CD^+ response.

Previous studies have concluded that measurable $CD4^+$ responses in CHC are rare, unless the patient is treated with anti-viral therapy *(Cramp 1999; Cramp 2000)*. Using one simple technique, the present study has demonstrated a significant HCV antigen $CD4^+$ response in 30% of CHC patients. These patients were discordant for severity of liver disease. In addition, assessment of antigen specific IFN-γ production has revealed that there is heterogeneity in the nature of the $CD4^+$ response in these patients. In 2 of the patients there was antigen specific IFN-γ secretion; although in one patient this was in the context of high background IFN-γ production. In the third patient there was no measurable IFN-γ secretion. This implies that there may be different mechanisms causing the ICAM-1 up-regulation in the assays from the three individuals.

In one patient with high HCV antigen specific IFN-γ secretion, the HSC cellular ELISA for ICAM-1 was repeated following pre-incubation of the conditioned supernatant with neutralising anti-IFN-γ antibodies. The effect of this pre-incubation was to completely abrogate the ICAM-1 up-regulation effect of the supernatant. This supports the conclusion that in this patient, the up-regulation of ICAM-1 in HSC was due to IFN-γ in the conditioned supernatant. This is supporting evidence for the

hypothesis that the interactions between the $CD4^+$ response and HSC are not due to a single, easily measurable factor such as IFN-γ secretion; IFN-γ can not be mediating the interaction in the patient where there is ICAM-1 up-regulation in the absence of IFN-γ, whilst it is clearly mediating the up-regulation in the supernatant used in the IFN-γ blocking experiment.

Unfortunately, further investigation of supernatants generated from the individual with IFN-γ independent ICAM-1 up-regulation was not possible as the patient underwent liver transplantation soon after the initial supernatants were generated, and was then started on treatment with immunosuppressive drugs.

IFN-γ is generally accepted as an anti-fibrotic cytokine *(Baroni 1996; Friedman 1999)*. HCV antigen specific IFN-γ production was a feature in PBMC from 2 patients included in the present study, and one of those 2 patients had severe fibrotic liver disease. Up-regulation of ICAM-1 in activated HSC is a surrogate marker of increased NF-κB activity and should render HSC less proliferative and less fibrogenic *(Gallois 1998; Elsharkawy 1999; Eng and Friedman 2001)*. 3 of the 10 patients studied had supernatants which produced ICAM-1 up-regulation in HSC, and 2 of those had severe fibrotic liver disease. These apparent inconsistencies were addressed by examining the effects of IFN-γ stimulation on primary HSC and then comparing this with the effect of conditioned supernatants on the same cells.
Activated primary human hepatic cells were stimulated for 16 hours with control medium or medium containing IFN-γ, the cells were then lysed and the RNA extracted. This experiment was undertaken using HSC from 3 different donors. A semi-quantitative gene array was used to assess mRNA levels using specific gene probes cross-linked to a membrane. The level of expression of the genes was normalized to β-actin expression. The genes investigated included markers of fibrotic activity, surface markers involved in possible immune interactions, and chemokines and cytokines. mRNA changes were confirmed in some cases at protein level using either FACS analysis, Western blotting or ELISA as appropriate.

Pooled results from the 3 populations of HSC revealed that the biggest changes produced by IFN-γ were in levels of mRNA for ICAM-1, HLA class II and some

chemokines. In the fibrosis associated genes, there was a slight reduction in Collagen 1 and Gelatinase A mRNA, and a slight increase in TIMP-1 mRNA. Surface molecule genes showing increased expression were CD40 and VCAM-1, whilst NCAM, TGF-β receptor and CD81 were all reduced. IL-6, -8, I-TAC, MCP-1, MIG and IP-10 mRNA were all increased and IL-10, MCP-3, MIP-1a, -1b, TARC, TGF-β and TNF-α were all reduced. The net effect of these changes in gene expression is likely to reduce fibrosis (reduction in collagen, Gelatinase A and TGF-β), increase recruitment of non-antigen specific lymphocytes, NK cells and monocytes (increase in IP-10, MCP-1, IL-8, ICAM-1, VCAM-1 and HLA class II) and suppress Th1 lymphocyte responses (increase in IL-6). *(Roitt 1997; Maher 2001)*

However, there was inter-individual variability in the IFN-γ induced changes in primary HSC gene expression between donors. These inter-individual variations were most evident in the genes associated with fibrosis, although there were differences also in IL-6, TARC and IP-10. This suggests that HSC from different individuals react differently to the same stimulus. This could be interpreted as giving the donor in array 1 a greater tendency to develop fibrosis and recruit inflammatory cell subsets than the other 2 donors, given the right activation stimulus.

Finally, we investigated whether the up-regulation in ICAM-1 expression in the passaged cells used in the screening ICAM-1 ELISA assay was purely an IFN-γ effect in CHC patient H. The reactive supernatant (conditioned with HCV NS3 protein), with high IFN-γ levels, was applied to primary HSC overnight, with the protein buffer conditioned supernatant as a negative control (low IFN-γ levels). The HSC were then harvested and used in the semi-quantitative gene array experiment. In parallel, primary HSC from the same donor were stimulated with conditioned media from patient F (CHC patient with non-reactive, low IFN-γ containing NS3 and buffer conditioned supernatants), and the same from a non-HCV exposed individual. A positive control using the same HSC was the standard IFN-γ containing or control medium.

There were differences in the gene expression pattern produced by the IFN-γ containing conditioned medium and the positive control IFN-γ containing medium.

These differences were most marked in the change in expression in Collagen 1 (up-regulated by the PBMC conditioned medium and down-regulated by IFN-γ), and the expression of ICAM-1 and IL-6. Although this supernatant was identified as reactive by up-regulating ICAM-1 expression in passaged HSC, the NS3 conditioned supernatant from patient H actually down-regulated the mRNA for this gene in primary HSC (ICAM-1 was up-regulated by IFN-γ containing positive control medium as expected). In addition, there was discordance in the effect of the positive control medium and conditioned medium in the effect on expression of IL-6 mRNA; the PBMC conditioned medium up-regulated IL-6 expression while the positive control medium down-regulated the same gene.

The 2 negative control PBMC conditioned supernatants had slightly variable effects on gene expression in the primary HSC. The HCV negative patient (K) produced effects similar to the positive control IFN-γ containing medium, although generally the changes were of a lower magnitude. The supernatants generated from the PBMC of the CHC patient was generally similar in effect on gene expression to the responses see with patient H, with the notable exception of Collagen 1 which was down-regulated with the non-reactive supernatant of patient F. Interestingly, although the supernatant from patient H was chosen because of the up-regulation in ICAM-1 expression it produced in passaged HSC, the mRNA for ICAM-1 was actually reduced by this supernatant in the primary cells. This suggests that the ICAM-1 effects may be controlled at a translation level rather than transcription.

The results from stimulation of primary HSC with reactive and non-reactive PBMC conditioned supernatants suggest that the effect of IFN-γ containing supernatants is not entirely due to IFN-γ. In addition, there are effects on HSC gene expression with supernatants found to be non-reactive by all of the preceding assays (lymphocyte proliferation, cytokine ELISA, ICAM-1 cellular ELISA). The exact nature of the constituents of the supernatants which may be exerting these effects is not yet clear, and will require further investigation.

There is variability in the severity of liver disease resulting from chronic hepatitis C infection. The liver damage is believed to be immune mediated. It has been difficult to

explain the phenomenon of discordance in severity of liver disease, as there have been no studies which confirm discriminatory anti-HCV immune responses between groups with varying degrees of liver damage. The present study develops a new assay which can increase the sensitivity for identifying CHC patients with detectable HCV specific $CD4^+$ responses from 20% to 30%. In addition, this study shows that the effects of these HCV specific responses are mediated by more than one factor, some may be primarily mediated by IFN-γ. Although IFN-γ is important in the responses generated by some individuals, it is not implicated at all in others with a detectable HCV specific immune response. Finally, we have demonstrated that primary HSC from different donors will respond differently to stimulation with IFN-γ. The most striking difference in activation pattern we have demonstrated between the HSC from different donors is in the genes associated with fibrosis. Although the differences in these genes are of low magnitude, HCV causes liver damage over decades, and thus small differences may have profound effects if they are given long enough to accumulate.

In summary, the present study demonstrates:

- There is a detectable $CD4^+$ response to HCV proteins in 30% of CHC patients
- There is heterogeneity in the nature of this $CD4^+$ response
- There is heterogeneity in the responses of HSC from different donors to the same stimulus

Taken together, these important findings may begin to explain the heterogeneity in the severity of liver disease found in CHC. The amount of fibrosis in any individual will depend upon the nature and magnitude of the immune response, and the character of the response it generates in the hosts HSC.

Chapter 7

Chapter 7

Discussion

7.1 Introduction

Hepatitis C is a major healthcare problem, affecting an estimated 170 million people worldwide. It is spread parenterally, and causes a chronic hepatitis in approximately 85% of those infected. The virus is not believed to be cytopathic, and the liver damage that results from chronic infection is thought to be immune mediated. A vigorous immune response against multiple viral epitopes is associated with viral clearance in the acute phase, but these responses must be maintained beyond 6 months for sustained virological clearance *(Diepolder 1997; Koziel 1997; Di Bisceglie 1998)*. In chronic hepatitis C (CHC) there a detectable, but inadequate, immune response to the virus. I have examined the hypothesis that the magnitude and character of this response dictates the severity of the resultant liver damage, with particular reference to $CD4^+$ T lymphocytes and their interactions with hepatic stellate cells.

7.2 Summary of Findings

7.2.1 HLA Associations

Antigen is presented to $CD4^+$ T cells through the MHC class II, and several studies have demonstrated an association between certain HLA alleles and clearance of acute HCV infection. HLA-DQB1*0301 has been shown to be associated with viral clearance in ethnically similar populations to Southampton's *(Tibbs 1996; Alric 1997; Thursz 1999)*. The phenotype of the immune response in acute HCV viral clearance is a brisk Th1 response to multiple epitopes *(Hoffmann 1995; Cramp 1999)*. One study examined frequency of HLA DQB1*0301 in end-stage HCV related liver disease requiring liver transplantation. It was found that this allele occurred at lower frequency in end stage liver disease when compared with the background frequency in the local healthy population. The conclusion drawn from this observation was that HLA DQB1*0301 is protective from severe liver disease in chronic hepatitis C infection *(Tillmann 2001)*. This might be explained by the effect of the Th1 cytokines in CHC.

The cytokines associated with a Th1 response are anti-fibrotic and therefore if present in the sub-optimal immune response in chronic disease, might be expected to produce less fibrosis and less severe liver disease *(Friedman 1999; Maher 1999)*. In the present study, I have attempted to demonstrate an association between HLA-DQB1*0301 and severity of liver disease.

I established the background frequency of HLA DQB1*0301 in the local population by genotyping samples from a local group of healthy solid organ donors. I compared the frequency of this allele in a population of CHC patients and found that there was no statistical difference between allele frequency in the 2 groups.

The CHC patients were then categorised according to severity of liver disease on liver biopsy. There were positive correlations between heavy alcohol intake and increasing age with increased severity of liver disease. This finding is in keeping with the current literature. However, there were no statistical differences between frequency of HLA DQB1*0301 and severity of liver disease. The demographics of the 2 groups were slightly heterogeneous, but multiple logistic regression analysis with correction for confounding variables including age and sex did not reveal a statistically significant difference between the groups discordant for severity of liver disease.

Liver biopsy results were used to further sub-divide the CHC patients, looking at inflammatory and fibrotic changes in isolation. There were no statistically significant differences in HLA DQB1*0301 frequency between the groups using any of these factors. However, there was a trend towards under-representation of the allele in severe fibrosis, but numbers were too small to achieve significance. This trend if it continued to statistical significance in a larger group would be in keeping with the findings of Tillmann *et al.*, as cirrhosis is the end stage of advancing fibrosis and is the commonest indication for transplantation in CHC *(Tillmann 2001)*.

Finally, sub-group analysis of the CHC patients was undertaken to test the hypothesis that Th1 $CD4^+$ T cell responses produced by antigen presentation through DQB1*0301 inadequate to produce viral clearance will result in chronic liver disease characterised by less fibrosis and more inflammation. Numbers of patients with discordant fibrosis and inflammatory indices were low, and thus the analysis was somewhat limited. There was a statistically significant absence of HLA DQB1*0301 in patients with severe fibrosis and mild inflammation, whilst I was unable to demonstrate over-representation of the allele in the group of patients with mild fibrosis and severe inflammation. However, previous alcohol abuse is a confounding

factor influencing degree of fibrosis in this group. The phenotype of mild inflammation with severe fibrosis would be more in keeping with a Th2 dominant $CD4^+$ response *(Friedman 1999)*.

The immune response in chronic hepatitis C is complex and not solely mediated by $CD4^+$ T lymphocytes. Thus it is perhaps not surprising that I have been unable to show clear associations between HLA DQB1*0301 and severity of liver disease in CHC with the limited numbers of patients available to study. However, the trends I have demonstrated towards increasing fibrosis and under-representation of this allele suggest that the $CD4^+$ of response is involved in dictating the phenotype of the liver disease that results from CHC infection.

7.2.2 $CD4^+$ Phenotype Correlated with Severity of Liver disease

Several groups have previously demonstrated clear differences in the magnitude and phenotype of the $CD4^+$ T lymphocyte response between patients with resolved acute HCV infection and those with CHC *(Diepolder 1995; Hoffmann 1995; Diepolder 1997; Cramp 1999; Gerlach 1999; Rehermann 1999; Rosen 1999; Takaki 2000)*.

In the present study we aimed to characterise the $CD4^+$ T cell responses of CHC patients and correlate the magnitude and phenotype of the response with the severity of the resultant liver disease.

Using standard lymphocyte proliferation assays and cytokine secretion assays in peripheral blood mononuclear cell (PBMC) cultures, I demonstrated that responses to non-HCV recall antigens such as influenza and tetanus toxoid were equivalent between normal controls, resolved acute HCV patients and CHC patients. When the same assays were performed using recombinant HCV antigens there were clear differences between resolved acute HCV patients and CHC patients, both in terms of magnitude of proliferative response and frequency of cytokine responses. Proliferative responses to recombinant HCV antigens in CHC patients were indistinguishable from those elicited from healthy individuals who had not encountered HCV. However, it remains possible that low magnitude proliferative in responses to at least one HCV epitope in the CHC patients were present but that this was being masked by the responses to many other, less discriminatory epitopes in a very "noisy" system.

The CHC patients were then categorised according to the severity of liver disease on liver biopsy. Using standard lymphocyte proliferation assays with HCV antigens, there were no statistically significant differences between the groups discordant for severity of liver disease. Assessing cytokine secretion by HCV stimulated PBMC revealed that antigen specific interferon-gamma (IFN-γ) production was more frequently seen in patients with more severe liver disease. This finding achieved statistical significance for the groups as a whole, but could not be used to discriminate between individuals discordant for liver disease severity. IFN-γ is generally believed to be an anti-fibrotic cytokine, and the observed association between production of this cytokine and more severe liver disease might seem to be a contradiction. However, IFN-γ is also involved in inflammatory cell recruitment, and it may be through this mechanism that its production is ultimately associated with more severe liver disease.

Fluorescence Activated Cell Scanning (FACS) analysis was used to try and further characterise the differences between the nature of the $CD4^+$ responses in resolved acute HCV and CHC infection. This technique allows responses from individual cells to be assessed. In particular, after stimulation with an antigen, the frequency of cytokine secretion can be assessed in the $CD4^+$ population of PBMC, when it co-exists with CD69 expression (a marker of activation). Using this technique some interesting trends were revealed. Resolved acute patients had a trend towards an increase in IFN-γ and IL-10 expression on stimulation with HCV antigens, whilst there was a trend towards a decrease in IL-2 and IL-4 expression. CHC patients had a trend towards an increase in all cytokine expression, most marked in IL-10 and IL-4. Although these results do not achieve statistical significance as numbers are small, they support the hypothesis that resolved acute infection is associated with a predominantly TH1 $CD4^+$ T cell response, and CHC with a Th2.

$CD4^+$ depletion / enrichment experiments were used to confirm that the proliferative and cytokine responses measured in the assays represented a $CD4^+$ response. The experiments were undertaken in resolved acute patients with measurable proliferative and IFN-γ responses to HCV antigens. The positive responses were confirmed to be generated by $CD4^+$ T lymphocytes. In addition, it was noted that the IFN-γ responses in the whole PBMC cultures were of lower magnitude than in the $CD4^+$ enriched fraction, and absent in the $CD4^+$ deplete. This suggests that there may an unidentified

factor in the non-$CD4^+$ fraction which inhibits IFN-γ production by $CD4^+$ cells. In a preliminary effort to identify a possible candidate factor for this effect, the supernatants were tested for IL-10 (a Th2 cytokine). Levels of IL-10 were high in the $CD4^+$ deplete fraction, and not detectable in the $CD4^+$ enriched fraction. Unfortunately, technical difficulties prevented measurement of IL-10 to sufficiently low levels to see if this could be implicated in the inhibition of IFN-γ production in the whole PBMC cultures. This interesting observation warrants further investigation and elucidation in more resolved acute HCV patients and in the CHC patients with no measurable $CD4^+$ responses.

7.2.3 $CD4^+$ Responses During Treatment for Chronic HCV Infection

Treatment for CHC with Interferon-α and ribavirin leads to sustained virological clearance in around 40% of patients treated. It has been reported by Cramp *et al.* that during treatment $CD4^+$ HCV specific lymphocyte proliferative responses increase in magnitude and that if maintained, this is associated with sustained virological clearance *(Cramp 2000)*. The newer treatments for HCV infection include the use of PEGylated interferon-α (PEG), and result in much improved sustained virological response rates, with combination treatments including ribavirin achieving spontaneous virological response in 54-56% of cases *(Heathcote 2000; Zeuzem 2000; Fried 2001; Manns 2001)*.

I have undertaken serial $CD4^+$ lymphocyte proliferation assays and FACS analysis of HCV specific PBMC responses in the patients on treatment with PEGylated-interferon-α and ribavirin. These results have been correlated with virological response. A pilot study of HCV specific lymphocyte proliferative responses in patients on treatment with standard IFN-α and ribavirin produced results comparable with those of Cramp *et al.* During treatment with PEG and ribavirin, the $CD4^+$ T cell proliferative responses did increase in magnitude, but this increase occurred later than demonstrated by Cramp *et al.* using standard IFN-α treatment. Also in contrast to the Cramp study, the increase in $CD4^+$ proliferative responses was not maintained in those who achieved a spontaneous virological response. There was no significant difference in serial proliferative response between patients discordant for eventual virological outcome.

FACS analysis data also failed to show a statistically significant difference between the HCV specific responses of virological non- or sustained-responders. However, in this small study, there was a trend for a switch from a dominance of Th2 type $CD4^+$ T cell response to a Th1 dominant response in the sustained responders. This may reflect that the patients who eventually responded to the combination treatment did have a Th response polarized towards a Th2 response, which could be switched to a Th1 response to clear the virus. The non-responders had very little evidence of HCV specific cytokine secretion at baseline, suggesting that there may be $CD4^+$ T cell unresponsiveness which is cannot be switched to a Th1 dominant response.
The most striking finding in the treatment study was the rate of virological clearance. The absence of viraemia at 4 weeks was 100% predictive of a sustained virological response after the end of treatment. This finding is consistent with other groups, although 4 weeks is earlier than other groups have reported. This early polarization of the 2 groups suggests that future studies should be concentrated at careful analysis of the immune responses during the first 4 weeks of treatment.

7.2.4 Interactions between $CD4^+$ T Cells and Hepatic Stellate Cells

The final common pathway of chronic liver injury is hepatic fibrosis, leading to cirrhosis. This is a significant problem in a large proportion of CHC patients and is responsible for most of the mortality and serious morbidity associated with the condition. The cells believed to be responsible for the deposition of abnormal extra-cellular matrix in chronic liver disease are hepatic stellate cells (HSC) *(Friedman 1993; Friedman 2000; Bataller and Brenner 2001)*. In their quiescent state, these fat storing cells are found n the space of Disse in the normal liver, but can proliferate and take on a myofibroblast-like phenotype with an appropriate stimulus. In CHC, there has been extensive study of the various aspects of the immune response to the virus, but little attention has been given to potential interactions between these immune responses and the effector cells in the hepatic fibrotic process.

In the present study I have developed a simple assay to screen PBMC HCV specific $CD4^+$ T cell responses for interaction with HSC. I have examined the responses of human stellate cells to IFN-γ, and then compared the effect of IFN-γ as a sole cytokine on HSC with the effect of IFN-γ containing supernatants generated from HCV stimulated PBMC cultures from CHC patients.

Using a novel assay to detect changes in the level of expression of ICAM-1 by activated, passaged human HSC, I was able to identify HCV antigen-specific immune responses in the supernatants generated from PBMC cultures. This assay detected HCV specific responses in 30% of CHC patients (50% higher than using standard proliferation and cytokine responses alone). In 2 out of the 3 patients HSC activation was associated with IFN-γ production. In the third patient, up-regulation of ICAM-1 could not be attributed to IFN-γ. This suggests that the interactions between the $CD4^+$ T cell response and the HSC are complex and are not associated with IFN-γ alone.

IFN-γ stimulation of primary human hepatic stellate cells produced interesting results. There was heterogeneity in the changes in mRNA expression of the fibrosis associated genes in HSC obtained from different donors. IFN-γ stimulation produced up-regulation of Gelatinase A and Collagen 1 mRNA in 1/3 donors, whilst the same stimulus produced down-regulation of the same genes in the other 2/3. However, IFN-γ induced changes in mRNA levels for cytokine, chemokine and immune active surface-markers were much more homogeneous across the 3 donors.

When conditioned supernatants from HCV antigen stimulated PBMC cultures were applied to primary HSC obtained from a single donor, there was further heterogeneity in the mRNA responses of the HSC. Supernatant from a CHC patient with no identifiable HCV specific responses exerted measurable effects on mRNA expression of fibrosis genes, surface markers, chemokine and cytokine genes. These responses were similar to those seen with supernatant from a patient with a measurable HCV specific IFN-γ response, but different from those obtained from an HCV naïve individual. The mRNA responses generated by the conditioned media that contained detectable IFN-γ were different from those produced by stimulation of the HSC with recombinant IFN-γ alone.

I have developed a set of novel functional assays of immune induction of HSC activation. We have used these assays to begin to investigate the nature and diversity of host responses to HCV. The results show that there may be a significant disease specific $CD4^+$ T cell response in patients with CHC than are not entirely attributable to IFN-γ and which may not be detectable using conventional assays. In addition, there are multiple levels at which heterogeneity may influence the outcome of the immune response and the resultant fibrotic response in the liver. This heterogeneity

may occur at the level of the immune response and at the level of the fibrotic response.

7.3 Summary of the Key Points Identified by the Investigation

The current study has investigated the $CD4^+$ T cell response in CHC. The first aspect of the study examined HLA class II associations to try and identify any correlation between the clearance associated allele DQB1*0301 and disease severity in chronic infection. There were no definite associations found, but sub-group analysis suggested that there may be a link between DQB1*0301 and certain phenotypes of CHC associated liver disease, in particular with severe fibrosis and minimal inflammation. This suggests that nature of the HCV specific $CD4^+$ T cell response could be implicated in dictating the nature and severity of the resultant liver disease in CHC. To follow this, functional studies of the HCV specific $CD4^+$ T cell response in CHC were undertaken. Patients were categorised according to severity of liver disease and the magnitude and phenotype of their $CD4^+$ T cell response in PBMC was assessed. There were no differences in proliferative responses to HCV, but there was a difference in HCV specific cytokine production between groups of patients discordant for disease severity. This suggests that in CHC the $CD4^+$ T cells do not proliferate in response to HCV antigen stimulation, but may secrete cytokines. These cytokines may influence inflammatory cell recruitment, and also have a direct effect on hepatocytes and other liver parenchymal cells, such as hepatic stellate cells. These secondary events may be the level at which the $CD4^+$ T cell response influences the severity of the resultant liver disease in CHC.

The effect of the $CD4^+$ T cell response on hepatic stellate cells was the next phase of the investigation. HCV specific $CD4^+$ T cell responses influence hepatic stellate cells, producing effects on; genes associated with fibrosis, expression of cell surface molecules involved in immune activation and expression of chemokines and cytokines. These effects were not solely attributable to IFN-γ, and were produced by supernatants generated by HCV protein stimulation of PBMC from CHC patients. Some of the donors deemed to be responsive to HCV in these assays did not have responses detectable in any other conventional assay. It may be at the level of $CD4^+$ T

cell interactions with hepatic stellate cells that the discriminatory factors which dictate severity of liver disease are finally identified.

The final phase of the study was to investigate the functional changes in the HCV specific $CD4^+$ T cell response during the course of treatment with anti-viral therapy, and correlate these changes with final virological outcome. This was a small study and the results did not achieve statistical significance, but they do suggest that patients with a Th2 polarized $CD4^+$ T cell response prior to treatment are more likely to respond to anti-viral therapy, as their responses can be switched to a more effective Th1 phenotype. However, effective viral clearance as a result of treatment was not seen to result in the restoration of HCV specific $CD4^+$ T cell responses comparable to those of patients who have spontaneously resolved acute infection.

7.4 Contribution to the Current Literature

Hepatitis C virus was first identified in 1989 and there has been a reliable diagnostic assay available since the early 1990s *(Choo 1989; Garson 1992; Crawford 1994)*. Early investigations focused on assessing the prevalence and natural history of the disease, and identified an 85% chronicity rate and an estimated 170 million people infected worldwide *(Di Bisceglie 1998)*. The wide variation in severity of the resultant liver disease in CHC suggested an immune mediated mechanism for the liver damage, and thus immune responses became the focus of further investigation: $CD4^+$ and $CD8^+$ T cell responses have been studied in resolved acute HCV infection and compared with those in chronic infection *(Diepolder 1995; Diepolder 1997; Christie 1998; Gerlach 1999; Naoumov 1999; Chang 2001; Lancaster 2002)*. More recently dendritic cells and antigen presentation in HCV infection have been investigated *(Akbar 2001; Auffermann-Gretzinger 2001; Bain 2001; Galle 2001; Ito 2001)*. HCV specific $CD4^+$ T cell responses have been characterised during anti-viral treatment have been serially analysed in a few studies *(Zhang 1997; Cramp 2000)*. In addition, there have been several studies examining HLA class II associations with acute and chronic hepatitis C infection *(Alric 1997; Asti 1999; Diepolder 1999; Thursz 1999; Thio 2001; Tillmann 2001)*. Previous work suggested that $CD4^+$ T cell responses in CHC might determine the severity of disease.

The present study is the first to undertake a comprehensive investigation of all aspects of the $CD4^+$ T cell response in patients with chronic hepatitis C infection discordant for severity of liver disease and search for correlates with severe liver damage. Although not conclusive it appears that Th1 responses are associated with clearance in acute infection or more liver fibrosis in CHC, and Th2 responses are associated with a greater likelihood of sustained virological response to treatment. This approach to characterizing the magnitude and phenotype of the $CD4^+$ T cell response in sub-groups of HCV positive patients is novel, and may lead to refinement of prognostic markers and the identification of processes that can be manipulated in CHC infection and influence the progression of liver disease in CHC.

To date, there has been no examination of the $CD4^+$ T cell response and its interaction with hepatic stellate cells in chronic hepatitis C. The study has involved the development of a set of novel assays for T cell function with respect to the induction of fibrosis. We have undertaken preliminary experiments which may reveal novel avenues of investigation aimed ultimately at identifying mechanisms of reducing or reversing hepatic fibrosis in CHC.

This is the first longitudinal study of $CD4^+$ T cell responses in CHC during treatment with PEGylated IFN-α and Ribavirin. The results differ from those published based on standard IFN-α and ribavirin treatment. Data in this field is still emerging and there is yet to be consensus about the nature of the changes in HCV specific $CD4^+$ T cell responses whilst undergoing combination anti-viral therapy.

The HLA study provides evidence to support the hypothesis that the $CD4^+$ T cell response is implicated in dictating the severity of liver disease in CHC. The sub-group analysis shows interesting trends which may suggest that HLA DQB1*0301 is associated with extreme polarization of the $CD4^+$ T cell response. There is no literature analyzing HLA associations in such specialised sub-groups.

7.5 Conclusions

- Standard assays for assessing $CD4^+$ T cell responses are inadequate for characterizing T cell responses in chronic hepatitis C infection

- There are differences in the magnitude of the HCV specific cytokine response in $CD4^+$ T cells from patients with CHC discordant for severity of liver disease

- There is inter-individual heterogeneity in responses to HCV at every level;
    - the immediate and on-going immune response to HCV
    - the extent to which the immune response causes activation of hepatic stellate cells
    - the variation in the response of HSC to similar immune stimuli

- Clearance of Hepatitis C with anti-viral therapy is more likely in individuals with a Th2 polarised immune response to the virus prior to treatment, and will be sustained if virological clearance occurs in the first 4 weeks of treatment

- There are clear differences in the HCV specific $CD4^+$ T cell response between individuals who have previously resolved HCV infection acutely and those with chronic infection, but viral clearance in response to successful anti-viral therapy does not result in restoration of responses comparable to those of individuals who have cleared HCV spontaneously

7.6 Suggestions for Future Work

The HLA study should be continued with recruitment of more numbers. This will ultimately allow regression analysis which may reveal a more concrete association between frequency of HLA DQB1*0301 and severity of liver disease. However, it will also generate a genomic DNA bank on the CHC patients allowing investigation of other further possible genetic linkages influencing severity of disease outcome in CHC i.e. TAP polymorphisms and cytokine polymorphisms, of which HLA class II associations may be a surrogate marker because of linkage disequilibrium.

Emerging molecular techniques such as TAQman PCR allows the generation of quantitative data on small numbers of cells. This technology could lend itself to a further analysis of the $CD4^+$ T cell response in CHC, focusing on the liver infiltrating lymphocytes rather than PBMC responses. To date, liver derived lymphocytes have required clonal or mitogenic expansion to obtain large enough numbers of cells to study from liver biopsy material, this leads to uncertainty about the validity of the data generated. TAQman technology would allow further investigation of the HCV specific $CD4^+$ T cell response in patients discordant for severity of liver disease in CHC.

Further work on the factors which may suppress the immune response in CHC could easily be continued using the techniques established in this thesis. The $CD4^+$ enrichment / depletion experiments should be repeated in CHC patients to see if $CD4^+$ cells in isolation from these patients can generate measurable proliferative and cytokine responses to HCV antigens. If this is not the case, then the original experiments could be revisited in resolved acute patients, with a more exhaustive search for the factor(s) causing suppression of the cytokine response in the whole PBMC cultures.

Studies of the $CD4^+$ T cell response during treatment with anti-viral therapy in CHC should focus on the early phase of treatment, during the first 4 weeks. This will help to clarify whether the action of the anti-viral therapy is primarily directly anti-viral or acting via manipulation of the immune response to achieve viral clearance.

Finally, perhaps the most exciting avenue of investigation is the interaction between the $CD4^+$ T cell response and hepatic stellate cells. More conditioned media should be generated from HCV antigen stimulated PBMC and screened for interactions with hepatic stellate cells. The nature of any activating factors can then be investigated. In addition, the effects of the conditioned media on target genes can be documented. Ultimately, it should be possible to develop co-culture assays for $CD4^+$ cells and HSC allowing study of direct cell to cell interactions. This is a particularly exciting prospect given the range of immune active surface markers that HSC express when activated. In this way the effect of HSC on $CD4^+$ cells could also be examined. Extension of this work would involve examining the hepatic stellate cells during their activation stage, rather than looking in changes in level of activation in fully activated cells. The same experimental techniques could be applied, but would need to be undertaken on freshly isolated HSC. This would give more insight into the effect of the immune response on fibrotic processes in a previously healthy liver.

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