Stimulation requirement and pathogenetic significance of T cells during chronic viral hepatitis

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Submitted for the degree of Doctor of Medicine, September 2006
ABSTRACT

Hepatitis B virus (HBV) and Hepatitis C virus (HCV) are important causes of liver inflammation and fibrosis. More than 500 million people are persistently infected worldwide and consequently are at increased risk of developing chronic liver disease, cirrhosis and hepatocellular carcinoma. Both viruses share some similarities; however, disease severity varies greatly from subject to subject and the variation in clinical course is a hallmark of viral hepatitis. The failure of the immune system to prevent persistent infection and chronic necroinflammatory change is central to understanding the disease caused by these human viruses.

The Introduction to this Thesis reviews our current understanding of the immunopathogenesis and natural history of both viruses in the context of the clinical and scientific challenge that chronic viral hepatitis represents.

The body of this work was to focus on immunological events which might shape and possibly dictate disease outcome in viral hepatitis. In this thesis I set out to investigate areas of cellular immunity which may contribute to pathology or protection in HBV and HCV. I have focused on the impact of cross-reactive T cells activated by HCV peptides and report that this may constitute a common occurrence in human viral infections. I explore the role of the NK cell receptor, NKG2D, in the modulation of CD8+T cell response in type B and C chronic viral hepatitis and report the impact of this novel receptor in human liver disease for the first time.
This Thesis concentrates on areas of T cell immunology which I feel will add to our understanding of viral hepatitis. It is this better definition of the basic immunology of these two important human viruses which will form the bedrock in the development of more efficient preventive and therapeutic strategies in the future.
Statement of originality

The studies that form the content of this thesis were designed and performed by myself.

Collaborations

Simonetta Urbani, Barbara Amadei and Paolo Fisicaro at the Azienda Ospedaliero Universitaria di Parma, Italy collaborated with me through patient recruitment and data analysis for the work in Chapter 3. Rebecca Moses collaborated with me throughout all the early ELISpot work. I established collaboration with Jilly Lloyd at the Department of Obstetrics and Gynaecology, UCL, who kindly provided me with the cord blood samples.

Liver tissue specimens for the work in Chapter 4, came from a number of collaborations at the Royal Free Hospital. Dr David Patch, the transplant co-ordinators, Clare Selden and Professor Dhillon in the Histopathology Department all contributed to this study.
Acknowledgements

I am greatly indebted to Dr Antonio Bertoletti and Professor Geoffrey Dusheiko for their help and guidance throughout the course of this thesis. Antonio has provided me with constant supervision over the two years of this work. His guidance and valued opinion have navigated me through the experimental work in the laboratory and enabled me to develop the scientific ideas in this thesis. The high standards he sets in terms of the quality and novelty of the research will be a benchmark for me to aspire to throughout my future career in viral hepatitis. Geoff provided me with the opportunity and support to work in viral hepatitis to begin with and has acted as my mentor throughout the time I have worked with him at the Royal Free. This work would not be possible without his supervision and assistance. I would also like to thank Adam Gehring, post-doctoral scientist within the laboratory who provided me with technical support, critical comment and assistance throughout the course of this work.

I express my gratitude to all those who I collaborated with in this thesis, unfortunately too many to mention individually. Furthermore, I cannot overlook the willingness of the many patients who participated in these studies, for their time and efforts I am truly grateful.

Finally, my wife Anthie Papadopoulou, deserves a special mention for her endless support.
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ABBREVIATIONS

AA  amino acid
Ab  antibody
ALT alanine amino transferase
APC antigen presenting cell
CMV cytomegalovirus
CTL cytotoxic T lymphocyte
DHV duck hepatitis virus
DMSO dimethylsulphoxide
dNA deoxyribonucleic acid
ds double stranded
E1 envelope 1
E2 envelope 2
EBV Epstein Barr virus
EDTA ethylene diamine tetracetic acid
ELISA enzyme linked immunosorbant assay
ELSpot enzyme linked immunospot
FACS fluorescence activated cell sorter
FCS fetal calf serum
FITC fluorescein isothiocyanate
g gram
HAV hepatitis A virus
HBSS hanks balanced salt solution
HBV hepatitis B virus
HCV hepatitis C virus
HHV human herpes virus
HLA human leucocyte antigen
HVR hypervariable region
ICS intracellular cytokine staining
IFN interferon
IL interleukin
IRF-1 interferon regulatory factor 1
ITAM immunoreceptor tyrosine based activation motif
IU international unit
L litre
LCMV lymphocytic choriomeningitis virus
m milli
M molar
MHC major histocompatibility complex
MHV mouse hepatitis virus
MIC MHC class-I related chain
mm millimetre
MUL-1 murine ULBP-like transcript 1
NANBH non-A, non-B hepatitis
NC negative control
NK natural killer
NS non-structural
ORF open reading frame
PBMC peripheral blood mononuclear cell
PBS phosphate buffered saline
PCR polymerase chain reaction
PE phycoerythrin
PEG polyethylene glycol
RAE I retinoic acid early inducible I
RNA ribonucleic acid
SFC spot forming cell
SP sexual partner
SVR sustained virologic response
TCR T cell receptor
Th T helper
TNF tumour necrosis factor
ULBP UL-16 binding protein
UTR untranslated region
VSV vesicular stomatitis virus
VV vaccinia virus
CHAPTER 1

GENERAL INTRODUCTION
1.1.1 HEPATITIS B VIRUS (HBV)

Hepatitis B virus (HBV) is a non-cytopathic, hepatotropic DNA virus that causes acute and chronic necroinflammatory liver diseases (Seeger and Mason, 2000). Acute infections may be clinically silent or cause an acute liver inflammation that may produce serious illness, and in 0.5% of subjects can lead to fatal fulminant hepatitis. The vast majority of adults acutely infected with HBV control virus replication, develop a long lasting immunity and recover from the disease. In contrast, neonatal infections are rarely resolved and most of these children become chronically infected (Stevens et al., 1975). Chronic infections can also occur in about 5% of infected adults. As in the acute infection such chronically infected individuals can be asymptomatic or experience varying grades of chronic liver injury that may lead to the development of hepatocellular carcinoma (HCC) (Beasley, 1988). It has been calculated that the risk of developing liver cancer in HBV infected subjects is over 100-fold that of age-matched non HBV infected individuals (Beasley et al., 1981). Worldwide deaths from liver cancer caused by HBV infection are thought to exceed one million per year (Pisani et al., 1999) (Figure 1.1).
1.1.2 Biology of HBV

HBV is a member of a family of viruses referred to as Hepadnaviridae (Figure 1.2). Viruses closely related to HBV have been found in woodchucks (Summers et al., 1978) and ground squirrel (Testut et al., 1996). These viruses have about 70% homology with HBV but do not infect humans or other primates. More distantly related viruses, with a similar genetic organisation are found in ducks, geese and primates. Due to the limited host range of HBV (namely humans and great apes) and the lack of in vitro systems to
infect normal human hepatocytes, these related viruses are currently used as a model system to characterise the mechanisms of hepadnavirus replication and to explore various disease profiles.

Hepatocytes are the only confirmed site of replication for HBV. Bile ductule, epithelial cells or subsets of cells in the pancreas, kidney or lymphoid cells may also be targets of infection, but the evidence of replication in these extrahepatic sites is controversial and at the moment these sites are not considered in discussion of viral replication and pathogenesis. The replication of hepadnaviruses is characterised by the synthesis of a ~3-Kb partially double stranded, relaxed circular DNA (rcDNA) genome by reverse transcription of an RNA intermediate, the pregenome (Summers and Mason, 1982). At the initiation of infection the viral rcDNA genome is converted into cccDNA. The cccDNA serves as the template for the transcription of viral mRNAs. One of these mRNAs, called the pre-genome, is used to synthesise the core protein (nucleocapsid subunit) and the viral reverse transcriptase. The reverse transcriptase binds to its own mRNA templates and is packaged into nucleocapsids where viral DNA synthesis occurs.

Mature nucleocapsids containing rcDNA are then enveloped in the endoplasmic reticulum and exported from the cell via the Golgi (Ganem et al., 1994; Seeger and Mason, 2000).

The genome of HBV contains four open reading frames that encode the viral nucleocapsid, polymerase, envelope and X proteins. Core and polymerase genes are
essential for viral DNA replication. The envelope open reading frame encodes for polypeptides (S, M and L), all of which are essential for envelopment of nucleocapsids.

The function of hepatitis X protein is unknown, but the protein is required for the establishment of infection in vivo (Chen et al., 1993) but is dispensable for viral replication in transfected cells (Blum et al., 1992).

The nucleocapsid open reading frame contains two start codons that define two overlapping proteins. The shorter of these proteins (hepatitis B core antigen, HBcAg) is the viral capsid protein that assembles in the cytoplasm of the hepatocytes to form the icosahedral subviral particles that package the viral reverse polymerase and the pre-genome (Crowther et al., 1994). The longer protein (pre core) is translocated into the endoplasmic reticulum where it undergoes truncation of its carboxy and amino terminal residues and is secreted into the blood as HBeAg (Ou et al., 1986; Roossinck et al., 1986).

The presence of HBeAg in the serum of HBV infected patients is a serological marker of viral replication, but since HBeAg is dispensable for in vivo infection (Chang et al., 1987), its exact function is unknown. Experiments in mice suggest that HBeAg can cause depletion of Th1 helper cells (Milich, 1997; Milich et al., 1990) thereby suppressing the anti-viral immune response. A similar tolerogenic effect may also be present in neonatal infection, although this possibility has not been proven in the natural host of hepadnaviruses.
All the hepadnaviruses express the three envelope components S, M and L. All three proteins contain the smallest (226 amino acids long) S domain referred to as hepatitis B surface antigen (HBsAg). The two larger proteins contain S plus an amino acid extension containing the pre-S2 antigen (M protein, 226 +51 AA long) or pre S2 and pre S1 antigens (L protein, 226 + 51 + 163 AA long) (Heermann et al., 1984; Stibbe and Gerlich, 1983a; Stibbe and Gerlich, 1983b). All three envelope proteins are found as components of the 42 nm-diameter infectious viral particle (Dane particle) (Figure 1.2) (Neurath et al., 1985). L and M constitute roughly 30% of the envelope protein content of the virus particle (Heermann et al., 1987). S by itself and together with the larger envelope proteins also forms filamentous and spherical “surface antigen” particles that are secreted from infected cells in at least 100-fold excess over complete virions. Lacking viral nucleic acid, these particles are not infectious, but can reach concentrations of several micrograms per millilitre of blood of HBV infected patients (Kim and Tilles, 1973). The reasons for maintaining high levels of HBsAg production are still uncertain, but may be implicated in tolerisation of the immune response and may help absorb neutralising antibodies during the progression of infection. Complexes made up of these particles with their cognate antibodies are probably responsible for the immune complex syndromes that can occur during HBV infection.

As already mentioned, the function of X protein is poorly defined, but it has been shown to be essential for virus replication in vivo (Chen et al., 1993). Antibodies against X have been found in sera of HBV infected individuals, demonstrating its expression during natural infection. In vitro experiments from cell culture, have demonstrated that X can
activate the transcription of host genes (Balsano et al., 1991; Hu et al., 1990; Twu and Schloemer, 1987), however since X does not seem to be a DNA-binding protein, its effects on transcription are thought to be indirect. Apart from the transactivation activity, X has been proposed to stimulate signal transduction or binding to protein targets such as p53 (Feitelson et al., 1993; Takada et al., 1995; Wang et al., 1994) or proteasome subunits (Sirma et al., 1998). Therefore, it is clear that X-defective virus is unable to initiate infection in vivo but the physiological role of X in the life cycle of HBV remains unresolved.

One other important point in the replication of HBV is that the use of reverse transcriptase (Summers and Mason, 1982) results in a high rate of DNA mutations, due to the lack of proofreading function of this enzyme. The presence of these viral mutations can lead to the selection of viruses able to escape recognition by the different arms of the adaptive immune response (Bertoletti et al., 1996).
1.1.3 Host-virus relationship: parameters influencing different outcomes.

The variability in the outcome of infection with viruses may depend on the balance between viral parameters that determine the ability of the virus to propagate and persist on one hand, versus the facets of the immune system that determine its efficiency in controlling virus replication (Zinkernagel, 1996).

Viral parameters include the initial infectious dose, the kinetics of viral replication and its ability to spread. These viral features are balanced by the variables of the immune
system: kinetics, specificity and duration of the humoral and cellular mediated immune response and other non antigen specific effector mechanisms such as activation of the adaptive immune response and cytokine production.

How these variables influence outcome are usually difficult to study in humans, but such work has been addressed in greater detail in the animal models of hepadnavirus infection. Epidemiological data have shown that the age of infection is a critical parameter that influences the outcome of HBV, with infection of neonates being associated with the development of persistent infection (Stevens et al., 1975). Experimental work performed in ducks and woodchucks confirm this epidemiological data. Persistent infection with woodchuck hepatitis virus (WHV) followed the transmission of virus to neonatal animals whilst infection of older animals is usually transient (Cote et al., 2000). Identical results were reported in ducks infected with DHBV, where persistent infection results following experimental inoculation of newly hatched animals (Jilbert et al., 1998).

The effect of the dose of virus in the outcome of infection is also supported by the experiments in ducks and woodchucks. Higher doses of virus generally induced high rates of chronicity (Cote et al., 2000; Jilbert et al., 1998). However, the data here are less consistent. Infection with a single viral particle is sufficient to initiate persistent infection in ducks (Jilbert et al., 1996) and low doses of WHV have been shown to induce a persistent infection in woodchucks (Cote et al., 2000). It is possible that kinetics of viral replication, not tested in these studies, can influence the outcome of infection. Mathematical models of the relationship between kinetics of virus replication and CTL
expansion have indeed suggested that slower replicating viruses could induce a weaker CTL response (Bocharov et al., 2004). Therefore, it is possible that the replication speed of HBV, in addition to age at exposure and quantity of initial inoculum, can all influence the pathogenesis of HBV infection. Accordingly with this possibility, HBV strains with enhanced viral replication have been demonstrated to be responsible for an epidemic of fulminant hepatitis (Baumert and Liang, 1996), reinforcing the possibility that speed of viral replication may influence the pathogenesis of HBV infection. A further important point of the work performed in animals infected with hepadnaviruses is the fact that persistent infection is not the evolution of a classical acute hepatitis. Chronicity in woodchucks developed in animals that, after infection, mounted a diminished immune response with reduced cytokine production and had a mild form of acute hepatitis (Nakamura et al., 2001). This suggests that the initial vigour of the immune response is a key factor in determining the outcome of infection.
1.1.4 Immune parameters correlating with HBV control

There are clear differences in the adaptive immunity of patients with chronic and resolved HBV infection. HBV-specific CD4 and CD8 T cell responses with a T helper (Th) type 1 profile of cytokine production are detectable and quantitatively stronger in peripheral blood mononuclear cells of subjects with a favourable outcome. Patients with chronic infections are characterised by weaker or undetectable virus-specific T cell responses (Ferrari et al., 1990; Jung et al., 1991; Maini et al., 1999; Penna et al., 1996; Penna et al., 1997; Rehermann et al., 1995; Webster et al., 2004). Whether the association between different outcomes of HBV infection and the vigour and breadth of the HBV-specific T cell response has a causative effect has been difficult to demonstrate.

CD8+ T cell deletion experiments performed in HBV infected chimpanzees have provided strong support for the concept that CD8+ T cells are the main cellular subset responsible for viral clearance (Thimme et al., 2003). Direct analysis of the frequency of CD8+ T cells in patients who clear HBV infection have shown that HBV-specific CD8+ T cells can reach ~2% of circulating CD8+ T cells during the acute phase of disease (Maini et al., 1999; Webster et al., 2000). Furthermore, the quantity of circulating HBV-specific CD8+ T cells is even higher years after recovery in subjects with acute hepatitis (0.2-0.5% of circulating CD8+ T cells) than in subjects with chronic infection (HBeAg+ patients), in whom such cells are barely detectable (Maini et al., 2000). These results are consistent with previous studies showing a very weak CTL response in patients with chronic HBV infection, usually only detectable during spontaneous (Rehermann et al., 200...
or therapy induced clearance of HBeAg (Boni et al., 2001). In addition, studies conducted in patients during the incubation phase of acute HBV infection, have demonstrated the expansion of virus-specific IFN-γ+ CD8+ and CD4+ T cells. Virus clearance was preceded by the expansion of both helper and cytotoxic T cells and present only in subjects who controlled the infection (Webster et al., 2000). This underlines the necessity of a coordinated helper and cytotoxic T cell response in host defence against HBV infection (Figure 1.3).

**Figure 1.3.** The host defence mechanisms against virally infected cells. The coordinated immune response involves type I IFNs recruiting the innate immune responses for viral containment and finally the adaptive immune responses providing persistent control of infection.
The importance of CD4 helper T cells in promoting CTL survival and effector function is supported in HBV infection by the fact that CD4 responses are only consistently detectable in acute infection (Ferrari et al., 1990) and in chronic patients undergoing HBeAg seroconversion (Tsai et al., 1992). In acutely infected HBV patients who have the ability to mount a protective multispecific CTL response, the HBV-specific T cell response is both strong and Th1-like (Penna et al., 1997). Furthermore the responses are also directed to a number of different epitopes within the structural proteins investigated (Ferrari et al., 1991). This multispecificity of the CD4 response may also be highly relevant to viral control. Consistent with this interpretation, heterozygosity of class II alleles has been shown to be associated with more favourable HBV control (Thursz et al., 1997).

The recent analysis of one HBV-HCV acutely co-infected patient who developed chronic HBV infection has provided further evidence of the significance of the coordinated activation of CD4 and CD8+ T cells. Longitudinal analysis of HBV-specific T cell responses, from the time of infection to chronicity, shows the presence of a multi-specific CD8+ T cell response in the absence of a CD4+ T cell response (Urbani et al., 2005b). It is likely that the absence of CD4 help prevented the maturation of a functionally efficient CD8+ T cell response.

Despite the cellular immune response being a major contributor to HBV clearance, humoral responses also play a role in controlling HBV. HBV clearance is associated with the production of anti-HBs antibodies (Alberti et al., 1978) and sera with high levels of
anti-viral antibodies (specific for the viral envelope) can control HBV infection (Grady et al., 1978).

It is also important to stress that effective viral control does not necessarily imply viral eradication. It is now accepted that patients who recover completely following acute hepatitis do not eradicate HBV infection. Several studies have shown that HBV is present (Cacciola et al., 1999; Chazouilleres et al., 1994; Michalak et al., 1994) and retains the ability to replicate in the liver of patients who have cleared serum HBsAg (Mason et al., 1998). Such persistence of low level HBV replication may in fact be critical to maintain an efficient HBV-specific T cell response that has been shown to persist for several years after acute HBV infection (Penna et al., 1996; Rehermann et al., 1996c).

Thus, it seems clear that subjects who are able to control HBV replication are able to express a co-ordinated T cell-immune response characterised by the expansion of a functionally active multispecific CTL response (Chisari and Ferrari, 1995). However, we still do not have a clear understanding as to why chronic patients are unable to mount a comparable HBV-specific T cell immune response. In addition to the factors already discussed; from patient age to mode of transmission (vertical infection) (Stevens et al., 1975), genetic factors of the host (Thursz et al., 1997) and viral parameters (dose and kinetics); the role of viral proteins produced by HBV (Milich et al., 1990) and their ability to alter the magnitude of the T cell response should not be overlooked.
The production of HBeAg, the secreted non-particulate form of nucleoprotein, has been demonstrated to mediate immunoregulatory function (Milich et al., 1998). In a murine model system, HBeAg can cross the placenta and establish an antigen-specific T cell tolerance (Milich et al., 1990). Furthermore, HBeAg is able to delete HBeAg-specific Th1 cells and skews the HBV-specific T cell response towards Th2 (Milich, 1997; Milich et al., 1998). Thus, high production of HBeAg could be responsible for the inhibition of an efficient anti-viral immune response, thereby contributing to viral persistence.

1.1.5 Non-cytopathic or cytopathic control of HBV

Several studies have shown HBV infected hepatocytes are more likely to be controlled by intracellular inactivation mediated by cytokines than by direct killing (Guidotti et al., 1999). This mechanism has been studied extensively in HBV transgenic mice where HBV replication is completely abolished in the hepatocytes by cytokine-dependent pathways that do not require cell death (Guidotti and Chisari, 1996; McClary et al., 2000). IFN-γ and TNF-α are able to selectively degrade replicating genomes of HBV; thereby clearing the virus without the need to kill infected cells (Guidotti and Chisari, 1996). This non-cytopathic mechanism of viral clearance is not peculiar to HBV. Control of murine cytomegalovirus (CMV) infection (Tay and Welsh, 1997) and Lysteria monocytogenes (Kagi and Hengartner, 1996) in the liver seems to be primarily mediated by IFN-γ production rather than perforin dependent (direct killing) pathways. Lymphochoriomeningitis virus (LCMV) is also cleared from hepatocytes by non-cytopathic mechanisms that are not operative in non-parenchymal
cells or splenocytes (Guidotti et al., 1999). Therefore, clearance of viruses from infected hepatocytes does not require massive lysis of infected cells, perhaps constituting a strategy to preserve the functional integrity of a vital organ from massive immune-mediated cytodestruction. The fact that clearance of HBV infection from the liver occurs before the peak of acute liver damage in chimpanzees (Guidotti et al., 1999) and also in humans (Webster et al., 2000) seems to demonstrate that non-cytopathic anti-viral pathways mediated by cytokines contribute substantially to viral control in the natural HBV infection.

However, because hepatocytes are capable of activating intracellular events leading to viral control after cytokine stimulation, this does not imply that 1) they are resistant to direct CTL mediated lysis, or 2) that cellular necrosis or apoptosis is not important in resolution of acute hepatitis B. Lysis of hepatocytes by Fas has been clearly demonstrated (Kondo et al., 1997) and an incremental change in the level of transaminases is always present during HBV clearance in patients with acute hepatitis (Webster et al., 2000). Furthermore, a degree of apoptosis and regeneration of hepatocytes occurs in acute and chronic liver damage in woodchuck hepatitis virus infection (Guo et al., 2000), showing that hepatocyte lysis is also occurring during virus control in infected hepatocytes.
1.1.6 Pathogenesis of liver damage during HBV infection

Since HBV is a non-cytopathic virus, it has been assumed that the extent of liver damage is proportional to the recognition of infected hepatocytes by CTL (Figure 1.4). However, the demonstration that HBV can be almost cleared by non-cytopathic mechanisms mediated by cytokines has challenged this model (Bertoletti and Maini, 2000).

Role of HBV-specific CTL in liver damage and virus control

HBV is not directly cytopathic
HBV-specific CD8 cells not only protect but also damage?

HBV infected hepatocytes

Figure 1.4. Role of virus-specific CTL, protection or pathology?

The ability to identify antigen-specific T cells directly *ex vivo* using HLA-class I tetramers (Altman *et al.*, 1996) has provided the opportunity to investigate the relationship between HBV-specific T cells and liver damage in humans. Direct *ex vivo*
quantification of HBV-specific CD8+ T cells, combined with the demonstration of the peculiar capacity of hepatocytes to clear viral infections by non-cytopathic mechanisms, have led to a new interpretation of the pathogenesis of liver damage during chronic viral infections (Bertoletti and Maini, 2000). Using HLA-class I tetramers it has been shown that chronic HBV infected patients lacking evidence of liver damage but controlling HBV replication possess functionally active HBV-specific CD8+ T cells both in the circulation and in the liver. By contrast, patients with a high level of HBV replication and evidence of liver inflammation show a different pattern of virus-specific CD8+ T cells. The frequency of intrahepatic CD8+ T cells specific for core 18-27, representing the immunodominant core epitope, were much lower in these patients due to their dilution in a large infiltrate of apparently non-antigen specific T cells. Interestingly, the actual number of intrahepatic HBV-specific CD8+ T cells was similar to that seen in patients without liver disease, taking into account the difference in the size of the total CD8 infiltrate. These results in chronic HBV infection show that comparable numbers of intrahepatic virus-specific CD8+ T cells could be associated with either protection or pathology (Maini et al., 2000) (Figure 1.5).
Patients HBV-ALT↓

Inhibition of HBV replication independent from liver damage

Liver

T cell recruitment

Blood

HBV-Specific CD8 cell

CXCR-3

CCr-5

Figure 1.5. Virus-specific CTL controlling viral replication independent of liver damage

These data demonstrate that the quantity of virus-specific cells does not appear to be the variable directly determining the extent of virus-induced liver pathology. Indeed, the presence of liver-infiltrating HBV-specific CD8+ T cells in the absence of liver inflammation suggests that control of viral replication and the occurrence of liver damage may be independent events. Hepatic pathology could in part be the consequence of the large infiltrate of antigen non-specific mononuclear cells, since this is the one variable, which correlates with the extent of liver inflammation. The importance of non-antigen specific T cell recruitment in the pathogenesis of liver damage has been shown in a transgenic mouse model of fulminant hepatitis (Ando et al., 1993) and in the concanavalin A-induced model of hepatitis (Kusters et al., 1996; Tiegs et al., 1992)(Figure 1.6).
Figure 1.6. The recruitment of a large volume of non-virus specific CTL with resulting liver damage, where virus specific CTL fail to control the virus.

The recruitment of non-antigen specific CD8+ T cells seems to be mediated by IFN-γ (Moskophidis and Kioussis, 1998). This cytokine should therefore be seen not only as an anti-viral cytokine able to clear infection without causing liver damage, but also as a typical inflammatory cytokine, causing activation of macrophages (Young and Hardy, 1995), increased susceptibility to TNF-mediated hepatic damage (Morita et al., 1995) and initiating the recruitment of T cells (Cook et al., 1995; Shields et al., 1999; Taub et al., 1993), NK cells (Salazar-Mather et al., 1998) or NKT cells (Kaneko et al., 2000) through the release of chemotactic cytokines.

A functional efficient CTL response recruited to “the right place at the right time” in an infected liver may be able to control HBV replication without causing massive or chronic
hepatocyte destruction. Virus-specific CTL can persist in the liver, controlling and inhibiting viral replication in the hepatocytes without causing biochemical or histologically evident liver disease. A circulating reservoir of virus-specific CD8+ T cells able to clonally expand, migrate to the liver, produce the correct anti-viral cytokines and mature to effector from memory cell phenotype will be necessary to maintain viral control and to respond quickly to alterations in the rate of viral replication. In line with this interpretation patients controlling HBV infection present a strong and multispecific CD8+ T cell response that persists for several decades after acute hepatitis (Rehermann et al., 1996c).

In contrast, chronic HBV patients present a defective CD8+ T cell response. The presence of a CTL response unable to control HBV could then cause the chronic recruitment of inflammatory cells that will sustain the necro-inflammatory activity promoting the long term complications of HBV infection, namely fibrosis, cirrhosis and hepatocellular carcinoma.
1.1.7 Natural History of Hepatitis B

Primary HBV infection in the naive host is usually asymptomatic. Symptomatic infection can occur, and is usually associated with a self limited course. Whereas vertical transmission of HBV from mother to neonate almost always results in chronic hepatitis, infection during adulthood usually results in resolution of the acute illness and lifelong immunity to reinfection (Wright and Lau, 1993). Despite this, 1-5% of HBV infection in healthy adults does not resolve spontaneously but develops into persistent infection. Persistent infection can be subclinical, with normal liver enzymes and unremarkable liver histology and patients with this profile of chronic HBV infection are termed asymptomatic carriers. Despite this, some asymptomatic carriers of HBV with minimal liver injury have been reported to have significant levels of intrahepatic viral replication (de Franchis et al., 1993). Patients with persistent infection with abnormal liver enzymes and liver histology are referred to as having chronic hepatitis B infection. Chronic HBV carriers, defined as persons positive for HBsAg for more than 6 months, are at increased risk of developing cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC) (Beasley, 1988; Lok and McMahon, 2001; McQuillan et al., 1989). There is a 4-10 week incubation period before HBsAg becomes detectable in the peripheral blood at which time viraemia is usually well established. This is followed by the detection of antibodies to the HBV core antigen (anti-HBc antibodies) and the presence of the IgM isotype reflects early/recent infection (Hoofnagle, 1981). The emergence of HBeAg in the blood has been shown in animal studies to coincide with hepatocyte infection rates of between 75 and 100% (Kajino et al., 1994). All of these factors contribute to the high
levels of infectivity in acute HBV infection, with both vertical and horizontal transmission being documented (Koff \textit{et al.}, 1977). The development of abnormal liver function tests, with elevated aminotransferases coincides with the establishment of the cell-mediated immune response as previously discussed in the pathogenesis section. The loss of HBeAg, HBsAg and the emergence of anti-HBe & anti-HBs-antibodies is associated with undetectable or low levels of HBV DNA. This low level HBV DNA is reported in some patients to persist for life (Prince \textit{et al.}, 2001), but is considered to be of low infectivity in the absence of detectable HBsAg.

1.1.8 Clinical picture of persistent infection

Persistent HBV infection is reflected clinically by detectable HBsAg in the blood and continued viral replication. The level of HBV DNA is usually dependent on the presence of HBeAg with high titres of HBV DNA being associated with HBeAg positivity. In patients with circulating HBeAg, the levels of virus can be as high as or exceed $10^9$ virions per ml, and such patients remain highly infectious (Weinberger \textit{et al.}, 2000). The development of anti-HBe antibodies is associated with a significant drop in the levels of viraemia. However, even with chronic HBV infection, there is continued loss of HBeAg from the blood and this is reported to occur at a rate of 5-10\% per year (Ribeiro \textit{et al.}, 2002). With loss of HBeAg and the development of anti-HBe antibodies there is often a transient hepatic flare reflecting immune mediated destruction of infected hepatocytes. Seroconversion is usually accompanied by up to a 5 log drop in the HBV DNA titre. This
picture reflects the dynamic nature of HBV infection with a continued immune challenge modulating the course of the disease. Even with HBeAg seroconversion, it is accepted that there is continued HBV replication in up to 85% of patients with anti-HBe antibodies, typically at lower levels (less than $10^5$ molecules per ml) (Tedder et al., 2002).

HBeAg-negative subjects usually have low levels of viral DNA, normal aminotransferases and tend to have a good prognosis (de Franchis et al., 1993). However, this picture is often different in Southern Europe and Asia, where up to 20% of such carriers have high levels of HBV DNA and significantly elevated aminotransferases (Sung et al., 2002). The majority of this group of patients are believed to have mutations in the pre core region of the virus and so do not express HBeAg (Carman et al, 1990; Brunetto et al., 1991).

### 1.1.9 Hepatitis B and Therapy

An understanding of viral clearance or eradication of HBV (undetectable viral DNA as measured by stringent PCR techniques) remains a challenge for scientists and clinicians alike. The loss of HBsAg marking the end of chronic HBV carrier status, as an end-point is unfortunately rare. 1-2% of patients per annum are reported to achieve loss of HBsAg with current treatment modalities, or spontaneously. This stresses the difference in disease outcome and goals of treatment in type B and C viral hepatitis.
Despite the argument for broadening the population of patients where treatment may be indicated, HBeAg positive patients with active disease remain the core population where indications for therapy are unequivocal. This specific population have an increased risk of progression to chronic hepatitis and cirrhosis (Liaw et al., 1988) and they also have an increased risk of hepatocellular carcinoma above that of other carriers (Yang et al., 2002). At the other end of the clinical spectrum, asymptomatic HBeAg-negative chronic carriers with viral loads below $10^5$ genomes/ml and normal liver enzyme values, tend to have a relatively stable course with low rates of progression, either clinical or pathological (de Franchis et al., 1993). At present this group of patients is largely excluded from therapy. However, there is a growing argument even for treatment of these patients, because of the possible risk of the development of hepatocellular carcinoma, as this risk may be higher in certain ethnic groups (Yang et al., 2002; Yuan et al., 2005).

HBeAg-negative patients with substantial viraemia and elevated liver enzymes should be offered anti-viral therapy, though the most effective treatment strategy remains unclear (Hadziyannis et al., 2003; Lok and McMahon, 2001).

Nor is it clear, which markers best measure a response to therapy in this patient population. Reduction in viral load is routinely analysed and a downward trend interpreted as a response, but there are few studies detailing the clinical interpretation of results. On the contrary, HBeAg seroconversion in HBeAg positive individuals and reduction in viral load are all features which are known to be associated with improvement in liver histology and survival (Niederau et al., 1996).
Therapy for HBV has been limited by side-effects (largely related to interferon alfa) and by the development of resistance to nucleoside analogues. Interferon alfa first licensed for the treatment of chronic HBV in 1992, and administered subcutaneously three times per week resulted in HBeAg loss, development of anti-HBe antibodies and a decline in serum alanine aminotransferase levels, in about 30% of patients who tolerated the drug. Such a response to interferon is also considered to be durable long after discontinuation of therapy (Wong et al., 1993). The side effects of interferon alpha have frequently deterred patients from embarking on a course of interferon as a first choice in the treatment of HBV. Fevers, myalgia, thrombocytopaenia and depression are all well documented and are often intolerable to many patients. Moreover, interferon’s immunomodulatory effect, namely an augmented immune responsiveness, is suggested by the flares of liver enzymes, which can be induced by its administration. However, such flares may precipitate overt liver failure in patients with advanced liver disease and so interferon remains contra-indicated in this patient group.

Lamivudine, a nucleoside analogue reverse transcriptase inhibitor, with the ability to directly block replication of the HBV genome was initially used in the treatment of HIV and was approved for the treatment of HBV in 1998. The initial use of lamivudine in the treatment of HIV and HIV/HBV co-infection revealed substantial reductions in the level of HBV DNA and these findings were replicated in patients with chronic HBV mono-infection (Dienstag et al., 1999; Puoti et al., 2002). Treatment with lamivudine is associated with a 3 to 4 log reduction in circulating levels of HBV DNA in the early stages of therapy. There is an associated rapid loss of HBeAg and seroconversion to anti-
HBe positive status in up to 20% of patients at 48 weeks therapy; a reduction in serum aminotransferases usually ensues. Lamivudine is generally well tolerated with few side-effects; furthermore it does not possess the overt immunomodulatory properties of interferon and so can be used in patients with advanced liver disease and even in decompensated cirrhosis (Villeneuve et al., 2000). As a result, lamivudine is now the most widely used first line agent in the treatment of HBV infection.

However, there is evidence that lamivudine requires an adequate host immune response to be effective and the link between HBeAg clearance and high pre-treatment values for alanine aminotransferases has been demonstrated (Chien et al., 1999). Pre-treatment values of aminotransferases greater than five times the upper limit of normal were associated with a HBeAg seroconversion rate of 65% compared with only 5% seroconversion rate for those with pre-treatment aminotransferases of less than twice the upper limit of normal, a rate similar to that for spontaneous loss of HBeAg. The exact interplay between lamivudine and the immune response is still being investigated, though it is hypothesized that by reducing the viral load lamivudine enables the immune response to deal more effectively with the remaining infected hepatocytes in the host. This ability of lamivudine to bolster or reverse the CTL hyporesponsiveness has also been reported (Boni et al., 2001).

The limitation of lamivudine is the high rate of viral resistance. There is an ongoing need for newer and more effective agents (Leung et al., 2001; Liaw, 2000). Drug resistance in lamivudine monotherapy is mediated largely by point mutations in the YMDD motif of
the catalytic centre of the viral reverse transcriptase. By the end of the first year of lamivudine therapy 15-20% of patients have resistant variants in the circulation and this rises to 40% at 2 years and up to 67% after 4 years (Liaw, 2000). Despite the development of resistance, 40-50% of patients have undergone HBeAg seroconversion by the end of 4 years of therapy. How best to tackle lamivudine resistance is still a subject for debate. Discontinuation of antiviral therapy can be associated with exacerbations of liver injury (Honkoop et al., 2000) and this pattern of liver injury has also been observed on the withdrawal of other antiviral agents (Marcellin et al., 2003).

Adefovir dipivoxil, approved by the FDA for the treatment of HBV in 2002, is a monophosphate nucleotide analogue that requires intracellular conversion by cellular kinases to its virologically active diphosphate metabolite. Adefovir diphosphate inhibits viral DNA polymerase enzymes of various hepatitis and herpes viruses as well as retroviruses including HIV. Incorporation of the drug into viral DNA results in DNA chain termination and eventual impairment of viral replication (Marcellin et al., 2003). At lower doses than previously used in HIV, Adefovir (10mg per day) can lead to a 3-4 log reduction in HBV DNA in HBeAg positive patients and there is associated histologic improvement in the liver (Marcellin et al., 2003). Efficacy in the treatment of HBeAg negative patients with elevated liver enzymes and HBV DNA is well documented (Hadziyannis et al., 2003). Furthermore, it has shown potency in inhibiting the replication of lamivudine resistant HBV mutants, both in-vitro and in-vivo, making it the drug of choice in cases of lamivudine resistance (Perrillo et al., 2000; Ying et al., 2000).
Newer agents are emerging for the treatment of HBV. The oral nucleoside, entecavir and the long acting formulation of interferon, peginterferon alfa-2a have been available since 2005. Entecavir is extremely potent and highly selective for HBV polymerase with no activity against HIV (Lai et al., 2002). Again it is reported to be active against lamivudine resistant HBV. Tenofovir, presently licensed for the treatment of HIV, has not yet been approved by the FDA for the treatment of HBV infection. This adenine nucleotide analogue is active against the HBV polymerase and has reported reductions of 4 logs in the circulating levels of HBV DNA with activity also extended to patients with lamivudine resistance (Nelson et al., 2003; Ristig et al., 2002).

Despite the growing number of antivirals at our disposal and more importantly a very effective vaccine, HBV continues to evade current management strategies. This is primarily as a consequence of drug resistance. The best strategy to overcome this obstacle is still unclear. With an increasing number of antiviral agents, some of which I have discussed, only randomized controlled clinical trials will address what the best agent or combination of agents will be to treat chronic HBV in the future. However, the sub-optimal therapies currently at our disposal, challenges our understanding of HBV at a molecular level. Clearer understanding of the immunopathology of HBV is a requisite for improved finite treatment of HBV. Better definition of the host’s immune response and the pathogenesis of HBV will also provide much needed insight into how the immune system can be manipulated to tackle this virus. This thesis will examine the role of the NK cell activating receptor NKG2D in the immunopathogenesis of chronic HBV.
infection. The definition of this novel receptor and its place in governing CD8+ T cell response in chronic viral hepatitis will be investigated in humans for the first time.
1.2.1 HEPATITIS C VIRUS

Hepatitis C virus (HCV) is a non-cytopathic, hepatotropic RNA virus. HCV can cause both acute and chronic hepatitis, but is believed to cause chronic infection in 60-80% of infected adults (Figure 1.7) and as many as 200 million people are estimated to be persistently infected worldwide. Chronic HCV infection is a major cause of liver damage leading to liver fibrosis, cirrhosis and hepatocellular carcinoma. Host immune responses are critical in determining the outcome of infection, with efficient helper and cytotoxic T cell responses resulting in viral control, while failure to mount such responses results in the insidious disease that is chronic HCV infection.

Several factors have impeded progress in the study of hepatitis C, all of which relate to the limitations of studying the virus in man, its natural host. Furthermore, the lack of reliable and efficient cell culture systems has compounded this problem. Despite this, experimental data is providing greater insight into the mechanisms of type C viral hepatitis. Studies in the chimpanzee, the only animal that can be infected with HCV have provided a clearer understanding of the in vivo immunological response in HCV infection (Rehermann and Nascimbeni, 2005).

This Introduction will provide an overview of our current understanding of HCV immunopathogenesis in the context of the increasing clinical challenge that HCV represents. This essential background information will provide a platform for the work of
this thesis which examines specific areas of T cell immunology in chronic HCV infection.

1.2.2 A brief history of HCV

The identification of HCV in 1989 (Choo et al., 1989) ended a protracted search for the major cause of hepatitis arising from blood transfusions. Since the development of diagnostics for the hepatitis A virus (HAV) and HBV in the 1970s, it was apparent that there was a blood borne, so called non-A, non-B hepatitis (NANBH) virus which had

Figure 1.7. The natural history of HCV infection.
remained elusive (Feinstone et al., 1975). The difficulty in detection, identification and verification of this NANBH agent, as what is now known as type C viral hepatitis, reflects the difficulty in reliably isolating this virus. Although readily transmissible to chimpanzees, progress in studies of the virus was limited by the failure of conventional immunological methods to identify specific viral antibodies and antigens (Bradley, 1985). Subsequent to the designation of this NANBH agent as HCV, the same group reported the development of a recombinant protein based assay for HCV antibody detection (Kuo et al., 1989) and in 1990 a PCR assay was developed that could specifically detect HCV RNA genomes in liver and serum (Weiner et al., 1990).

1.2.3 Epidemiology

Prevalence rates vary considerably with geographical location throughout the 130 countries worldwide reported to be affected (Figure 1.8). The prevalence of HCV seropositivity in healthy blood donors in the U.K is of the order of 0.01-0.02%. Prevalence rates in Equatorial Africa are recorded at up to 6.5% and up to 28% in Egypt, where parenteral antischistosomal therapy is thought to have contributed to the high rates of antibodies against HCV (Frank et al., 2000). In the United States, 1.8% of the population is positive for HCV antibodies with 74% of this group being positive for HCV RNA, equating to 2.7 million chronically infected persons. Illegal drug use, more specifically injection drug use, and high risk sexual behaviour are factors strongly associated with HCV infection (Alter et al., 1999).
Prior to the introduction of screening, a significant proportion of infected subjects acquired HCV through the transfusion of contaminated blood or blood products (Alter, 1994; Schiff, 1994; Tong et al., 1995). The screening of donor blood products for anti-HCV antibodies has reduced the risk of infection to less than 1 in 103,000 transfused units (Schreiber et al., 1996). This is still nearly five times the estimated risk of transfusion related infection with HIV-1 infection (1 in 493,000) but about half the equivalent risk for transfusion related infection with HBV (1 in 63,000) (Schreiber et al., 1996). The more recent introduction of screening pooled samples by polymerase chain reaction (PCR) will ensure that the risk associated with blood transfusion will now be even lower than previously estimated (Beld et al., 2000).

In some cases of HCV infection, no apparent risk factors can be identified (Alter et al., 1992). This is an important point as it clearly may suggest an underestimation of absolute numbers of infected individuals if all risk factors have not been identified. Alter et al. reported a number of associations identified as increasing the risk of HCV infection namely poverty, high risk sexual behaviour, less than 12 years education and having been divorced or separated (Alter et al., 1999). Skin piercing procedures, tattooing, traditional practices (using non-sterilized knives) have also been shown to transmit the virus (Hayes and Harkness, 2001). Sexual transmission is considered to be an inefficient means of transmission, and whether this is a consequence of low levels of the virus in the genital fluids or tissues or due to the lack of appropriate receptors in the genital tract remains unclear. What is clear is that sexual transmission of HCV is less efficient than is the case for HIV-1 (Wyld et al., 1997). However, sexual transmission is reported to be facilitated
by co-infection with HIV-1 but the precise mechanism of this is poorly understood. Vertical transmission is also increased significantly in the presence of maternal co-infection with HIV (Thomas et al., 1998). By contrast, vertical transmission of HCV in the mono-infected mother is rare and this represents one of the major differences between type C and type B viral hepatitis.

HCV virions have also been detected in saliva of infected persons, but this and casual household contacts such as shared razors and toothbrushes all appear to be inefficient modes of transmission of HCV (Couzigou et al., 1993).

Figure 1.8. Geographic distribution of HCV infection.
1.2.4 Biology of HCV

HCV is a single stranded RNA virus, which belongs to the Flaviviridae family; with hepatitis G virus, yellow fever virus and dengue virus being the most closely related human viruses (Robertson et al., 1998).

The HCV genome is a single stranded positive sense RNA with 9,500 nucleotides and is comprised of a single long open reading frame (ORF) flanked by two untranslated regions (UTRs). HCV encodes a polyprotein of approximately 3,000 amino acids which is cleaved into 10 polypeptides, including three structural (core, E1 and E2) and non-structural proteins. The envelope protein 2 (E2) contains the two hypervariable regions (HVR 1 and HVR 2) which show marked sequence variability owing to a high rate of mutation, the result of selective pressure by virus-specific antibodies. E2 also contains the CD81 binding site. CD81 is located on hepatocytes and B lymphocytes and is considered the cellular receptor or co-receptor for the virus (Pileri et al., 1998). The nonstructural proteins (NS) encode the virus-specific protease, helicase and polymerase and because of the central role of these proteins in the virus life cycle, they are targets for antiviral therapies (Kolykhalov et al., 2000). Likewise, the highly conserved untranslated regions at both ends of the viral RNA are also potential targets for antiviral therapies as they play a critical role in polyprotein translation and viral replication (Tang et al., 1999).
There are six known genotypes (G1-6) and more than 50 subtypes of HCV. Different HCV genotypes have distinct geographical distribution in their prevalence. Genotype 1a is the most common in the U.S.A and Northern Europe, while genotype 1b has a worldwide distribution. Genotype 2 represents 10-30% of all HCV types but is particularly common in Italy and Japan. More recently genotype 3a, with its origins in the Indian subcontinent and Southeast Asia, has spread widely and rapidly through the intravenous drug using community. Genotypes 4, 5 and 6 are rare in the West, but are commonly found in Africa and the Middle East (G4), South Africa (G5) and Southeast Asia in the case of genotype 6. HCV genotype is the principal determinant of response to anti-viral therapy and also dictates treatment strategy in terms of duration of therapy (Tang et al., 1999).

1.2.5 HCV pathogenesis

Early events

HCV infection results in strong activation of innate immunity and has been shown to significantly induce Type 1 interferons (IFN), IFN-α/β. These cytokines possess both antiviral functions and immunomodulatory effects (Biron, 2001). Despite the resulting vigorous innate immune response, chronic HCV infection appears to develop in the majority of individuals and thus underlines the inability of the innate immune response alone to control virus (Bigger et al., 2001; Su et al., 2002).
HCV infection in the chimpanzee model has demonstrated changes in the IFN-α response genes within 2 days of HCV inoculation and these changes parallel virus replication kinetics (Bigger et al., 2001). HCV replicates immediately in man and viraemia is detectable within 1-2 weeks after exposure (Thimme et al., 2001) and within 3 days in chimpanzees (Bassett et al., 2001). The ability of HCV to act as a type 1 IFN-α inducer is supported by the enhanced expression of IFN-α/β genes in patients with chronic HCV (Patzwahl et al., 2001). Studies to date have reported the antiviral effects of IFN may be limited to the early stages of HCV infection and this is supported by the efficacy of IFN-α monotherapy in the treatment of patients with acute HCV infection (Jaeckel et al., 2001). The role of type 1 IFNs appears to be the restriction of excessive viral replication in the early phase of infection and not HCV clearance. The study of viral kinetics supports this interpretation where the initial rapid and early peak of viraemia is followed by a 4-6 week period where the levels of HCV RNA remain stable or augment minimally (up to 10⁶ RNA copies/ml in chimpanzees and 10⁷ in humans)(Bassett et al., 2001; Bigger et al., 2001; Major et al., 1999). This period of infection is remarkable for the lack of HCV-specific T and B cell induction and the absence of liver inflammation. NK and NK-T cells are cellular components of the innate immune system that produce IFN-γ and kill infected cells (Biron and Brossay, 2001). IFN-γ production has antiviral effects but also mediates the recruitment of inflammatory cells to the liver (Kaneko et al., 2000; Liu et al., 2000; Trobonjaca et al., 2001). However, the ability of HCV to evade the innate immune response cannot be overlooked. Recent experimental data suggest that the NS3/4A complex cleaves the viral polyprotein but also cellular proteins and interferes
with the signal cascade of RIG-1 and CARDIF which play a role in interferon expression and signalling (Li et al., 2005; Meylan et al., 2005).

The overt lack of liver inflammation in the first 4-6 weeks of infection suggests that the contribution of NK and NK-T cells is minimal in this period (Bassett et al., 2001; Bigger et al., 2001; Major et al., 1999). The HCV protein E2 has been shown to directly inhibit global NK cell function (Crotta et al., 2002; Tseng and Klimpel, 2002) which could explain the absence of liver inflammation in the presence of HCV replication and IFN-α mediated NK cell recruitment to the liver. Bigger et al. also reported no change in the expression levels of Interferon regulatory factor-1 (IRF-1), a transcription factor essential for NK cell activity in vivo (Duncan et al., 1996), in the livers of acutely infected chimpanzees (Bigger et al., 2001).

The first signs of liver inflammation, namely elevated aminotransferases, heralds the involvement of the adaptive immune response with the detection of HCV-specific T cells in the circulation and liver (Thimme et al., 2002; Thimme et al., 2001).

**HCV kinetics & the cellular immune response**

Differences in the timing and detection of the cellular immune response in HCV may contribute to the difference of disease profile in HBV and HCV infection. Despite this, patients who spontaneously recover from HBV and HCV infection demonstrate the ability to mount an efficient T and B cell response (Figure 1.9). On the contrary, patients
who develop chronic infection are found to have weak or narrowly focused T cell responses that are delayed and sometimes transient (Rehermann and Nascimbeni, 2005).

**Figure 1.9.** The control of HBV & HCV infections is dependent on the same facets of the host’s immune response

Type C viral hepatitis differs from HBV in that high HCV titres are usually detected in the blood within 1-2 weeks of infection. Despite this, there is a major delay between the appearance of high viral titres and the detection of HCV-specific T cells in the blood of infected patients. As mentioned, it can take from 5-9 weeks for such responses to be detected in humans, as demonstrated by the analysis of T cell responses in a subject exposed to HCV through needlestick injury (Thimme et al., 2001). Sequential liver biopsies from experimentally infected chimpanzees showed that virus-specific CD4 and CD8 T cells accumulate in the liver 8-14 weeks after infection, coinciding with viral
clearance and liver disease (Cooper et al., 1999; Thimme et al., 2002). Furthermore, it has now been established that such virus-specific CD4 and CD8 T cell responses persist after viral clearance (Ferrari et al., 1994) and can be detected up to two decades after recovery, while HCV-specific antibodies are lost in some individuals (Takaki et al., 2000).

A prospective study in intravenous drug users has provided evidence for protective immunity in some individuals. Previous HCV infection was associated with a reduced risk of developing a new viraemia or persistent infection on re-exposure to HCV (Mehta et al., 2002). Studies in chimpanzees support this clinical observation and animals that had previously recovered from HCV infection were found to have a milder course of liver disease and achieved viral clearance within a shorter timeframe when re-challenged. Viral clearance correlates with strong peripheral T cell responses and intrahepatic IFN-γ production (Bassett et al., 2001; Weiner et al., 2001). CD4 and CD8 T cell depletion studies have demonstrated the need for CD4 help in the maintenance of protective immunity while CD8 T cells have been shown to be the principal effectors of protective immunity (Grakoui et al., 2003; Shoukry et al., 2003). Furthermore, these studies suggest CD4 help appears to enable the CD8+ T cells to keep pace with the evolution of viral escape mutations.
1.2.6 T cell failure in HCV infection

HCV-infected patients who develop chronic infection are characterized by weak, narrowly focused or absent virus-specific CD4 and CD8 T cell responses in the acute and chronic phase of HCV infection (Neumann-Haefelin et al., 2005). The lack of HCV-specific T cell response is a consequence of either primary T cell failure or T cell exhaustion/deletion. The analysis of the early phase of HCV infection in healthcare workers after accidental needlestick injury reported that 2/4 patients who developed persistent infection had no demonstrable HCV-specific CD4 T cell response throughout the course of infection, indicative of primary T cell failure. The CD4 T cell response in the other two patients was transient or episodic (Thimme et al., 2001) and the loss of the CD4 T cell responses coincided with re-emergence of viremia, suggesting T cell exhaustion.

The mechanisms of primary T cell failure remain unclear. There are reports of dendritic cell (DC) dysfunction in chronic hepatitis C, where both DC and macrophages exhibit impaired antigen presentation, but this remains controversial. However, reduced IL-12 production is reported in chronic HCV and this correlates with weak HCV-specific T cell responses (Anthony et al., 2004). Deletion of virus-specific T cells in the presence of continuous high viral loads in LCMV infected mice (Moskophidis et al., 1993) may help to explain T cell exhaustion resulting in chronic HCV infection. In the LCMV model, high viral loads driving the activation and expansion of antigen-specific CD8 cells can lead to functional inactivation resulting in irreversible anergy and/or deletion.
1.2.7 Viral escape mutations

The appearance of the adaptive immune response is associated with the selection of quasispecies and escape mutants (Erickson et al., 2001). Viral escape mutations result in inefficient antigen recognition by impairing antigen binding to the MHC molecules and/or T cell receptors. The high rate of HCV replication together with its quasispecies nature and lack of proof reading capacity of the RNA polymerase contributes to the rapid diversification of the viral population. The same studies report that progressive evolution of viral diversity in hypervariable region 1 of E2 results in chronically evolving hepatitis. T cell escape is reported to be an early event (Erickson et al., 2001) and it is noteworthy that escape mutations detected in chronically infected patients did not diversify further during several years of follow-up (Chang et al., 1997). The selection of escape variants in the presence of weak as opposed to robust HCV-specific T cell response (Cooper et al., 1999; Erickson et al., 2001) suggests that it is the weak T cell responses which make viral escape possible.

1.2.8 T cell dysfunction

T cell dysfunction is a reported phenomenon in the early stages of acute HCV in all patients, irrespective of outcome. However, in those with a self limited course of infection, recovery of CD8+ T cell function was associated with a decline in viremia and resolution of disease (Thimme et al., 2001) while CD8+ T cell function remained
suppressed in those who progressed to chronic infection (Urbani et al., 2002). Urbani et al also reported functional differences in virus-specific CD8+ T cells between acute hepatitis B and C, thus supporting a role for virus-specific CD8 cells in successful viral clearance. HCV-specific CD8 cells in the acute phase of infection express lower levels of perforin and display reduced functional capacity in terms of proliferation, lytic activity and IFN-γ production in contrast to the functional efficiency reported in HBV. This suggests the functional defect is present irrespective of the final outcome of HCV, but may only be a transient effect.

Studies in mice have indicated that high viral loads may lead to T cell unresponsiveness, referring to the inability of CD8+ T cells to produce IFN-γ upon antigenic stimulation (Neumann-Haefelin et al., 2005). Data is limited on the effect of viral load on T cell function during the natural course of infection. However, studies have demonstrated the enhancement of the proliferative responses of HCV-specific CD4+ T cells in chronically HCV infected patients during combination antiviral therapy (Barnes et al., 2002), again suggesting that high viral loads may inhibit virus-specific T cell responses.

T cell dysfunction is not limited to the circulatory compartment, nor is it restricted to the CD8 T cell population (Ulshenheimer et al., 2003). Furthermore, it has been reported that the effects of HCV on T cell maturation are not limited to HCV-specific CD8+ T cells. CMV-specific T cells were shown to express a less mature phenotype in chronic HCV infection (Lucas et al., 2004), but no functional impairment in vitro was reported in these
patients. Regulatory T cells described in the blood and livers of individuals with chronic HCV may also contribute to the suppression of HCV-specific T cell function.

Viral proteins have also been shown to directly modulate immune responses to HCV. HCV core protein expressed in hepatocytes and lymphocytes interacts with TNF receptor 1 (TNFR1) and Fas, modulating the apoptosis of these cells (Neumann-Haefelin et al., 2005). Extracellular core protein interacts with the complement receptor gC1qR on naïve T cells, dendritic cells and macrophages and so impairing T cell activation, proliferation and IFN-γ production (Kittlesen et al., 2000). Circulating HCV core protein has been shown to inhibit IFN-γ secretion by CD8+ T cells. The addition of exogenous IL-2 to in vitro T cell culture has been demonstrated to reverse the inhibitory effects of HCV core on T cell function (Accapezzato et al., 2004).

1.2.9 Humoral immune responses

HCV-specific antibodies do not reflect specific stages of disease as is the case in HBV. The appearance of HCV-specific antibodies is variable in infected patients; no antibodies appear early after infection and may be delayed for up to 2-3 months or not appear at all. HCV-specific antibodies are not maintained for life and can disappear 10-20 years after recovery from infection (Rehermann and Nascimbeni, 2005). Antibodies specific for the HCV envelope glycoproteins (E1 and E2) have been shown to neutralize in vivo infectivity of HCV in chimpanzees and modulate HCV RNA levels in vaccinated and re-
challenged chimpanzees (Forns et al., 2000). The development of tissue culture based assays has allowed the identification of antibodies that block HCV entry into hepatocytes.

The use of replicon systems, although incapable of supporting virus production, have advanced our understanding of the HCV viral life cycle. This work has recently led to the isolation of JFH-1 which is capable of viral particle production in permissive cells, and thus expediting studies of HCV replication (Bukh and Purcell, 2006). Pseudoparticles are retroviral nucleocapsids surrounded by lipid envelope into which HCV glycoproteins can be embedded, enabling studies of possible antibody neutralisation.

Antibodies that neutralize HCV pseudotypes in vitro have been found to be strain specific and present at very low levels in vivo during the first six months of HCV infection. Patients who recover within the first months of HCV infection test negative with this assay (Bartosch et al., 2003; Logvinoff et al., 2004). Logvinoff et al also reported that it may take up to 6-12 months until antibodies with increased neutralization titres and crossreactivity with E1 and/or E2 of different HCV quasispecies appear. While recovered patients test negative, the highest antibody titres are found in patients with established chronic HCV infection, which is indicative of the emergence of escape mutants (Farci et al., 2000).
1.2.10 Clinical Course of HCV infection

Acute HCV infection, as described, is characterized by detectable viraemia in the serum of almost all patients within 1-2 weeks of exposure. However, the acute phase of HCV infection is usually asymptomatic and is rarely diagnosed at this time. As a consequence, there is a lack of experimental data investigating the host cellular immune response during this critical phase of the infection. Clinical symptoms such as jaundice, which are attributed to T-cell mediated liver injury, are rare in HCV but when present appear 6-8 weeks after exposure to the virus. There are also reports of severe acute hepatitis and a fulminant hepatitis related to HCV, but again, this is extremely rare (Farci et al., 1996).

HCV is reported to establish chronic hepatitis in 60-80% of infected adults (Seeff, 2002). Chronic HCV infection is associated with a prolonged symptom-free period with the interval between primary infection and the development of cirrhosis lasting between 20 and 30 years. Once persistent infection is established, which is associated with hepatic necroinflammation and a varying degree of fibrosis, spontaneous clearance of viraemia is rare (Seeff et al., 2000) and the major long-term complications of chronic hepatitis C are cirrhosis, end-stage liver disease and hepatocellular carcinoma. Death due to chronic HCV infection is most frequently observed in persons with cirrhosis, and cirrhosis is estimated to develop in 20% of infected individuals.

Despite the many studies reporting natural history of HCV, chronicity and the development of cirrhosis, there are a number of conflicting reports regarding disease progression. Studies reporting the outcome of patients infected with HCV through
contaminated anti-D immune globulin in Ireland and Germany, suggest the development of cirrhosis is as low as 2%, twenty years after exposure (Kenny-Walsh, 1999; Wiese et al., 2000). How best to interpret this discrepancy is unclear.

One explanation relates to the study population, the patients included in these studies shared a common number of characteristics reported to be associated with a favorable outcome to HCV (or slow rate of progression of disease); these include female sex and young age at infection; and therefore may underestimate the true percentage of patients developing clinical sequelae. It is also argued that the homogenous nature of these study populations is unrepresentative of HCV infection in the general population where the development of HCV related chronic liver disease is known to be highly variable and unpredictable.

In contrast male sex, older age at infection and co-infection with HIV-1 or HBV are associated with a predicted accelerated clinical progression (Poynard et al., 1997; Zarski et al., 1998). Alcohol intake is also reported to have a pronounced effect on the course of disease.

The development of hepatocellular carcinoma (HCC) in HCV is also reported to differ from that in HBV. It is reported that HCC can develop in the absence of cirrhosis in patients with chronic hepatitis B, but this is extremely rare in patients with hepatitis C. On the contrary, established HCV related cirrhosis is associated with a 1-4% risk per year
of developing HCC, (Tsukuma et al., 1993) necessitating increased supervision of cirrhotic patients and cancer screening programmes.

The changing landscape of HCV infected individuals; where injection drug use is now the major risk factor for HCV infection has brought new challenges to the management of hepatitis C virus. Co-infection with HIV-1 (and other viruses) will add to the burden of already sub-optimal treatment strategies. The gravity of this situation has been reported in a European cohort, where 75% of HIV-1 positive patients with a history of intravenous drug use were reported to be co-infected with HCV (Soto et al., 1997).

This fell to 33% in HIV-1 patients in the absence of injection drug use, highlighting the possible increased sexual transmission of HCV in the presence of HIV-1. With or without injection drug use, HIV co-infection is associated with an accelerated course of HCV, with a reported risk of cirrhosis of 25% after 15 years in this cohort, compared with 6.5% in HCV mono-infected persons (Sanchez-Quijano et al., 1995). A similar pattern of accelerated disease is also reported for individuals with HBV/HCV co-infection (Zarski et al., 1998).

1.2.11 Hepatitis C and Therapy

Treatment of hepatitis C was initially based on the administration of interferon alfa, with its ability to alter host-virus interactions in a way that is not fully understood. The
The antiviral effect of interferon is mediated through the host response (Pawlotsky, 2000) and it appears to be most effective in the setting of early HCV infection. Interferon is a natural cellular protein capable of 1) induction of cytokine secretion, 2) induction of an antiviral state in their target cells, 3) recruitment of immune cells and 4) induction of cell differentiation (Baron et al., 1991; Lengyel, 1982).

However, it has been the addition of ribavirin, a synthetic guanosine analogue, which has potentiated the antiviral effects of interferon alfa (Pawlotsky, 2000) and dramatically changed response rates in HCV. Ribavirin selectively inhibits viral RNA polymerases in vitro and in the past was used primarily for the treatment of severe respiratory syncitial virus infection in infants. When given alone, ribavirin has not proven to be effective in the treatment of chronic HCV (Di Bisceglie et al., 1995). The mechanisms of how ribavirin potentiates the immunomodulatory effects of interferon are not well defined. It has been reported that ribavirin could alter the Th-1/Th-2 balance by causing a shift towards Th-1 responses (Hultgren et al., 1998), alternatively it may relate to the ability of ribavirin to suppress HCV-specific IL-10 production (Cramp et al., 2000). With the combination of ribavirin and interferon, given for 24 weeks (genotype 2 & 3) or 48 weeks (genotype 1 & 4), 40% of all patients achieve a sustained virologic response (SVR) and the associated potential longterm benefits (Poynard et al., 1998).

The addition of the polyethyleneglycol (PEG) molecule to interferon produced a biologically active molecule with a longer half-life and improved pharmacokinetics compared with the natural molecule. This led to the introduction of the more convenient
once-weekly administration of interferon (Glue et al., 2000a; Glue et al., 2000b). PEG-interferon, when given alone for 48 weeks, was reported to double the SVR rate seen with standard interferon in the treatment of chronic hepatitis C (Lindsay et al., 2001; Zeuzem et al., 2000). Despite this, relapse rates remained high with pegylated interferon monotherapy and most patients with genotype 1 HCV failed to achieve SVR. The natural progression from here, to optimize treatment strategies was to give pegylated interferon in combination with ribavirin (previously shown to decrease the risk of relapse when given in combination with standard interferon).

This combination of antiviral therapy has increased the SVR rate compared with that of previous combination therapy over the same timeframe (Manns et al., 2001). The improved response rates of upto 80% in genotypes 2 & 3 and 50% in genotypes 1 & 4 coupled with the more convenient once weekly dosing with pegylated interferon in combination with daily ribavirin has led to the introduction of this regimen as the standard for the treatment of hepatitis C virus.
CHAPTER 2

MATERIALS AND METHODS
2.1 GENERAL MATERIALS AND METHODS

The Materials and Methods employed in this thesis vary in the two different studies undertaken. Materials and methods specific for each project are discussed in more detail in the respective chapters.

2.2 Ethics

Ethics approval was sought and granted by the local Ethics committee at the Royal Free Hospital, London for all patients recruited from the Viral Hepatitis Clinic at the Royal Free. Collaborative work with the Department of Obstetrics and Gynaecology, UCL was approved by the local Ethics committee at University College London Hospitals. Additionally, patients recruited for inclusion in the cross-reactivity study by our collaborators in Parma, Italy had approval for this study by formal submission to their local Ethics committee. Patients were invited to partake in the study and if agreeable, informed written consent was obtained before inclusion. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.
2.3 Viral testing at the Royal Free Hospital

Specific clinical viral testing was performed using the following:

Viral serology for hepatitis B virus was performed with the HBsAg-Ultra (Biomedieux, France). The remaining serological markers (HBeAg, anti-HBe etc.) were tested with Vitros ECI Analysers (Clinical Diagnostics, UK). HBV DNA was measured using the Roche Cobas Taqman HBV assay (Genotypes A-G). The dynamic range of HBV DNA measurement is 12 IU/ml-100 x10^6 IU/ml. Viral loads by quantitative PCR at The Royal Free were given in both IU and copies/ml, with the value for IU being multiplied by a factor of 5.82 to obtain the level in copies/ml.

HCV antibodies were tested with Vitros ECI ELISA (Ortho-Clinical Diagnostics, USA). Anti-HCV positive subjects but HCV RNA negative were then tested with a Chiron HCV RIBA (Chiron, USA) to confirm anti-HCV positive status.

At present HCV RNA is tested with the automated Abbott system. Initially this was Abbott LCX HCV PCR (Abbott, USA) with a lower limit of detection of 50 IU/ml. Currently Abbott Real Time, HCV PCR with a lower limit of detection of 23 IU/ml. Prior to March 2004, HCV RNA was tested with Bayer branched DNA or Bayer TMA assay (Bayer, Germany) with a lower limit of detection of 650 IU/ml. HCV RNA positive samples were routinely tested for genotype. This was determined using a Bayer AutoLiPA (Bayer, Germany).
2.4 Clinical Definitions

1. Negative Controls

Individuals without viral or biochemical evidence of viral hepatitis. Specifically these individuals had no history of exposure to or indeed risk factors for viral hepatitis. None of the negative controls were laboratory or hospital workers.

2. Exposed non-infected individuals

Individuals exposed to virus but with no clinical/diagnostic parameters of infection. In this study all of these subjects were the sexual partners (SP) of chronically infected patients.

3. True naives

Cord blood was obtained from infants born to HCV negative mothers and the immunological profile in this instance was considered as a truly naïve T cell population against which all other responses were measured.

4. Chronic HCV

Chronic HCV infection is characterised by elevated liver function tests (LFTs) and defined as the detection of HCV RNA by PCR (detection limit as described above) on at least two consecutive occasions six months apart.
5. Spontaneously resolved HCV infection

Spontaneously resolved HCV infection is defined as the absence of detectable levels of HCV RNA by PCR (using the assay described above), in the presence of HCV antibodies on at least two occasions six months apart.

6. Chronic HBV

HBsAg is the hallmark of hepatitis B virus infection and its persistence in the blood for more than six months implies chronic infection. Tests for HBV DNA in the serum (described above) are used to assess the level of HBV replication.

7. Normal liver tissue

Liver tissue acquired at the time of hepatic resection for liver metastases. Liver specimens acquired were negative for chronic viral hepatitis and were from subjects with no known history of chronic liver disease. Liver tissue was acquired from the tumour free margins at the time of metastasis resection.

8. Orthotopic Liver Transplantation (OLT)

Liver retrieved at the time of OLT for chronic viral hepatitis. Explant tissue acquired from patients undergoing transplantation for end-stage chronic viral hepatitis. Liver tissue was cut and removed from the explant under the supervision of the histopathologists.
2.5 Peripheral blood samples

Peripheral blood samples were obtained from patients and negative controls recruited for the study. This included the acquisition of blood from patients with chronic viral hepatitis (HBV and HCV infection), and in some instances the sexual partners of patients attending the viral hepatitis clinic at the Royal Free. Blood was usually drawn in the clinic and between 40-50ml of blood was collected for the purpose of the study at the time of venepuncture. In patients recruited for the NKG2D study, blood was taken at the time of liver biopsy, usually performed on the ward and described below. Blood samples were collected in heparinised vacutainers and then taken to the laboratory (Institute of Hepatology, Chenies Mews) where the isolation of peripheral blood mononuclear cells (PBMC) was performed.

PBMC were isolated by density gradient centrifugation. The whole blood was layered onto lymphoprep (Oslo, Norway) taken from a 4°C fridge and allowed to reach room temperature. The blood to lymphoprep ratio was 2:1. This was then centrifuged at 2100rpm for 20 minutes with no brake. The PBMC fraction was removed with a sterile Pasteur pipette and washed once at 1800rpm for 10 minutes. One further slower wash was then performed (1100rpm) for 9 minutes. PBMC were then resuspended and counted. The majority of experiments were set up directly *ex vivo* using the fresh PBMC. Where this was not possible, PBMC were resuspended in freeze medium, transferred to cold freezing vials (Nunc, U.S.A) and immediately transferred to the -80°C freezer for
analysis at a later time. PBMC were frozen in aliquots of 10-15x10^6 cells/vial. Short term storage was at -80°C and long term storage was in liquid nitrogen.

To defrost frozen PBMC, vials of cells were rapidly thawed in a water bath and immediately washed in RPMI before resuspension and use.

### 2.6 HCV peptides and Matrix design

The experimental work in chapter 3 centres on the use of a large panel of synthetic HCV peptides covering the HCV polyprotein. 601 15-mer peptides based on the sequence of HCV-1 [genotype 1a covering all structural (core, E1, E2) and non-structural proteins (NS3, NS4, NS5)] and overlapping by 10 residues were grouped in pools of 12 in a matrix array, such that each peptide was present in two pools only. 106 peptide pools were used in total, and the matrix for pools A1-A24 (peptides 1-144) are shown in Table 2.1.

A positive response in two peptide pools containing the same peptide could then be used to identify the single putative response, which was then confirmed by repeating the experiment with the individual peptide.

The full list of all peptides used is given in Appendix 1.
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Table 2.1. The HCV matrix, peptides 1-144
2.7 Intrahepatic T cells

The experimental work in Chapter 4 is based primarily on the acquisition of human liver tissue and the harvesting of intrahepatic lymphocytes. Patients being investigated in the viral hepatitis clinic at the Royal Free were invited to partake in the study. In the first instance, written informed consent was obtained and only where sufficient core material was attained at the time of biopsy was liver tissue used as part of the study. Generally speaking, only liver tissue in excess of the 1.5cm in length was used for the purpose of the study. Tissue was fixed in 10% zinc formalin for routine histological examination and surplus liver tissue was taken directly from the hospital ward to the laboratory for ex vivo analysis.

Further specimens were obtained at the time of orthotopic liver transplantation and at resection of liver metastases. OLT for end-stage chronic viral hepatitis (HBV & HCV) provided the opportunity to acquire greater volumes of liver tissue and so run parallel experiments to compare and optimize methodology. It also enabled us to comprehensively study the effects of various cytokines on the same population of intrahepatic lymphocytes (IHL). The importance of this can not be overstated as, the acquisition and study of human tissue is becoming increasingly challenging.

Diseased tissue was washed extensively in Hanks balanced salt solution (HBSS) and then manually disrupted in RPMI 1640 (Autogen Biotech) plus 10% fetal calf serum. Resection of liver metastases provided us with the opportunity to acquire “normal” liver
tissue. This tissue, from patients with no known liver disease, was resected away from the tumour margins, washed and disrupted in the same manner as the virally infected livers.

Harvesting of IHL from fresh liver tissue is described in Chapter 4 and patient peripheral blood lymphocytes (PBL) were prepared in parallel by standard density gradient centrifugation as described previously.
CHAPTER 3

ANALYSIS OF T CELL RESPONSE AND THE INFLUENCE OF CROSS-REACTIVITY IN HEPATITIS C VIRUS
3.1.1 INTRODUCTION

CD8+ T cells recognize processed peptides presented in the antigen binding sites of MHC class1 proteins. Presented peptides are usually 8 or 9 amino acids in length with distinct motifs that require two or three residues of the peptide to fit into the MHC groove (Falk et al., 1991). The T cell Receptor (TCR) binds to the peptide-MHC complex by means of only a few contacts with the peptide side-chains that project out of the MHC groove (Bjorkman, 1997).

These features are the basis of the specificity of T cell recognition, but T cells also present an intrinsic degeneracy in their recognition of peptides. It has been calculated that a given TCR has the ability to recognize a million different peptide-MHC combinations (Mason, 1998). For this reason, single T cells can recognize different peptides that present variable degrees of amino acid (AA) homology (Anderson et al., 1992; Kuwano et al., 1991) and this degeneracy of T cell recognition represents the structural basis of the phenomenon of cross-reactivity (Welsh and Selin, 2002).

The analysis of T cell clones that had unexpected crossreactivity, such as vesicular stomatitis virus (VSV)- and influenza virus-specific CTLs, and were found to cross-react with uninfected allogenic targets (Braciale et al., 1981a; Braciale et al., 1981b; Sheil et al., 1987), was explained by T cell degeneracy. Similarly, EBV infection in humans has shown high degrees of allospecific CTL activity being generated during infection (Burrows et al., 1997; Tomkinson et al., 1989; Yang and Welsh, 1986). This was initially
attributed to, non-specific, polyclonal bystander activation, a term referring to the activation of T cells in which the TCRs are not being triggered by the antigens that are driving the immune response, but through a cytokine dependent process. However, it is now considered to be secondary to T cell clones that are crossreactive with virus-infected syngenic targets and uninfected targets that express allogenic MHC antigens (Burrows et al., 1999; Nahill and Welsh, 1993; Selin et al., 1994).

Importantly, CD8+ T cell cross-reactivity between HCV and influenza A virus determinants have recently been demonstrated (Urbani et al., 2005a; Wedemeyer et al., 2001).

3.1.2 The phenomenon of T cell cross-reactivity presents two important implications in the study of viral immunopathogenesis:

The presence of potentially cross-reactive T cells can alter the clinical and immunopathological profile of viral infection. Cross-reactivity could also account for the reported presence of virus-reactive T cells in subjects who lack all serological markers of viral infections. Historically these results were interpreted as indicative of previous viral exposure.

We have analyzed this immunological phenomenon during HCV infection. Before reporting the experimental data, we will provide a short overview of the impact of cross-reactivity during viral infection.
3.1.3 Heterologous immunity during viral infection

The clinical and pathological profiles of viral infections are variable. Differences in the infection dose, viral strain and genetic make-up of the host have been invoked to explain the variability of outcomes and pathological manifestations. A further possibility is that the variability in the pathology caused by viral infections strictly reflects different profiles of T cell immunodominance and different kinetics of T cell responses. This phenomenon has been demonstrated in mice and it is caused by the presence of a large repertoire of memory T cells from earlier infections that can cross-react with a second infecting viral pathogen, leading to a massive recruitment of pre-existing memory cells into a primary immune response (Brehm et al., 2002; Chen et al., 2001; Welsh and Selin, 2002).

3.1.4 Viral infections in the mouse model

Chen et al reported a potent role for memory CD8+ T cells in heterologous immunity using a respiratory model system for viral infection (Chen et al., 2001). Mice were intranasally infected with two unrelated viruses, LCMV and vaccinia virus (VV). The mice were first immunised with LCMV, an RNA virus, and then challenged with the unrelated VV (a DNA virus). This study demonstrated that a heterologous virus can induce the activation of cytotoxic function and IFN-γ production of memory T cells specific to a previously encountered virus. They also report the selective expansion and
modulation of the original T cell repertoire as well as the organ-dependent redistribution of these antigen-specific heterologous memory T cells. Intracellular staining of lymphocytes isolated from VV-infected LCMV immune mice provided evidence that LCMV-specific memory CD8+ T cells were activated in vivo to produce IFN-γ. This resulted in a reduction in the viral load and altered immunopathology, as anti-IFN-γ could enhance VV titres in the LCMV immune mice and blocked the infiltration of the lung with LCMV-specific memory T cells upon VV challenge. The absence of activated memory T cells resulted in severe lung pathology (acute inflammatory responses and fulminant pulmonary oedema) which undoubtedly contributed to the high mortality associated with VV infection in control mice.

However, it is noteworthy that the accumulation of LCMV-specific CD8+ T cells in the lung could also produce architectural changes, which again could be ablated by treatment with anti-IFN-γ. The development of bronchiolitis obliterans in some of the VV-infected LCMV-immune mice was also attributed to this phenomenon. This study also reports the selective expansion and accumulation of certain epitope-specific populations of the LCMV-specific memory CD8+ T cells on exposure to VV. Chen et al attribute this activation and selective expansion in the lung to cross-reactive T cell responses. Three VV peptides are reported to share homology with LCMV NP205 and are capable of stimulating LCMV-specific CD8+ T cells to produce IFN-γ. The complexity of the memory CD8+ T cell response is also highlighted by this study, where exposure to VV was associated with a differential accumulation of LCMV-specific CD8+ T cells to different anatomical sites. The selective expansion of specific epitopes in each site
resulted in the development of different repertoires. It has been proposed that the functional type of each LCMV epitope-specific memory T cell population may influence where they traffic during the resting memory state and during acute VV challenge.

### 3.1.5 Viral infections in humans

Many viruses in humans, such as Epstein-Barr virus, varicella-zoster and even the 1918 influenza virus strain have been reported to cause much more severe infections in teenagers and young adults compared with the disease profile in young children (Simonsen et al., 1998; Welsh and Selin, 2002). Such variations may be attributable to immunopathology occurring as a consequence of the reactivation of memory T cells which may be more prominent in an immunologically mature individual. Infection with Dengue virus is of importance in considering this phenomenon. The gravity of infection with different Dengue serotypes in a dengue virus immune individual can lead to severe immunopathological responses. These responses are considered the consequence of potent reactivation of cross-reactive T cells in the absence of protective antibody responses (Welsh and Selin, 2002).

Urbani et al. in a recent study demonstrated the potential significance of heterologous immunity in the pathogenesis of hepatitis C virus infection (Urbani et al., 2005a). HCV typically has an asymptomatic onset but persistent infection is reported to develop in the
majority of infected persons (60 to 80%) despite the presence of a CD8+ T cell response (Lechner et al., 2000b; Thimme et al., 2001).

Clinical symptoms (usually mild) may be present in up to one third of adults with acute HCV infection. Despite this, a severe acute hepatitis can develop but is rarely fulminant. The mechanisms responsible for such variability in the course and outcome of infection are poorly understood. Urbani et al. reported an association between a peculiar hierarchy of immunodominance of HCV-specific CD8+ T cell responses, cross-reactivity between HCV and influenza-specific CD8 cells and a severe clinical course of hepatitis C. The results of this study suggest a role for CD8+ T cell cross-reactivity in influencing the severity of the HCV associated liver pathology. Furthermore, it illustrates a model of disease induction that can be applied to different viral infections in which immunopathology are sustained by the anti-viral immune response (Urbani et al., 2005a).

In the Urbani study, the global HCV-specific T cell response was analysed in eight patients with acute HCV with variable outcomes. Two of the eight patients developed a severe clinical course of acute HCV with extremely elevated aminotransferases, rapidly rising bilirubin levels and prolonged prothrombin time. The remaining six patients had a mild course of liver disease in keeping with the usual course of HCV infection. The comprehensive analysis of the HCV-specific T cell repertoire revealed T cell responses narrowly focused on a few peptide pools in the 2 patients with severe hepatitis, while a multispecific T cell response was detected in the remaining patients. Analysis with intracellular cytokine staining revealed only a single sequence containing the previously
identified HLA-A2 restricted NS3 1073-1081 CD8 epitope (Wedemeyer et al., 2001) to be present in the two patients with severe hepatitis. *Ex vivo* tetramer staining confirmed the ELISPOT data, with a robust but narrowly focused CD8+ T cell response against the NS3 1073-1081 epitope described. The vigour of this response was unprecedented; with NS3 1073-1081 tetramer + CD8+ T cells reaching values of 36% and 12% of total CD8+ T cells from the respective patients. These frequencies exceed those previously reported in acute HCV infection (Thimme et al., 2001; Urbani et al., 2002) and those detected in the patients with a mild course of infection also included in this study.

The peculiar vigour of this response and the resulting different clinical course is thought to be related to differences in the T cell repertoire in each individual patient. This theory is supported by the high degree of sequence homology between HCV NS3 1073-1081 and the influenza A neuraminidase epitope NA 231-239. The demonstration of cross-reactivity between these two HCV and influenza sequences in humans and in HLA-A2+ positive mice (Wedemeyer et al., 2001) adds to the argument for a private repertoire of memory T cells with the ability to cross-react with heterologous viruses, in this case HCV. Despite the rapid and robust expansion of the NS3 1073-1081 specific CD8+ T cells, the virus was not controlled and liver pathology was severe. Thus, a robust but isolated response is inadequate for viral control. This has implications in terms of antiviral vaccine development where a CD8+ T cell response focused on a single immunodominant epitope with poor antiviral activity may in fact have severe pathological consequences.
3.1.6 The influence of T cell cross-reactivity on HCV-peptide specific T cell response

Based on these observations we set out to determine whether cross-reactive T cells activated by HCV-peptides is a common finding in the human T cell response. We investigated whether such HCV-specific T cell responses previously considered a sign of prior HCV exposure could be accounted for by cross-reactivity. In order to address these questions we comprehensively analysed the global HCV-specific response in a heterogeneous group of subjects using the most advanced techniques at our disposal.

Newer immunological tools have improved in vitro analyses of the prevalence and function of antigen-specific CD4+ and CD8+ T cell responses in persistent viral infections. The most significant of these advances have been the capability to directly visualise epitope-specific cells (MHC-class I and class II tetrameric complexes) (Altman et al., 1996; Novak et al., 1999) and the ability to measure the secretion of T cell cytokines [intracellular cytokine staining, ELISPOT (Miyahira et al., 1995; Schauer et al., 1996)].

Furthermore, the understanding that the in vivo T cell stimulatory complex, formed by MHC molecules and processed viral peptide, can be mimicked in vitro by the addition of synthetic peptides (Germain, 1994) to PBMC has evolved into their direct use in the analysis of specific T cell responses. Measurement of T cell reactivity using large pools of synthetic peptides spanning different pathogenic antigens is becoming the standard for the study of pathogen-specific T cell repertoire in humans. This method is particularly
useful in the analysis of T cell responses against viruses that do not easily infect antigen presenting cells, such as hepatitis C virus (HCV) (Cerny et al., 1995; Cox et al., 2005; Lauer et al., 2004; Lauer et al., 2002; Urbani et al., 2005a).

Different patterns of HCV-specific T cell response are demonstrable in recovered versus chronically infected HCV patients. Historically this differential response was analysed using limited arrays of HCV proteins and peptides (Cerny et al., 1995; Diepolder et al., 1995; Koziel et al., 1995; Missale et al., 1996; Rehermann et al., 1996b). More recently the use of comprehensive panels of synthetic peptides spanning the entire HCV polyprotein have confirmed and detailed further the broader and stronger HCV-specific T cell response profile present in resolved HCV infection (Cox et al., 2005; Lauer et al., 2004). The contrast between this response and the profound quantitative and functional defects of HCV-specific T cells observed in chronically infected subjects has been emphasised by this technique.

However, the use of such broad panels of peptides could increase the possibility of detection of HCV-peptide reactive T cells which have not been initially primed by HCV virions, but are cross-reactive T cells triggered by unrelated antigens. Indeed, T cells present an intrinsic degeneracy in their recognition of peptides. Single T cells can recognize different peptides that present variable degrees of AA homology (Anderson et al., 1992; Kuwano et al., 1991) and this degeneracy of T cell recognition represents the structural basis of the phenomenon of cross-reactivity (Welsh and Selin, 2002). CD8+ T cell cross-reactivity between HCV and influenza A virus determinants have recently been
demonstrated (Wedemeyer et al., 2001) and could have a potential impact on the profile of disease associated with HCV infection (Urbani et al., 2005b).

Cross-reactivity could also account for the reported presence of HCV-peptide reactive T cells in subjects who lack all serological markers of HCV infection (Koziel et al., 1997; Scognamiglio et al., 1999). These results were interpreted in the past as indicative of previous HCV exposure since patients acutely infected with HCV can maintain long term HCV-specific cellular immunity still detectable after loss of humoral responses (Takaki et al., 2000). However, T cell cross-reactivity among homologous peptides covering sequences of different pathogens cannot be excluded. This phenomenon has obvious implications in data analysis but it can also influence the outcome of HCV infection if HCV infects an individual with pre-existing memory responses which can be rapidly and efficiently recalled by homologous HCV sequences. In this context, the protective efficacy of HCV-peptide reactive T cells found in HCV-exposed but ostensibly non-infected subjects may be more difficult to predict if these cells merely represent T cells specific for unrelated pathogens cross-reacting with HCV peptides but not primed by HCV.

In this Chapter, we set out to analyze whether cross-reactive T cells activated by HCV peptides is a common occurrence in humans. The magnitude, function and cross-reactivity of HCV peptide reactive T cells were studied in non-HCV infected newborns and adults. Comparisons were made with responses present in both resolved HCV infection and in chronically infected patients. We demonstrate that non-HCV infected adults possess HCV-peptide reactive T cells, which can react with peptide sequences of
common pathogens. The impact of cross-reactive T cells in the interpretation of the data found in HCV-infected subjects and their potential ability to alter the immunopathological profile of viral infection is discussed.
3.2 MATERIALS AND METHODS

3.2.1 Subjects

Thirty-two subjects were included in the study. Blood was obtained from ten healthy volunteers, comprising the negative control group (N.C, subjects 1-10), all were anti-HCV negative and without any HCV risk factors. A further six subjects, the sexual partners (S.P, subjects 11-16) of six chronically infected HCV patients were studied. Patients with resolved (subjects 17-22) and chronic (subjects 23-32) HCV infection were recruited from the viral hepatitis clinics at the Royal Free Hospital, London and Divisione Malattie Infettive, Parma. All patients with chronic HCV had raised serum alanine aminotransferase (ALT), detectable anti-HCV and HCV RNA for a period of more than six months. Patients with resolved HCV were anti-HCV positive (ELISA and RIBA) on two occasions over a six month period but HCV RNA was undetectable by PCR. The age, sex, disease profiles, serological status, risk factors and ALT levels for all subjects are outlined in Table 3.1. Cord blood from anti-HCV negative mothers (n=5) was provided through our collaboration with the Department of Obstetrics and Gynaecology, UCL. The study was approved by the local Ethics Committee at the Royal Free and University College Hospitals.
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Table 3.1: Characteristics of the study groups.
N.C. = negative control, S.P. = sexual partner, + denotes presence of known risk factors. N.C. had no exposure to or risk factors for HCV. None of the individuals recruited were laboratory or hospital workers.
3.2.2 Synthetic peptides and antibodies

Synthetic peptides representing a panel of 601 15-mer peptides overlapping by 10 residues and spanning the entire sequence of HCV-1 were purchased from Chiron Mimotopes (Victoria, Australia). Peptides homologous to HCV reactive peptides covering selected sequences of human herpes virus (HHV1) and vaccinia virus (VV) were purchased from Primm It (Milano, Italy). Anti-CD8 (conjugated with Quantum Red or fluorescein isothiocyanate - FITC) and anti IFN-γ FITC were purchased from SIGMA Aldrich (Saint Louis, MI). Anti-perforin (FITC) was purchased from BD Pharmingen.

3.2.3 Isolation of peripheral blood mononuclear cells (PBMC) and in vitro expansion of HBV and HCV-specific cytotoxic T cells

PBMC were isolated from fresh heparinized blood or cord blood by Ficoll-Hypaque density gradient centrifugation and suspended in RPMI 1640 supplemented with 25 mM Hepes, 2 mM L-glutamine, 50 µg/ml gentamycin and 8% human serum (complete medium) and used immediately in ELISPOT assays or expanded in vitro. For in vitro expansion, PBMC were dispensed in 96-well plates at a concentration 1.5 x 10⁶/ml in complete medium and stimulated with peptides at 1 µM final concentration. Recombinant IL-2 was added on day 4 of culture (50 IU/ml) and the immunological assays were performed on day 8-10.
3.2.4 IFN-γ intracellular staining

Freshly separated PBMC or cells obtained after in vitro expansion were incubated in medium alone (control) or with viral peptides (1 μM) for 1h; Brefeldin A (10 μg/ml) was added for an additional 4 h of incubation. After washing the cells were stained with anti-CD8 or anti-CD4 quantum red monoclonal antibody for 20 min at 4°C, and then fixed and permeabilized as described. Cells were stained with anti-IFNγ -FITC for 15 min at room temperature, washed again, and analyzed on a Becton Dickinson flow cytometer (FACScalibur) using the CELLQuest software.

3.2.5 ELISPOT assay

601 15-mer peptides based on the sequence of HCV-1 [genotype 1a covering all structural (core, E1, E2) and non-structural proteins (NS3, NS4, NS5)] and overlapping by 10 residues were grouped in pools of 12 in a matrix array (Lauer et al., 2004), such that each peptide was present in two pools only.

HCV-specific T cell responses ex-vivo were analysed after overnight stimulation with individual peptide mixtures. Each plate contained 1 PHA stimulated well as a positive control to confirm the validity of the assay. Briefly, 96 well plates (Multiscreen-IP-Millipore S.A.S., Malshelm, France) were coated overnight at 4°C as recommended by the manufacturer with 5 μg/ml capture mouse anti-human IFN-γ mAb (1 DIK, Mabtech,
Switzerland). Plates were then washed 7 times with PBS/0.05% Tween 20, and blocked with RPMI/10% FCS for 2 hours at 37°C. 2 x 10^5 PBMC were seeded per well, and peptides were added at a concentration of 10 μg/ml. After overnight incubation at 37 Celsius, 5% CO2, the plates were washed with PBS/0.05% Tween 20, and then 50 μL of 1 μg/ml biotinylated secondary mouse anti-human IFN-γ mAb (7B6-1, Mabtech, Sweden) was added. After 3 hours of incubation at room temperature, plates were washed 4 times and 100 μl goat alkaline phosphatase anti-biotin Ab (Vector Laboratories Inc. Burlingame, CA, USA) was added to wells, and the plates were incubated for a further 2 hours at room temperature. Plates were then washed 4 times, and 75 μl of alkaline phosphatase conjugate substrate (5-bromo-4-chloro-3-indolyl phosphate, Biorad Laboratories-Ercules, CA, USA) was added. After 4 to 7 minutes, the colorimetric reaction was stopped by washing with distilled water. Plates were air-dried and spots were counted using an automated ELISPOT reader (AID ELISPOT Reader System, Autoimmune Diagnostika GmbH, Strassberg, Germany). The number of specific IFN-γ secreting PBMC was calculated by subtracting the number of spots obtained in the non-stimulated control well from the stimulated sample. A positive control (PBMC stimulated with PHA) was included with each plate to validate the sensitivity of the assay.

3.2.6 Multiple Cytokine secretion assays

The production of multiple cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-6) in the supernatant of the HCV-peptide reactive T cell lines was tested using a BD™ Cytometric
Bead Array (BD bioscience). 100μl of supernatants of peptide stimulated or unstimulated HCV-peptide reactive T cell lines generated as described above was collected after 18 hours of peptide stimulation. Production of cytokines was tested simultaneously with the assay according to the manufacturers’ instructions.

3.2.7 Statistical Analysis

The Mann-Whitney U test was used where appropriate to determine the significance of differences between groups; a p-value of <0.05 was taken as significant.
3.3 RESULTS

3.3.1 Direct \textit{ex vivo} quantitation of ELISPOT response in cord blood of HCV negative infants

To exclude the interference by the repertoire of memory T cells specific for unrelated pathogens present in adults, overlapping peptides covering the entire HCV polyprotein were initially tested on lymphocytes derived from cord blood of infants born to anti-HCV-negative mothers. The 601 peptides covering the entire HCV genome were grouped in a matrix array into pools of 12 and used to directly stimulate IFN-\(\gamma\) production from T cells in a direct \textit{ex vivo} ELISPOT assay. Figure 3.1 shows the results of all five tests. PHA stimulated wells of cord blood detected at least 100 SFC/well. In contrast, the HCV peptide stimulated wells were predominantly negative. However, a number of individual mixtures did elicit a response higher than background, but this never exceeded 12 SFC/well in all the subjects analyzed.

We assume that cord blood represents the closest example of a truly naïve T cell repertoire, thus we based our calculation of what constitutes a positive result in adults on these results. The mean of the maximum responses obtained in each of the five different cord blood specimens was calculated to be 10.2 SFC/well. HCV-peptide reactive T cell responses in adults were considered positive if the number of spots per well minus the background was at least 3 times the mean value of maximum responses found in cord blood (3\(\times\)10\(^{-1}\) = 30 SPF/well, 150 SPF/10\(^6\) PBMC).
Figure 3.1: IFN-γ production by direct *ex vivo* ELISPOT analysis on cord blood. PBMC from cord blood from the 5 different indicated newborns were stimulated overnight with 106 pools of overlapping 15-mer peptides covering the whole HCV sequence and with PHA. SFC = spot forming cells.

### 3.3.2 Direct *ex vivo* quantitation of ELISPOT response in HCV infected and non-infected subjects

The pools of HCV peptides covering HCV proteins were then tested directly *ex vivo* in six patients with resolved HCV infection (anti-HCV positive, HCV-RNA negative on two different occasions 6 months after infection), ten patients with chronic HCV infection and sixteen subjects with no serological markers of HCV infection. Ten anti-HCV negative subjects had no classical risk factors for HCV infection (N.C. 1-10, Table 3.1) and were considered the control group. A further six subjects were anti-HCV negative but all had
reported exposure to HCV, (i.e. were sexual partners of HCV-RNA positive patients with chronic hepatitis, (S.P.11-16, Table 3.1)). The results of the direct \textit{ex vivo} ELISPOT of two representative subjects from each group are shown in Figure 3.2A.

HCV-peptide reactive T cells were easily detectable \textit{ex vivo} in resolved patients, (Figure 3.2, Sub.17) in keeping with several previous studies. The overall responses to all different peptide pools presented a median total magnitude of 850 (Range 710-2421) SFC/20x10^6 cells. Some pools were able to elicit a response reaching a magnitude of 277 SFC/well and further analysis with individual peptides revealed the presence of at least three T cell epitopes recognized in each of the patients with resolved HCV infection.

The overall quantity of IFN-\(\gamma\) producing T cells detected in PBMC from patients with chronic HCV infection and HCV-negative subjects was lower than that observed in resolved patients; median total magnitude of response to all peptide pools was 104 (Range 24-292) SFC/20x10^6 cells in chronic, 411 (Range 208-502) in N.C. and 479.5 (Range 328-548) in S.P.; but HCV-peptide reactive T cells could be detected in all tested subjects irrespective of their HCV infection status or risk factors. In keeping with previous studies, the T cell responses present in negative controls, sexual partners and chronically infected individuals lack the multispecificity found in patients with resolved HCV infection. The magnitudes of the individual responses were variable but in some cases (see Figure 3.2, Subject 2, where response to pool 14 was 106 SFC/2x10^5) HCV-peptide pools were able to elicit a magnitude almost identical to those found in resolved HCV infection. Interestingly, the direct \textit{ex vivo} frequency of HCV-peptide reactive T
cells found in patients with chronic HCV was lower than those present in HCV negative patients (Figure 3.2A, 3.2B). The difference in total SFC between these two groups (Figure 3.2B) was found to be statistically significant (p<0.0001).

Figure 3.2: IFN-γ production by direct ex vivo ELISPOT analysis on adults. PBMC were stimulated overnight with 106 pools of overlapping 15-mer peptides covering the whole HCV sequence. A) Results of 2 representative subjects from each group of HCV infected and non-infected patients are shown. The line corresponds to a value of 30 SFC/ 2x10⁵ PBMC. Responses superior to this value were considered positive. B) Quantity of SFC stimulated by all peptide pools covering the whole HCV polyprotein. Each dot represents an individual patient for each group. C) Number of positive peptide pools in each subject. Dots represent individual patients.
To confirm that the IFN-gamma production found by direct *ex vivo* ELISPOT analysis in HCV negative patients was sustained by CD4+ or CD8+ T cells we generated HCV-peptide reactive T cell lines. CD4+ or CD8+ T cells able to react to individual HCV peptides can be demonstrated in HCV-negative subjects and sexual partners of HCV infected patients (Figure 3.3). Their profile of cytokine production was Th1/Tc1, with the ability to produce IFN-γ and TNF-α but not IL-4, IL-5 or IL-2 after peptide specific stimulation. (Figure 3.3, B). These results show that HCV-peptide reactive T cells can be detected in the circulation of HCV-negative subjects at frequencies and magnitudes comparable to those in sexual partners of HCV-positive subjects and higher than those observed in chronically infected patients.

**Figure 3.3:** Characterization of HCV-peptide reactive T cells present in HCV-negative control subjects. A) Dot plot analysis of IFN-γ producing T cells specific for HCV-NS3 1436-50 and HCV-NS5B 2811-25 peptides. PBMC were stimulated with individual peptides and after 10 days, were re-stimulated with the stimulatory peptide. Frequency of IFN-γ producing cells was tested with Intracellular cytokine staining. B) Multiple cytokine analysis of supernatants of the peptide stimulated cells. Supernatants of T cell lines demonstrating presence of IFN-γ + cells were analyzed for the indicated cytokines. Supernatants were collected after overnight stimulation with HCV peptide. Pep = peptide.
3.3.3 HCV-peptide reactive T cells after in vitro expansion

The low quantity of HCV-peptide reactive T cells observed in chronically HCV infected patients is attributable to their low frequency in the blood and to the impaired IFN-γ production of these T cells (He et al., 1999; Lauer et al., 2004; Lechner et al., 2000a; Urbani et al., 2002). *In vitro* expansion of peptide stimulated PBMC is a strategy previously used (Lauer et al., 2002; Penna et al., 1991; Rehermann et al., 1996a; Webster et al., 2004) to detect peptide-specific T cells present at low frequencies in the circulation. Thus, to further evaluate the HCV-peptide reactive T cell repertoire present in HCV infected (resolved and chronic) and in HCV negative subjects, T cell responses against HCV peptide pools were analyzed after in vitro expansion. PBMC of HCV infected patients and HCV negative subjects (N.C. and S.P.) were stimulated with the different peptide mixtures and after 10 days of in vitro expansion, the presence of HCV-peptide reactive T cells was tested.

*In vitro* expansion increased the number of peptide pools able to elicit a T cell response in all groups. The subsequent identification of the individual peptides able to elicit a response shows that HCV negative (N.C.) subjects have T cells able to recognize a median of 4.5 (Range 3-6) HCV peptides (Figure 3.4). In contrast, broad HCV peptide-reactive T cell responses were detected in the blood of all six patients with resolved HCV infection, with a median of 21.5 (Range 15-25). In six patients with chronic infection, the HCV-specific repertoire was broader than that of HCV negative subjects. The median number of individual peptides recognized by chronic HCV patients analyzed was 7.
(Range 3-15) and comparison between this group and the negative controls (N.C.) was found to be statistically significant (p=0.0055). This demonstrates a significant enhancement of the HCV-peptide specific T cell repertoire in the circulation of patients with chronic HCV after *in vitro* expansion.

**Figure 3.4:** Quantification of HCV peptides recognized by T cells after *in vitro* expansion. Experimental plan: PBMC were stimulated *in vitro* with the 106 different peptide pools. After 10 days of *in vitro* expansion, cells were re-stimulated overnight with the initial stimulatory peptide pools in an IFN-gamma ELISPOT assay. Lines were considered positive when SFC of stimulated wells were at least double the SFC in un-stimulated wells. Positive lines were then tested with individual peptides present in the pools. A) The number of positive peptides found in the different patient groups using the experimental plan outlined is shown. B) *Ex vivo* and *in vitro* ELISPOT pattern of response against HCV mixtures is shown for one representative negative control and one chronic HCV patient.
3.3.4 Identification of HCV-peptide reactive T cells cross-reacting with homologous sequences of common pathogens

Having established the presence HCV-peptide reactive T cells in the peripheral blood of HCV-negative subjects, we investigated whether T cells cross-reacting with homologous sequences of common pathogens can sustain these responses.

We first searched the National Centre for Biotechnology Information Gene Bank database using the “Blast program” for peptides displaying a degree of homology with the eleven HCV peptides (Table 3.2) able to activate directly *ex vivo* PBMC of normal subjects without risk factors.

Variable degrees of sequence homology with different pathogens or self human proteins were found by the *in silico* analysis for each HCV peptide analyzed. We thus focused our functional analysis on the following 3 sequences: NS5B $^{281-25}$ (peptide no. 563) PLARAAWETARHTPV, NS5B $^{2936-50}$ (peptide no. 588) GRAAICGKYLFWAV and E1 $^{361-375}$ (peptide no. 73) YFSMVGNWAKVLVV, which were selected for the following criteria:

(1) the HCV peptides elicit high direct *ex vivo* frequency (NS5B $^{281-25}$ 91 SFC/ subject 3, NS5B $^{2936-50}$ 70 SFC/ subject 6, E1 $^{361-375}$ 48 SFC/ subject 4); (2) peptide reactivity was confirmed at different time points (not shown), (3) high sequence homology with common pathogens or antigens was noted (Table 3.3).
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<td>CD8</td>
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<tr>
<td></td>
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<tr>
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<td>CD4</td>
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<tr>
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<td></td>
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<tr>
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<td>CD8</td>
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<tr>
<td></td>
<td>GRAAICGKYLFNWAV</td>
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</table>

*Indicates < 6 shared AA
The peptides HCV NS5B \textsuperscript{2811-25} and HCV E\textsubscript{l} \textsuperscript{361-375} revealed clear homology respectively with the sequences of hepatitis B virus (HBV) envelope (GKYLWEWAS) and with vaccinia virus (VV) proteins (LVGNWDKNDV) respectively (Table 3.3). Peptides covering these homologous sequences were synthesised and tested both directly \textit{ex vivo} in PBMC and then on HCV-peptide reactive T cell lines but they consistently failed to activate PBMC and HCV peptide reactive T cells (not shown).

In contrast, evidence of cross-reactivity between the HCV-NS5B \textsuperscript{2811-25} specific CD8\textsuperscript{+} T cells with a human herpes virus (HHV1) sequence was found. The 15-mer peptides NS5B \textsuperscript{2811-25} shared several amino acids with the UL55 protein of HHV1 (Table 3). However, the

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequence*</th>
<th>Source</th>
<th>GenBank accession no.</th>
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<tr>
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<td>PLARAAWETARHTPV</td>
<td>HCV genotype 1B, NS5B</td>
<td>P26624</td>
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<tr>
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<td>ATWQAETAMHT</td>
<td>HHV1</td>
<td>P36296</td>
</tr>
<tr>
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<td>P26624</td>
</tr>
<tr>
<td>HBV Env</td>
<td>GKYLWEWA</td>
<td>HBV-Major surface Ag</td>
<td>Q05496</td>
</tr>
<tr>
<td>HCV E\textsubscript{l}</td>
<td>YFSMVGNWAKVLVVL</td>
<td>HCV genotype 1B, E\textsubscript{l}</td>
<td>P26624</td>
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<tr>
<td>VV 275-284</td>
<td>LVGNWDKNDV</td>
<td>Vaccinia Virus</td>
<td>BAD97827</td>
</tr>
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</table>

Table 3.3: HCV peptide sequence homology with common pathogens. Boldface indicates shared amino acids.
shared AA between the two sequences encompass an 11 AA stretch, a length that is not considered compatible with a classical CD8+ epitope. For this reason, we first determined the HCV-epitope contained within the 15-mer peptides. Overlapping 10- and 9-mers covering the 15-mer peptide NS5B \textsubscript{2811-25} region were tested directly \textit{ex vivo} (Figure 3.5) to determine the minimal sequence able to activate NS5B \textsubscript{2811-25} specific CD8+ T cells. Figure 3.5 shows that the 10-mer NS5B \textsubscript{2816-25} AWETARHTPV peptide elicited a direct \textit{ex vivo} IFN-\textgamma ELISPOT response similar to the NS5B \textsubscript{2811-25} PLARAAWETARHTPV peptide. In contrast, no response was activated by NS5B \textsubscript{2811-20} PLARAAWET and NS5B \textsubscript{2815-23}AAWETARHT peptides. Having established that the NS5B \textsubscript{2811-25} peptide specific T cells direct their recognition on the 10 mer NS5B \textsubscript{2816-25} AWETARHTPV, the corresponding homologous HHV1-peptide QAETAMHTSK was synthesized and tested. Figure 3.5b shows that the HHV1-peptide QAETAMHTSK can directly activate PBMC of the subject responding to HCV NS5B \textsubscript{2811-25} peptide. In addition, HCV NS5B \textsubscript{2811-25} peptide specific T lines can be stimulated with both NS5B \textsubscript{2816-25} AWETARHTPV and HHV1 QAETAMHTSK peptides.
**Figure 3.5:** Recognition of NS5B\textsubscript{2816-25} and HHV1 peptides by HCV-NS5B CD8\(^+\) T cells. a) *Ex vivo* IFN-\(\gamma\) ELISPOT analysis. PBMC of subject 3 were stimulated overnight with the indicated peptides. The results are expressed in spot forming cells (SFC)/\(2\times10^5\) PBMC. b) Plots represent IFN-\(\gamma\) producing CD8\(^+\) cells of the NS5B\textsubscript{2816-25} specific T cell line stimulated with no peptide, NS5B\textsubscript{2816-25} and HHV1 UL55\textsubscript{29-39} peptides. Numbers in the right upper quadrant indicate the percentages of IFN-\(\gamma\) producing CD8\(^+\) cells after 4 hours stimulation. Pep = peptide.
3.4 DISCUSSION

This study was designed to analyze whether *in vitro* evaluation of HCV-specific T cell responses in humans using large arrays of synthetic peptides is potentially altered by the presence of cross-reactive T cells specific for different pathogens but with a degenerate recognition of HCV peptides. The results confirm that this is the case, since monomeric HCV-peptide reactive T cells can be found in the blood of HCV negative adults without any classical risk factors of HCV infection (including past blood transfusion, sexual contact with HCV infected subjects and intravenous drug use). In all of these HCV negative subjects, HCV peptide reactive CD4+ and/or CD8+ T cells were detected when a large panel of synthetic peptides covering the whole HCV polyprotein (601 peptides) was employed. On the whole, T cell response against HCV peptides was clearly greater (both in magnitude and multispecificity) in patients who recovered from HCV infection than in HCV negative adults. Nevertheless, for selected individual peptide mixtures, the reactive T cells found in HCV negative adults present a direct *ex vivo* frequency comparable with HCV-specific T cells present in subjects who recovered from HCV infection. In addition, these cells had the potential to expand and produce Th1-like cytokines (IFN-γ, TNF-α) but not IL-4 or IL-5. Thus, at least functionally, these responses were indistinguishable from classical virus-specific effector/memory T cells present in patients with resolved viral infection.

It is difficult to ascertain whether the presence of these HCV-peptide reactive T cells in HCV-negative patients is indicative of previous exposure to HCV (thus, suggesting an
under estimation of HCV infection in the general population) or whether it is the consequence of a degenerate recognition of HCV-peptides by cross-reactive T cells.

We favour the latter interpretation since HCV-specific T cell responses present in subjects who recovered from HCV infection are often multispecific with HCV-specific T cells able to recognize multiple HCV epitopes located in the different HCV proteins, while those found in HCV-negative subjects show T cells reactive to 1 or 2 peptides out of the 601 used. Furthermore, this mono-oligospecific HCV-reactive T cell response does not broaden into a multispecific response after in vitro expansion, a method that increases substantially the number of HCV peptides recognized by T cells of recovered and chronic patients. More importantly, we were able to demonstrate that NS5B_{2816-25} specific CD8+ T cells found in HCV negative subjects were reactive to a homologous HHV1 sequence. Thus, in addition to the cross-reactivity between HCV and an influenza A virus determinant already demonstrated by Wedemeyer et al. (Wedemeyer et al., 2001), we found that a further human pathogen might induce cross-reactive HCV-specific T cells, suggesting that T-cell cross-reactivity between different pathogens is a common phenomenon in adults.

We cannot rule out occult hepatitis C in such subjects but in the absence of any indication of liver disease occult hepatitis C infection (Berasain et al., 2000; Castillo et al., 2004) is unlikely.
Although we failed to demonstrate the direct evidence of cross-reactivity of all the HCV peptide-reactive T cells found in the HCV-negative subjects studied, we should not forget that T cell cross-reactivity does not necessarily depend on identity of amino acids on the linear sequence of the peptide. T cell receptors of a given T cell clone can be activated by quite dissimilar peptides (Hausmann and Wucherpfennig, 1997; Hemmer et al., 1999). Thus, the strategy of identifying cross-reactive peptides based on sequence homology even though commonly used is bound to underestimate the extent of cross-reactivity. In addition, the search of homologous peptides is based on the search of known sequences, and thus unable to detect yet-unidentified homologous sequences.

What are the putative consequences and potential implications of the detection of HCV peptide-reactive T cells in HCV-negative adults? The pathological impact of cross-reactivity on virus infection has been analyzed in animal models, where it was shown that previous encounter with different pathogens can influence virus-related immuno-pathology. We have recently shown that severe cases of HCV induced hepatitis are characterized by a large expansion of HCV-specific CD8+ T cells able to cross-react with an influenza neuraminidase sequence (Urbani et al., 2005a) suggesting that virus-related immuno-pathology can be influenced by the presence of potentially cross-reactive T cells in humans also. The demonstration that all HCV-negative adults present at least one HCV-peptide reactive T cell response suggests that cross-reactivity can potentially influence the degree of immuno-pathology in HCV infection. Moreover, rapid expansion upon HCV exposure of memory responses originally primed by unrelated pathogens may
favour protection against HCV if cross-reactivity leads to expansion of T cell specificities which are normally involved in HCV control.

The identical quantitative detection of HCV-peptide reactive T cells in HCV-negative patients with or without risk factors suggests that HCV peptide reactive T cells from sexual partners of HCV chronic patients are not necessarily primed by HCV.

Since we cannot distinguish between HCV-primed or HCV cross-reactive T cells, the potential protective effect of isolated HCV-peptide reactive T cells found in sexual partners of patients with chronic HCV infection is questionable. Cross-reactive T cells detected in vitro may be primed in vivo by unrelated pathogens and simply recalled in vitro by HCV epitopes irrelevant to virus control (since they are not generated by HCV antigen processing during natural infection). However, our data do not contest the idea that low level HCV inoculation can prime an HCV-specific cellular immune response. It is noteworthy that when HCV contact is more likely to occur (i.e. sexual partners of patients with acute HCV infection and high viremia) (Kamal et al., 2004) these subjects develop a multispecific CD4+ and CD8+ response, a profile different from the oligo-reactivity found in our HCV-negative subjects.

A further interesting observation revealed by our comprehensive analysis of HCV-peptide reactive T cell response in different groups of HCV infected and non-infected individuals is that the overall quantity and the individual ex vivo frequency of HCV-peptide reactive T cells is similar and frequently even lower in chronically HCV infected patients than in HCV-negative adults. This data further supports the evidence that
persistent HCV infection qualitatively alters the circulating and intrahepatic pool of HCV-specific T cells (Accapezzato et al., 2004; Lauer et al., 2004; Spangenberg et al., 2005). To what extent HCV–peptide reactive T cells not found in chronically infected patients are deleted, compartmentalized within the liver or suppressed in their functional capacity needs further investigation.

In conclusion, this study illustrates that HCV peptide reactive T cells can be demonstrated in adults irrespective of previous HCV exposure. Therefore, the presence of such isolated HCV-peptide reactive T cells does not necessarily equate to previous HCV exposure since cross-reactivity between HCV peptides and sequences of common pathogens, like HHV 1, was revealed. However, cross-reactivity instead of previous HCV priming does not exclude the potential for protection of cross-reactive T cells upon HCV exposure, provided the homologous HCV epitopes are involved in the induction of protective responses in natural infection. The demonstration that the comprehensive magnitude of HCV-peptide reactive T cells present in chronically HCV infected patients might even be lower than that of HCV-peptide reactive T cell response found in HCV-negative adults, adds further support to the argument for a profound collapse of virus-specific T cell response caused by HCV persistence.
CHAPTER 4

THE ROLE OF NKG2D MOLECULE ON HUMAN INTRAHEPATIC CD8+ T CELLS IN CHRONIC VIRAL HEPATITIS
4.1.1 INTRODUCTION

Chronic viral hepatitis (HBV & HCV) is characterised by a lymphocyte infiltration into the infected liver and progressive liver injury that evolves from chronic inflammation to the development of fibrosis, cirrhosis and hepatocellular carcinoma (Beasley, 1988; Lok and McMahon, 2001; McQuillan et al., 1989; Seeff, 2002; Seeff et al., 2000). It is now believed that the outcome of viral hepatitis is determined by the host’s ability to mount efficient helper and cytotoxic T cell responses (Bertoletti and Maini, 2000). Many studies have investigated the population of intrahepatic lymphocytes (IHL) with greater emphasis on CD8+ T cells (Ferrari et al., 1990; Grabowska et al., 2001; He et al., 1999; Maini et al., 2000), but despite the volume of experimental data, the modulation of this CD8+ T cell population remains poorly defined.

It is now clear that CD8+ T cells control virus replication through both cytolytic and non-cytopathic mechanisms, mediated by IFN-γ and TNF-α (Guidotti and Chisari, 1996). The magnitude of the virus-specific CD8+ T cell response is considered to be the determining factor in virus control, while the quantity of HBV-specific CD8+ T cells has been shown to correlate with viral control and not liver damage (Maini et al., 2000). However, when the virus-specific CD8+ T cell response fails to control virus replication it may contribute to liver pathology through direct hepatocyte lysis and the recruitment of large numbers of non-virus specific CD8+ T cells to the liver (Ando et al., 1993; Kusters et al., 1996; Maini et al., 2000; Tiegs et al., 1992). The virus-specific CD8+ T cell response is diluted as a result of the massive cellular infiltration and it is this non-virus specific lymphocytic
infiltrate which is implicated in liver damage and inflammation in patients with persistent viral replication. The large non-virus specific component of the CD8+ T cell infiltrate demonstrated in chronic HBV patients with active liver disease is consistent with previous reports in animal studies. The recruitment of non-virus specific CD8+ T cells is mediated by IFN-γ in the transgenic mouse model of fulminant hepatitis (Ando et al., 1993) and similar work in HBV infected chimpanzees report liver damage to occur concomitantly with a massive infiltration of non-antigen specific CD8+ T cells (Guidotti et al., 1999).

Experimental work to date on IHL has focused primarily on the frequency and function of virus-specific CD8+ T cells (Ferrari et al., 1990; Grabowska et al., 2001; He et al., 1999; Maini et al., 2000). It has now been established that virus-specific CD8+ T cells are preferentially recruited to the infected liver (He et al., 1999; Maini et al., 2000), however these intrahepatic virus-specific CD8+ T cells are functionally defective in chronic hepatitis patients with an inability to produce antiviral cytokines (IFN-γ) and in some instances produce the regulatory cytokine IL-10 (Accapezzato et al., 2004). It is noteworthy that the reduced functional ability of effector CD8+ T cells in chronically HCV infected livers also extends to non-virus specific CD8+ T cells (Nisii et al., 2006). While these studies provide greater insight into the function of CD8+ T cells and their contribution to liver injury and viral persistence, little is known regarding the activation requirements of IHL.
This study sets out to dissect the possible mechanisms of liver injury mediated by non-virus specific CD8+ T cells and the factors that govern it. We investigated the activation requirements of intrahepatic CD8+ T cells in chronic viral hepatitis with particular emphasis on factors that modulate the classical antigen dependent T cell response. Furthermore, we explored the possibility of TCR independent CD8+ T cell activation and how this might function in chronic viral hepatitis. We investigated the role of the activating NK cell receptor, NKG2D, which is known to be present on CD8+ T cells, and analyse its impact in both TCR dependent and independent processes and its overall contribution to the immunopathology of viral hepatitis.

4.1.2 Classical TCR activation

CD8+ T cells are classically activated through their clonotypic T cell receptor (TCR) by MHC class I-peptide molecules (Figure 4.1). Naïve CD8+ T cells require two independent signals to become fully activated. The first is an antigen specific signal, sent via the TCR. The second signal, termed co-stimulation is independent of the antigen receptor and is critical to allow full T cell activation, sustain cell proliferation, induce differentiation to effector and memory status and allow cell-cell cooperation (Frauwirth et al., 2002; Frauwirth and Thompson, 2002).
Naïve lymphocytes stimulated through the TCR alone,

1) fail to produce cytokines
2) are unable to sustain proliferation
3) often undergo apoptosis or become non-responsive to subsequent stimulation.

Co-stimulation refers to a critical event in the activation process whereby interactions between receptor/ligand pairs on the responder lymphocyte and an "accessory cell", an antigen presenting cell (APC) in the case of T cell activation, serves to amplify antigen specific signaling through the TCR. CD28, the first cell surface molecule shown to have
co-stimulatory function (June et al., 1990) and the related inducible co-stimulator (ICOS) molecules appear to be the major co-stimulatory molecules for the activation of naïve/resting T cells or activated/effecter T cells respectively (Mueller, 2000). CTLA-4 and PD-1 share structural homology with CD28 and ICOS, but are inhibitory receptors and therefore are not described as co-stimulatory (Frauwirth et al., 2002; Frauwirth and Thompson, 2002).

More recently a new family of receptors previously thought to be restricted to NK cells, have been identified on effector and memory CD8+ T cells (Figure 4.2). These receptors, the natural killer group 2 D (NKG2D) in particular, have been intensely studied and are reported to play a key role in immune mediated diseases (Groh et al., 2003; Jabri et al., 2000; Ogasawara et al., 2004). NKG2D is an activating receptor on NK cells, CD8+ T cells and γδ-TCR+ T cells (Bauer et al., 1999; Jamieson et al., 2002; Wu et al., 1999), and experimental data supports an increasing relevance of these receptors in the activation of CD8+ T cell function. In addition, NKG2D has been shown to be important in T cell mediated, TCR independent epithelial damage in coeliac disease (C.D.), which resembles liver damage in chronic viral hepatitis. Both conditions are associated with a massive CD8+ T cell infiltrate and the resulting pathology is believed to be mediated in an antigen non-specific manner. In this chapter I set out to define what role NKG2D plays in the immunopathogenesis of chronic HBV and HCV infection.
NKG2D is an activating receptor expressed on effector CD8 T cells and interacts with its ligands MICA and MICB.

**Figure 4.2**: T cell activation; the classical pathway (TCR mediated) or alternatively activation through the NKG2D receptor.

### 4.1.3 The activating receptor, NKG2D

In order to understand the potential involvement of NKG2D in the activation of virus specific and non-virus specific CD8+ T cells in chronic viral hepatitis (Figure 4.3), we will first review the molecular biology of the NKG2D molecule.

*In humans NK receptors are divided into 3 groups,*

1) killer immunoglobulin receptors (KIR)
2) natural cytotoxicity receptors (NCR)
3) c-type lectin Receptors.
NKG2D is a member of the c-type lectin-activating receptor family. It differs from the other NKG2 members (NKG2A, NKG2C, and NKG2E) which are highly related in sequence, are present as heterodimers with CD94 and recognize a non-classical MHC-class I molecule known as HLA-E in humans (Braud et al., 1999). NKG2D is a promiscuous receptor which binds at least 6 different ligands including a family of cell surface glycoproteins with structural homology to MHC class I proteins, MHC class-I-related chain (MIC) A and MIC-B, and UL-16 binding proteins (ULBP) also known as RAET1 (Verneris et al., 2004).

![Diagram](image)

**Figure 4.3:** NKG2D providing activating or co-stimulatory signals to the CD8+ T cell.

NKG2D exists as a transmembrane glycoprotein expressed as a disulfide-bonded homodimer. It has no signaling motifs in the intracellular region, (Houchins et al., 1991)
but instead associates non-covalently with the adapter protein DAP 10 in mouse and human NK and T cells to form a hexameric complex on the cell surface. Mouse and human NKG2D differ, in that signaling occurs exclusively through DAP 10 in humans but can also occur through DAP 12 in activated mouse NK cells (Rosen et al., 2004). The cytoplasmic domain of DAP 10 contains a YxxM motif. This is noteworthy as the YxxM motif is also found in the cytoplasmic domains of the CD28 and ICOS molecules where it is reported to impart a co-stimulatory signal (Chambers, 2001; Chambers et al., 2001), supporting the role of NKG2D as a co-stimulatory molecule on CD8+ T cells, akin to that of CD28.

The YxxM motif recruits and activates the p85 subunit of phosphoinositide-3 (PI3) kinase and Grb2. This differs from DAP 12 which has an immunoreceptor tyrosine based activation motif (ITAM) in its cytoplasmic domain that recruits and activates the Syk and ZAP 70 protein tyrosine kinases (Lanier et al., 1998). Therefore it can be deduced that the NKG2D activity may well differ between mice and humans owing to this fundamental difference in individual activation pathways.

NKG2D activity may also differ between mice and humans owing to reported differences in the ligands, with the high affinity ligands retinoic acid early inducible 1 (RAE 1) family of glycoproteins, H60 and murine ULBP-like transcript 1 (MULT1) being identified in mice (Cerwenka et al., 2000; Diefenbach et al., 2000). The MICA, MICB, ULBP, RAE-1 and H60 genes are polymorphic and so within the human population or in different strains of mice, there is considerable diversity. NKG2D ligands in mice are
reported to be silent in normal, healthy adult tissues with the exception of \textsc{mult1} mRNA which is reported to be widely detected (Ogasawara and Lanier, 2005). MIC A/B ligands are stress inducible molecules (Groh \textit{et al.}, 1996) and their expression on the cell surface is under the control of a heat shock promoter which can be induced by cellular stress, including heat shock, transformation or infection (both viral and bacterial) (Das \textit{et al.}, 2001; Groh \textit{et al.}, 2001). Expression of the ligands can also be induced by the pathways involved in cellular responses to DNA damage and in human and mouse autoimmune diseases (Ogasawara and Lanier, 2005).

\subsection*{4.1.4 NKG2D and tumour immunity}

NKG2D ligands are frequently expressed on a substantial number of human and mouse tumours and could potentially act as danger signals to alert the immune system to the existence of these abnormal cells (Cerwenka \textit{et al.}, 2000; Diefenbach \textit{et al.}, 2000). This could provide a first line of defence against certain cancers, as tumours are known to be poorly immunogenic as they develop from self cells and so are inefficiently recognized by the adaptive immune system. Human NK cells and \(\gamma\delta\text{-TCR}^+\) T cells have been shown to efficiently kill tumours bearing NKG2D ligands (Bauer \textit{et al.}, 1999; Groh \textit{et al.}, 1999).

Furthermore the expression of NKG2D ligands on tumours has been reported to augment the generation of tumour-specific T cells (Diefenbach \textit{et al.}, 2001). The possibility that immunotherapies could be used to influence the outcome of such tumours has also been
raised by the reported enhanced NKG2D anti-tumour effects seen after the administration of IL-12 with resulting prevention of carcinogen induced sarcoma formation in a mouse model (Smyth et al., 2005).

The study of NKG2D in cancers has given further insight into its role, but also its limitations. Cancer patients with high concentrations of soluble MIC proteins in their serum were found to have reduced NKG2D expression on the CD8+ T cells (Groh et al., 2002). The chronic exposure of NK cells or CD8+ T cells to soluble MIC proteins arising from tumour cells or cell surface NKG2D ligands leads to functional impairment of NKG2D dependent activation (Groh et al., 2002; Ogasawara and Lanier, 2005; Oppenheim et al., 2005). Such prolonged exposure to the NKG2D ligands may desensitize the NK cells and CD8+ T cells and in doing so lose the anti-tumour effect.

4.1.5 NKG2D and viral immunity

NK cells kill targets which have lost or express insufficient amounts of MHC class-I (Moretta et al., 2002). This is a frequent event in tumour- or virus-infected cells and supports a role for NKG2D in the recognition of such cells. However, there are reported differences in how NKG2D functions in different cells types. Unlike NK cells, cross-linking NKG2D on antigen-specific cytotoxic T lymphocyte (CTL) clones does not induce cytokine production, calcium flux or trigger cytotoxicity (Jamieson et al., 2002). However, NKG2D signaling is reported to augment cytotoxic and proliferative responses
of T cells on antigen encounter, and so qualifying it primarily as a co-stimulatory molecule (Groh et al., 2001).

Cytomegalovirus (CMV) is of particular interest in this scenario. Spies and colleagues reported that NKG2D can serve as a co-stimulatory molecule on CMV-specific CD28-CD8+ T cells (Groh et al., 2001). Furthermore, they reported the expression of MIC proteins on the lung tissue sections from CMV-infected patients, thus confirming a central role for the NKG2D-MIC pathway during viral infections and T cell activation. Mouse and human studies have also demonstrated how CMV has evolved mechanisms to avoid recognition by NKG2D bearing NK and T cells (Ogasawara and Lanier, 2005). These studies have shown that human CMV (HCMV) is capable of up-regulating MIC in human lung tissue (Groh et al., 2001), but HCMV encodes a UL16 protein that intracellularly retains three of the six human NKG2D ligands (ULBP1, ULBP2 and MIC B) (Dunn et al., 2003; Rolle et al., 2003; Wu et al., 2003). This escape mechanism is highlighted in fibroblasts infected with a UL16-deletion HCMV, which were killed more efficiently than fibroblasts infected with wild type HCMV (Rolle et al., 2003).

MIC-B has been detected on human macrophages infected with Influenza A or Sendai virus and this is reported to be at least partially dependent on virus induced IFNα production (Siren et al., 2004). A further role for NKG2D has been described in mouse hepatitis virus (MHV) (Dandekar et al., 2005) where central nervous system pathology, secondary to the virus, is to some extent a consequence of the NKG2D dependent mechanism mediated by γδ-TCR+ T cells. These studies indicate a role for NKG2D in
immune responses against viral infection and the countermeasures taken by viruses to prevent this surveillance. The importance of this involvement cannot be underestimated when we consider that our understanding of the pathogenesis of viral hepatitis is continuously evolving. In this work I put forward an argument for a possible role for NKG2D in type B and C viral hepatitis.

4.1.6 NKG2D and TCR independent CD8+ T cell activation

The studies discussed so far report the ability of NKG2D to function as a link between the innate and adaptive immune system and demonstrate how NKG2D can modulate TCR signaling, effectively controlling antigen-specific killing (Groh et al., 2001; Groh et al., 2002; Jabri et al., 2000).

This link between innate and adaptive immunity serves to focus CTL killing on transformed targets, minimizing damage to unaltered cells expressing cross-reactive self antigen (Meresse et al., 2004).

Despite convincing data to support this co-stimulatory role, there is growing evidence that T cells could be activated directly through the NKG2D signaling pathway. TCR independent cytolytic activity of CTL cultured with IL-15 (or high doses of IL-2) have previously been reported (Gamero et al., 1995). The intraepithelial intestinal CTL express high levels of NK cell associated receptors and expand on exposure to IL-15 (Jabri et al.,...
2000) and thus represent a mechanism of immunopathogenesis in Coeliac disease (C.D.).

Infiltration by IL-15 (or high dose IL-2) activated CTL to the small intestinal epithelial compartment is associated with massive cell death, supporting a role for TCR independent NK-like killing (Green and Jabri, 2003) in C.D. Neither lower doses of IL-2 (30u/ml) nor IL-7 were capable of arming NKG2D in the same way (Meresse et al., 2004). Further experiments suggested that the differential effects of IL-15 were dependent on source and activation status of the CTL. Naïve and resting memory CD8+ T cells, even after 3 weeks of culture in IL-15, were incapable of arming NKG2D mediated cytolysis. However, stimulation with plate bound anti-CD3 mAb (24hrs) prior to incubation with IL-15 (or high dose IL-2) did arm NKG2D mediated lysis in naïve and resting memory cells (Meresse et al., 2004). Thus IL-15 primes the NKG2D signaling pathway, where its effects are most remarkable in the effector CD8+ T cell population.
4.1.7 Why is coeliac disease important in understanding the immunopathogenesis of chronic viral hepatitis?

The intestinal epithelium in C.D. is the site of a massive TCRαβ+ CTL infiltration, which is thought to contribute to epithelial damage in an antigen-nonspecific manner (Green and Jabri, 2003).

It has been demonstrated that the presence of IL-15 in the tissue microenvironment of the gut epithelium increased the level of CTL NKG2D expression and their ability to kill MIC expressing targets independent of TCR activation. This, in addition to reports that NKG2D and IL-15 reduced the TCR activation threshold of CTL, suggests that the NKG2D signaling pathway might promote deleterious autoimmune responses (Groh et al., 2003; Meresse et al., 2004; Roberts et al., 2001). The presence of IL-15 in the liver (Golden-Mason et al., 2004) coupled with the large infiltration of antigen-nonspecific CD8+ T cells in viral hepatitis make the case for the NKG2D signaling pathway as a previously unrecognized mechanism of liver damage. We will now examine the role of NKG2D in type B and C chronic viral hepatitis and define the mechanisms that control the cytolytic potential of intrahepatic lymphocytes.
4.1.8 The expression and function of NKG2D molecule on intrahepatic CD8+ T cells in chronic viral hepatitis

Chronic viral hepatitis is an inflammatory disease of the liver mainly caused by persistent HBV and HCV infection. The mechanisms causing the destruction of hepatocytes and the associated chronic inflammation are still elusive, though CD8+ T cells are likely to play a leading role over the various cell populations (macrophages, granulocytes, platelets) that are implicated in the chronic inflammatory process. Direct recognition of infected hepatocytes by virus-specific CD8+ T cells is considered to represent the main event responsible for initial virus clearance and hepatocyte damage. This antigen-specific mediated CD8+ T cell activation triggers the liver recruitment of inflammatory cells, which can sustain and amplify the hepatic damage (Bertoletti and Maini, 2000).

However, virus-specific CD8+ T cells are reported to represent, at least during chronic infection, a minority of the intrahepatic CD8+ T cell infiltrate, which is the hallmark of chronic active viral hepatitis (Colucci et al., 1983; Dienes et al., 1987; Yang et al., 1988). Several studies have shown that, both in HBV and HCV related chronic hepatitis (Grabowska et al., 2001; He et al., 1999; Maini et al., 2000), less than 10% of the intrahepatic CD8+ T cells are virus-specific. In addition we demonstrated that, during chronic active hepatitis B, virus-specific CD8+ T cells are diluted among a majority of apparently non-antigen-specific CD8+ T cells, a cell population where the pathogenetic impact has never been comprehensively evaluated (Maini et al., 2000).
An attractive hypothesis on the possible role of the bulk of intrahepatic CD8+ T cells in liver damage can be drawn from studies in coeliac disease (Green and Jabri, 2003; Jabri et al., 2000; Meresse et al., 2004), an intestinal inflammatory disorder induced by dietary gluten in genetically susceptible individuals. Here, gluten-specific T cells are considered the culprits in initiating the inflammatory process, but chronic epithelial damage is maintained by gut infiltrating CD8+ T cells which undergo a genetic reprogramming of natural killer like cell function and can be activated independent of the TCR stimulation.

The TCR-independent activation of intraepithelial CD8+ T cells in coeliac disease was reported to act through the crosslinking of NKG2D, with the ligands MIC-A and MIC-B, expressed by epithelial cells under conditions of stress. This has been reported to be an IL-15 dependent process, and it is noteworthy that IL-15 is produced in the infected liver (Golden-Mason et al., 2004). We thus hypothesized that the bulk of intrahepatic CD8+ T cells characteristic of the cellular hepatic infiltrate during chronic viral hepatitis might be involved in chronic liver damage through a process of CD8+ T cell activation independent of antigen-specific recognition. To investigate this possibility we analysed the expression of NKG2D on freshly isolated CD8+ T cells from chronically infected human livers (HBV & HCV). We also studied the expression of the natural ligands of NKG2D molecules, including MIC-A and MIC-B, on human hepatocytes. We then measured the functional impact of NKG2D and IL-15 on TCR-dependent and independent activation of intrahepatic CD8+ T cells.
4.2 MATERIALS AND METHODS

4.2.1 Intrahepatic and peripheral blood mononuclear cells

Patients undergoing liver biopsy as part of their work-up in the viral hepatitis clinic were invited to partake in the study. After informed consent was obtained, a liver biopsy was performed under ultra-sound guidance. Only biopsies in excess of 1.5cm in length were used for the purpose of the study. 1.5cm of liver tissue was fixed in 10% zinc formalin for histological examination and remaining tissue was analyzed directly ex vivo.

Further specimens were obtained at the time of liver transplantation for end-stage chronic viral hepatitis (HCV, n=12; HBV, n=7). Intrahepatic lymphocytes were harvested from fresh tissue using two different methods. Mechanical disruption of liver tissue and the yield of lymphocytes was performed as described previously (Curry et al., 2000). Briefly, liver tissue was dissected and homogenized through a 70μm nylon cell strainer (BD Falcon, USA) to remove tissue clumps. The filtrate was then washed with PBS at 1800rpm x 10mins and one further wash at 1500rpm x 8mins. The final pellet was resuspended in complete RPMI supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml 2-mercaptoethanol (2-ME).

Alternatively, excess liver tissue not required for diagnostic purposes was extensively washed in RPMI and then digested with collagenase (Sigma, St. Louis, Mo; 1 mg/ml) and DNAse (Sigma, 25 ng/ml) for 1 h at 37 °C. The cell suspension was washed twice and
mononuclear cells were recovered by centrifugation over a Ficoll-Hypaque density gradient.

Patient peripheral blood lymphocytes (PBL) were prepared in parallel by standard density gradient centrifugation as previously described. Briefly, PBMC were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and suspended in RPMI 1640 supplemented with 25 mM Hepes, 2 mM L-glutamine, 50 μg/ml gentamycin and 8% human serum (complete medium) and used immediately in phenotypic analysis or functional studies.

4.2.2 Phenotypic analysis

For phenotypic staining, mononuclear cells (PBL & IHL) were incubated with CD8 PE-Cy5 (BD Pharmingen, San Diego, CA) +/- CD3 FITC (BD Pharmingen, San Diego, CA) and the following antibodies for 30 min on ice; NKG2D-PE, CD28-PE, CD56-PE, IgG1-PE (BD Pharmingen, San Diego, CA). Following incubation cells were washed with PBS three times, resuspended in 2% paraformaldehyde and acquired using FACScan flow cytometer (BD Biosciences, San Diego, CA).

To assess the effect of cytokines on NKG2D expression, mononuclear cells (PBL & IHL) were cultured in; IL-2 (20-300U/ml), IL-7 (20-100ng/ml), IL-15 (20-100ng/ml), MIP-α (10μg/ml), MIP-β (10μg/ml) and IFN-γ (1,000 U/ml) for 24-72 hours. The use of high doses of cytokines in this manner, ie 300U/ml of IL-2, was aimed at generating NK like lines.
4.2.3 Generation of ex vivo expanded and activated T cells (NKG2D+ T cells)

PBMC were isolated from healthy donors by density gradient centrifugation. T cells were activated and expanded as described (Vemeris et al., 2004). Briefly, mononuclear cells were resuspended at 2x10^6 cells/ml in complete RPMI supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml 2-mercaptoethanol (2-ME) at 37°C, 5% CO₂.

On day 0, cells were incubated with IFN-γ (1,000 U/ml; AL-Immunotools) for 24 hours. Cells were then stimulated with anti-CD3 (25ng/ml; Serotec) and recombinant IL-2 (rIL-2; 300U/ml; R+D Systems). Thereafter, cells were stimulated with rIL-2 (300U/ml) every 3-5 days and fresh medium was added to maintain a cell density of approx. 2x10^6 for a total of 14-28 days.

4.2.4 Functional assay

CD107a-PE antibody (BD Pharmingen, San Diego) was used for degranulation assays. Functional assays were set up with plate bound antibody as follows; Isotype control, NKG2D only, CD3 only, CD3+NKG2D or CD3+CD28. Plates were blocked overnight @ 4°C with CD3 (1µg/ml), NKG2D (10µg/ml) or combination either of CD3/NKG2D or CD3/CD28 (CD28 10µg/ml) in 100 µl of 0.05 M carbonate buffer (pH 9.6). Plates were washed three times with RPMI to remove unbound antibody. Mononuclear cells were then transferred to the plate in 100µl complete RPMI plus CD107a PE and incubated for 6-16 hours (Betts and Koup, 2004) in the presence or absence of 20ng/ml IL-15.
Following the incubation, cells were washed and then labelled with Cy-chrome anti-CD8 (incubation 30min on ice) and analyzed by flow cytometry.

4.2.5 Generation of NK clones

NK clones were generated in Dan Davis’s laboratory at Imperial College and kindly provided to us for use in this project.

4.2.6 Purification of primary human hepatocytes

Normal human liver tissue was obtained from healthy tissue surrounding liver metastases following informed consent according to ethical and moral guidelines of the institution.

Tissue sections were collected in William’s E medium and maintained at 4°C for a maximum of 2 hours before cell isolation. Hepatocytes were isolated by enzyme perfusion described for use with human liver by Strain et al with some modifications (Strain et al., 1991). A section of liver of about 100-200g was cut and exposed vessels on a single surface were cannulated with 3 mm internal diameter tubing.

The tissue was perfused sequentially at 50 ml/min with 500 ml PBS-HEPES wash solution to remove William’s E medium, 500 ml PBS-Hepes-0.5 mM EGTA and again with 500 ml PBS-HEPES wash solution. Finally, 300 ml enzyme solution [0.05% collagenase (Roche, Indianapolis, IN), 0.012% hyaluronidase (Sigma, Dorset, UK), 0.025% dispase II (Roche), 0.005% DNAse (Roche) containing 5 mM CaCl$_2$], maintained at 41°C, was perfused with recirculation and enzymatic digestion continued
for 10 - 20 min until the liver was judged to be substantially softened. Tissue was mechanically dissociated in 200 ml HBSS (Invitrogen) containing 10% FBS, 5mM CaCl₂. The cell suspension was filtered through a 60 μM cell strainer and pelleted at 37xg for 10 min at 4°C. Cells were washed 3x in HBSS containing 10% FBS, 5mM CaCl₂ after which viability and yield were assessed. Purified hepatocytes were used immediately after isolation or from frozen stocks thawed from 70% University of Washington solution, 20% FBS and 10% DMSO freezing medium. Isolated hepatocytes did not contain lymphocytes/monocytes as assessed by flow cytometry and appeared greater than 90% pure by microscopic analysis and forward vs. side scatter by flow cytometry. Contamination with some endothelial cells could not be ruled out however, endothelial cell surface markers (VCAM-1, HLA-DR) were undetectable in FACS staining.

4.2.7 Cell Lines and medium

The hepatocyte-like cells, HepG2, used as targets to assess the role of NKG2D as a co-stimulator, were grown and maintained in RPMI 1640 (Autogen Bioclear, Wiltshire, UK) supplemented with 10% heat inactivated FBS, 20 mM Hepes, 0.5 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, MeM amino acids + L-glutamine, MeM non-essential amino acids (Invitrogen Ltd, Paisley, UK) and 5 μg/ml Plasmocin (InvivoGen, San Diego, CA) to prevent mycoplasma contamination.

HBe₁₈⁻₂₇ specific CD8+ T cell clones were generated from resolved HLA-A2+ HBV patients. PBMC were isolated from fresh heparinized blood by Ficoll-Hypaque density
gradient centrifugation. Short term lines were made by stimulating PBMC in Aim-V 2% hABs with 1μM of either HBc\textsubscript{18-27} or HBpolymerase\textsubscript{455-63} peptides + 20 U/ml IL-2 (R&D systems, Abingdon, UK) for 10 days.

HBc\textsubscript{18-27} specific CD8+ T cells were labelled with PE-conjugated HLA-A2 class I pentamers (Proimmune, Oxford, UK) bearing the HBc\textsubscript{18-27} epitope for 30 min at 37°C and purified via magnetic cell sorting using anti-PE microbeads (Miltenyi Biotech, Surrey, UK). CD8+ T cells were then cloned by limiting dilution assay and clonal populations were expanded in Aim-V, 2% hAB serum, 20 U/ml IL-2, 10 ng/ml IL-7, and 10 ng/ml IL-15 (R&D systems, Abingdon, UK) with 1.5 ug/ml PHA (Sigma-Aldrich, Dorset, UK) using allogenic irradiated PBMC as feeder cells.
4.3 RESULTS

CD8+ T cells infiltrating HBV and HCV chronically infected livers express high levels of NKG2D. Freshly isolated intrahepatic and circulating T cells were stained with fluorescent conjugated antibodies and analyzed immediately with flow cytometer. The majority of intrahepatic CD8+ T cells (anti-CD3+ and anti-CD8+) were CD28 negative in both normal and chronically infected livers. All intrahepatic and circulating CD8+ T cells expressed NKG2D molecules, but NKG2D expression was consistently higher in the liver-infiltrating CD8+ T cells than in their circulating counterpart, in all patients with chronic HBV (n=5 subjects) and HCV (n= 8 subjects) infected livers (Figure 4.4a,b). However, no correlation was found between levels of NKG2D expression and ALT values or inflammatory score (Table 4.1).

NKG2D up-regulation in intrahepatic CD8+ T cells was detected in both collagenase treated and mechanically disrupted biopsies, ruling out the purification process employed to harvest IHL, as an explanation for the increased NKG2D expression. NKG2D expression was higher in the CD28- than in the CD28+ population of CD8+ T cells. These data show that intrahepatic CD8+ T cells express higher levels of NKG2D molecules both in HBV and HCV related chronic hepatitis.
Figure 4.4. (a) Histograms representing NKG2D expression on PBL and IHL for normal liver, HBV and HCV infected livers. (b) NKG2D expression CD8+ T cells Δ immune fluorescence (I.F.) (liver-blood). NKG2D is preferentially expressed by intrahepatic CD8+ T cells in chronic viral hepatitis.
<table>
<thead>
<tr>
<th>PATIENT</th>
<th>VIRUS</th>
<th>ALT</th>
<th>PBL NKG2D expression</th>
<th>Liver NKG2D expression</th>
<th>Liver Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HBV</td>
<td>115</td>
<td>23</td>
<td>37</td>
<td>Moderate to severe hepatitis</td>
</tr>
<tr>
<td>2</td>
<td>HBV</td>
<td>80</td>
<td>25</td>
<td>29</td>
<td>Moderate chronic hepatitis, developing cirrhosis</td>
</tr>
<tr>
<td>3</td>
<td>HBV</td>
<td>45</td>
<td>25</td>
<td>55</td>
<td>Cirrhosis, moderate inflammatory activity</td>
</tr>
<tr>
<td>4</td>
<td>HBV</td>
<td>24</td>
<td>13</td>
<td>39</td>
<td>Marked fibrosis, mild inflammatory activity</td>
</tr>
<tr>
<td>5</td>
<td>HBV</td>
<td>66</td>
<td>22</td>
<td>30</td>
<td>Cirrhosis, moderate inflammatory activity</td>
</tr>
<tr>
<td>6</td>
<td>HBV</td>
<td>62</td>
<td>19</td>
<td>42</td>
<td>Mild hepatitis, developing cirrhosis</td>
</tr>
<tr>
<td>7</td>
<td>HCV</td>
<td>60</td>
<td>18</td>
<td>30</td>
<td>Mild chronic hepatitis, mild fibrosis</td>
</tr>
<tr>
<td>8</td>
<td>HCV</td>
<td>64</td>
<td>38</td>
<td>47</td>
<td>Mild chronic hepatitis, mild fibrosis</td>
</tr>
<tr>
<td>9</td>
<td>HCV</td>
<td>42</td>
<td>21</td>
<td>32</td>
<td>Cirrhotic liver, moderate chronic hepatitis</td>
</tr>
<tr>
<td>10</td>
<td>HCV</td>
<td>95</td>
<td>27</td>
<td>35</td>
<td>Moderate lymphocytic infiltrate, mild fibrosis</td>
</tr>
<tr>
<td>11</td>
<td>HCV</td>
<td>49</td>
<td>14</td>
<td>33</td>
<td>Cirrhosis, moderate inflammatory activity</td>
</tr>
<tr>
<td>12</td>
<td>HCV</td>
<td>66</td>
<td>21</td>
<td>46</td>
<td>Cirrhosis, mild inflammatory activity</td>
</tr>
<tr>
<td>13</td>
<td>HCV</td>
<td>57</td>
<td>24</td>
<td>37</td>
<td>Cirrhosis, moderate inflammatory activity</td>
</tr>
<tr>
<td>14</td>
<td>HCV</td>
<td>68</td>
<td>17</td>
<td>31</td>
<td>Mild fibrosis, non specific chronic inflammation</td>
</tr>
<tr>
<td>15</td>
<td>HCV</td>
<td>74</td>
<td>22</td>
<td>40</td>
<td>Chronic hepatitis, mild fibrosis</td>
</tr>
</tbody>
</table>

Table 4.1. Patient characteristics. NKG2D expression (I.F.) correlated with ALT values and histopathology.
4.3.1 The exclusive modulation of NKG2D expression by IL-15

IL-15 has been shown to enhance the surface expression of NKG2D molecules while other cytokines like TNF-α, IL-7, IL-10 and IL-12 have no detectable effect (Roberts et al., 2001).

We analyzed whether, in addition to IL-15 (Kakumu et al., 1997), other cytokines (IFN-γ, IP-10, MIP-α, MIP-β) known to be present in chronic viral hepatitis might influence NKG2D expression.

As shown in figure 4.5, only exposure to IL-15 rapidly increases the NKG2D expression in peripheral and intrahepatic CD8+ T cells. IP-10, MIP-α and MIP-β have no effect and only modest effects were detectable with IFN-γ.

These results suggest that the selected up-regulation of NKG2D molecules found in intrahepatic CD8+ T cells of HBV and HCV infected livers might be preferentially mediated by IL-15.
**Figure 4.5.** IL-15 enhances NKG2D expression on CD8+ T cells over time. The maximum effect was at 48 hours *in vitro* culture.

### 4.3.2 NKG2D-mediated activation of intrahepatic CD8+ T cells

Cross-linking of NKG2D molecules on CD8+ T cells does not normally induce T cell activation. However, recent reports have shown that CD8+ T cells maintained in culture in particular conditions or present in tissue with aberrant IL-15 production can be directly activated by NKG2D signalling alone. Because of the high NKG2D expression detected on liver-infiltrating CD8+ T cells present in chronic viral hepatitis (both type B and C), we investigated whether these cells can be directly activated by NKG2D cross-linking.
Activation of CD8+ T cells was tested by measuring CD107 expression, a marker of T cell degranulation in CD8+ T cells stimulated by plate-bound antibody. NKG2D is capable of directly stimulating NK-cells, but failed to directly stimulate peripheral T cells expressing high levels of NKG2D molecules after IL-15 incubation. (Fig 4.6a, b).

More importantly, we could not detect any direct in vitro NKG2D-mediated activation of intrahepatic CD3+CD8+ T cells purified from HBV (n=8) and HCV (n=12) infected livers. Experiments were performed both in the presence and absence of IL-15 (20ng/ml). Intrahepatic T cells had the ability to efficiently increase CD107 expression after anti-CD3 antibody mediated stimulation, on the contrary cross-linking with anti-NKG2D antibody alone was insufficient to activate the degranulation pathway in intrahepatic T cells purified from HBV and HCV infected livers. (Figure 4.6c, d).
Figure 4.6. CD107 degranulation assay was used to measure the direct activation through the NKG2D receptor of NK cells, NKG2D+ T cells, intrahepatic T cells (HBV & HCV).

4.3.3 NKG2D molecule: a co-stimulatory effect

Several studies have demonstrated that NKG2D molecules can act as co-stimulatory molecules for both mouse and human CD8+ T cells. To characterize whether NKG2D can act as a potential costimulatory molecule in the CD8+ T cell response against infected hepatocytes, we first studied its effects on activation of HBV-specific CD8+ T cells clones selected from patients with resolved hepatitis B infection. The core 18-27
specific CD8+ T cell clone, used for the functional experiments, presents a phenotype compatible with fully differentiated effector CD8+ cells (CCR7 negative, CD62L-) and express NGK2D molecules to values similar to the bulk of intrahepatic T cells (Figure 4.7a).

**Core 18-27 Expression of NKG2D**  **Polymerase Expression of NKG2D**

![Histograms showing NKG2D expression on T cell clones](image)

*Figure 4.7a.* NKG2D expression on the T cell clones (core 18-27 & Polymerase). Filled histogram represents NKG2D expression, shown in the presence of an isotype control.

To test the effect of NKG2D ligation on the activation of core 18-27 specific CD8+ T cells, HLA-A2+ HepG2 cells were pulsed with different concentrations of core 18-27 peptide. After removal of unbound peptide with extensive washing, the peptide pulsed hepatocyte-like cells were mixed with CTL clones at an effector to target ratio of 1:1 in the presence of 1 or 5 μg/ml of anti-NKG2D antibodies or isotype control antibodies. Figure 4.7b shows that the addition of NKG2D antibodies inhibits substantially CD8+ T cell degranulation, particularly in the presence of limiting amount of peptide.

These results suggest that NKG2D molecules can act as a costimulatory molecule during the recognition of virus-infected hepatocytes by virus-specific effector CD8+ T cells.
Figure 4.7b. NKG2D co-stimulates HBV-specific CD8+ T cells in vitro. HepG2 cells known to express MIC-A/B are pulsed with core 18-27 peptide, co-cultured with core 18-27 CD8+ T cell clones in the presence of the NKG2D blocking antibody.

We then tested whether NKG2D molecules were able to co-stimulate the TCR-dependent activation of intrahepatic CD8+ T cells. Intrahepatic T cells were incubated overnight in wells coated with anti-CD3 alone, anti-CD3 and anti-NKG2D, or isotype control antibodies in the presence of IL-2 or IL-15. Anti-CD3 dependent activation of intrahepatic T cells was co-stimulated by NKG2D cross-linking only in the presence of IL-15 (Figure 4.8).

These results support the possibility that NKG2D molecules can act as co-stimulatory molecules on intrahepatic T cells, depending on the cytokine milieu present within the liver tissue microenvironment.
Figure 4.8(A), (B). NKG2D co-stimulates intrahepatic CD8+ T cells in the presence of IL-15. CD107 expression is measured as mean fluorescence (M.F.) intensity. X axis represents the various antibodies used.
Intra-hepatic T cells stimulated with:

- Isotype Ab
- Anti-CD3 Ab
- Anti-CD3 Ab + Anti-NKG2D Ab

Figure 4.8c. IHL (HBV) exhibiting NKG2D co-stimulation in the presence of IL-15.

Intra-hepatic T cells stimulated with:

- Isotype Ab
- Anti-CD3 Ab
- Anti-CD3 Ab + Anti-NKG2D Ab

Figure 4.8d. IHL (HCV) exhibiting NKG2D co-stimulation in the presence of IL-15.
4.4 DISCUSSION

In an attempt to further investigate the potential contribution of intrahepatic CD8+ T cells in liver damage we set out to analyse in this study whether liver infiltrating CD8+ T cells present phenotypic and functional characteristics of NK-like cells. The phenotypic comparison of NKG2D expression on intrahepatic and circulating CD8+ T cells demonstrates that intrahepatic cells in both HBV and HCV chronically infected patients display a consistently higher NKG2D expression.

It is likely that such up-regulation is mediated by the presence of intrahepatic IL-15. Among the different cytokines that are present in the liver during chronic inflammation (IFN-γ, MIP-α, MIP-β, IP-10 and IL-15) we demonstrated that only IL-15 has the capacity to up-regulate NKG2D expression in peripheral blood. IL-15 does not only up-regulate NKG2D expression, in addition its production in the intestinal epithelium of patients with coeliac disease is instrumental in T-cell receptor independent T cell activation of NKG2D expressing T cells.

However, despite the enhanced phenotypic expression and the production of IL-15 in infected human livers, we could not demonstrate T-cell receptor independent T cell activation of intrahepatic NKG2D expressing T cells. Freshly purified intrahepatic T cells failed to be directly activated by cross-linking with anti-NKG2D antibodies, while this assay was robust enough to activate classical NK-cells. Furthermore, we found no correlation between NKG2D expression and ALT values, an indirect marker of the
possible involvement of NKG2D-expressing CD8+ T cells in liver damage. However, the potential limitations of our methods need to be underlined before excluding the possibility that intrahepatic T cells are not endowed with NK-like features.

The purification of intrahepatic T cells through enzymatic means might have altered the responsiveness of the cells mediated by NKG2D alone. Harvested intrahepatic T cells were viable and could be activated by anti-CD3 cross-linking, however NKG2D signals through a different pathway, involving DAP-10 adaptor protein, which might be more sensitive to the robust T cell handling involved in intrahepatic T cell purification. More importantly, our NKG2D mediated activation assay, involves exclusive NKG2D cross-linking, since anti-NKG2D antibodies were directly bound to the plates.

In contrast, previous reports demonstrate an NKG2D-mediated activation of T cells using redirected cytolytic assays, in which NKG2D antibodies were bound to the Fc receptor of P815 cells. It is possible that adhesion molecules present on the target cells are necessary to stabilize the NKG2D interaction and thus improve the sensitivity of the assays. Consistent with this possibility is the fact that the anti-NKG2D plate-bound activation assay used in our work, activated only 16% of true NK cells.

In contrast to the inability to stimulate CD8+ T cell degranulation alone, engagement of NKG2D increases the CD8+ T cell degranulation of TCR-stimulated cells. This co-stimulatory activity was detected both in the in vitro expanded HBV-specific CD8+ T cells activated by recognition of MHC class I viral peptide complex and in the ex vivo
purified intrahepatic CD8+ T cells activated by anti-CD3 antibody engagement in the presence of IL-15. It is thus likely that NKG2D expression on intrahepatic virus-specific CD8+ cells and the presence of the IL-15 in the intrahepatic microenvironment might play a role in the recognition of virus infected hepatocytes. Hepatocytes express low levels of HLA-class I molecules. NKG2D expression on virus-specific CD8+ T cells and IL-15 might act in concert to lower the activation threshold of effector CD8+ T cells, and permit efficient recognition of hepatocytes expressing lower levels of viral antigens.

The demonstration of increased expression of the NKG2D ligands on virally infected hepatocytes will be a critical step to the progression and development of this work. This is work in progress and although we have detected increased expression of ULBP-3 on HCV infected hepatocytes, this ligand or any of the other NKG2D ligands for that matter were not consistently detected on virally infected human hepatocytes. However, it must be pointed out that our data are too few at this time to draw any concrete conclusions regarding the expression of NKG2D ligands in chronic viral hepatitis. The whole series of NKG2D ligands will require detailed analysis in future studies with particular emphasis on their expression in different pathological conditions (and at various stages of such conditions) in order to fully elucidate the impact of NKG2D expression on CD8+ T cell activation.

In conclusion, in this work we analysed the phenotypic expression and functional role of the NKG2D molecule during chronic viral hepatitis in humans. We report a consistently higher expression of NKG2D molecule on intrahepatic CD8+T cells. However, the
functional significance of this phenotypic finding remains uncertain. Increased NKG2D expression does not seem to allow a T-cell receptor independent, NK-like, CD8+ T cell activation, but does provide a co-stimulatory effect in TCR-mediated CD8+ T cell activation. A greater understanding of the potential pathogenetic role of the intrahepatic CD8+ T cell infiltration present in chronic viral hepatitis is likely to necessitate a more detailed analysis of the NKG2D-mediated CD8+ T cell activation, coupled with a comprehensive study of the expression of NKG2D ligands on purified hepatocytes present in different pathological conditions.
CHAPTER 5

DISCUSSION AND FUTURE WORK
In this Chapter, I address the broader implications of the data presented in this thesis and outline where I envisage this work will go in the future.

Chronic type B and C hepatitis is a major cause of morbidity worldwide. The mechanisms responsible for the development of chronic disease as opposed to viral clearance and lifelong immunity are ill-defined, but it is established that the different components of innate and adaptive immune response contribute to these variable outcomes.

In this Thesis we have primarily focused on the role of T cells in chronic viral hepatitis, which represent a component of adaptive immunity necessary for both viral clearance and disease pathogenesis.

The main body of work presented in Chapter 3 examines the influence of T cell cross-reactivity on HCV-specific T cell responses. We start with the premise, “that no one is naïve”, but instead each individual has a lifelong exposure to numerous pathogens which shapes and moulds their own exclusive T cell repertoire. Such variation in the immunological make-up is likely to contribute to different interpretations of the T cell response but may also influence disease outcome. In keeping with this, the presence of cross-reactive T cell response between common human pathogens and HCV-peptides alter the analysis and the consequent interpretation of the role of HCV-specific T cell response. Studies to date have pursued a line of investigation to detect potential protective HCV epitopes by testing HCV-negative subjects with known risk factors for
HCV infection. The presence of HCV-specific T cells in this HCV-negative population has been assumed to represent a "protective T cell response". We argue that this is not necessarily accurate, as our data demonstrates that HCV-peptide specific reactive T cells can be the result of cross-reactivity with other pathogens and not exclusively the result of direct HCV priming. However, our data does distinguish between the broad repertoire seen after recovery from acute hepatitis C, and the narrow cross reactive focus we observed.

Importantly, our data support the possibility that cross-reactive T cells can potentially alter the clinical profile of HCV infections. Urbani et al have reported that the presence of a robust and vigorous single CD8+ T cell response directed against a single HCV epitope cross-reacting with an influenza epitope was associated with a severe hepatitis and did not result in viral clearance (Urbani et al., 2005a). This work demonstrates how cross-reactivity might in some selected cases influence the clinical and pathological profile of HCV-disease. In this Thesis the demonstration of cross-reactivity between HCV peptides and sequences of common pathogens, such as human herpes virus suggest that cross-reactivity is not an unusual event. Furthermore, we can deduce that if cross-reactivity is a frequent occurrence, it is almost certainly another variable contributing to the different clinical outcomes of HCV infection.

Lastly, our analysis of the whole HCV-specific T cell repertoire in such diverse groups from non-HCV infected newborns and adults to chronic carriers of HCV has provided further evidence of the ability of the virus to qualitatively alter the circulating pool of
HCV-specific T cells. It was important to demonstrate that the comprehensive magnitude of HCV-peptide reactive T cells present in chronically HCV infected patients is similar and in some cases even lower than that of HCV-peptide reactive T cell response found in HCV-negative adults.

The conspicuous deficit of HCV-peptide specific T cells in chronically HCV-infected patients found in our work confirms the profound collapse of virus-specific T cell responses caused by HCV persistence previously reported by Spangenberg and others (Spangenberg et al., 2005, Accapezzato et al., 2004, Lauer et al., 2004). It is noteworthy that such responses are not necessarily restored with combination antiviral therapy, or in some cases only transiently improved, stressing the limitations of the current drug regimens for the treatment of HCV.

The work presented in Chapter 4 deals with the impact of the activating receptor NKG2D on the modulation of CD8+T cells in chronic viral hepatitis. We asked whether this novel receptor is a player in the mediation of liver damage in hepatitis B and C viral infection. If so, can the role of this receptor be more clearly defined to enable us to manipulate such factors for the treatment of chronic viral hepatitis in the future?

In this Thesis, I have focused on the destruction of hepatocytes and the development of chronic inflammation leading to progressive liver damage. I used the work of Bertoletti and Maini as a benchmark to develop one of the central hypotheses of this study. Liver damage is largely mediated by CD8+ T cells and direct recognition by virus-specific
CD8+ T cells is considered to be the main event for initial viral clearance and hepatocyte damage. The dilution of this virus-specific component in the massive CD8+ T cell infiltrate is a hallmark of chronic viral hepatitis. It is this non-virus specific infiltrate which appears to be the variable in chronic disease, with almost equivalent absolute numbers of virus-specific CD8+ T cells in both self limited infection and in chronic hepatitis. The role of this population of non-antigen specific CD8+ T cells is poorly understood. More precisely, what modulates this population of cells and what their pathogenetic impact is, remains unclear. These are the questions we set out to tackle in this work.

Studies in coeliac disease, discussed in detail in this Thesis, provide an exciting line of investigation as to how this population of cells may be implicated in chronic viral hepatitis. It has been reported in Coeliac disease that the intestinal epithelial damage is mediated by the large infiltrate of non-antigen specific CD8+ T cells and that these cells are activated in a TCR-independent manner (Meresse et al., 2004). The requisite for such activation appears to be the right inflammatory environment and more specifically this refers to the presence of IL-15. If we take these conditions and apply them to the virally infected liver, we find the optimum tissue microenvironment to support the activation of a large population of non-antigen specific CD8+ T cells to mediate liver damage in a similar way.

More importantly, the TCR independent activation of CD8+ T cells in Coeliac disease is through the activating receptor, NKG2D. We were able to demonstrate the increased
expression of this activating receptor on the IHL of chronically infected human livers (HBV & HCV) for the first time. Having established this, we then set out to investigate whether these liver infiltrating CD8+ T cells present both phenotypic and functional characteristics of NK cells. Increased expression of NKG2D was found consistently on intrahepatic CD8+ T cells in chronic viral hepatitis. In keeping with previous reports in coeliac disease, we show that it is likely that IL-15 is responsible for this upregulation of NKG2D (Green and Jabri, 2003, Moresse et al., 2004). However, unlike the reports in coeliac disease we could not demonstrate that these conditions in the virally infected liver could support T cell activation of intrahepatic NKG2D expressing CD8+ T cells. This study reports that freshly purified CD8+ T cells could not be directly activated by cross-linking with anti-NKG2D antibodies. Consistent with these findings, NKG2D expression could not be directly correlated with ALT values, a crude marker of NKG2D-expressing CD8+ T cell mediated liver damage.

However, as I have stressed in Chapter 4, this does not preclude a role for NKG2D in the mediation of liver damage in chronic viral hepatitis. We accept that our assay, the CD107a degranulation assay, may not be sufficiently robust and so our methodology has inherent weaknesses, namely the reliance on exclusive NKG2D cross-linking with plate bound anti-NKG2D antibodies. Moreover, it is noteworthy that other work on NKG2D has used the P815 tumour cell line and this is something that we will look at in the future. We will also use this work as a springboard to comprehensively analyse the expression of the NKG2D ligands on virally infected human hepatocytes. This is something we previously investigated, but our data are too limited at this time to draw any firm
conclusions. Future work will focus on the presence or absence of the NKG2D ligands, MIC-A, MIC-B, ULBP-1, 2, & 3 on virally infected hepatocytes, but more importantly we will have to consider the timeframe of such an evaluation. HBV and HCV are dynamic diseases, in a constant state of evolution from liver inflammation to fibrosis and cirrhosis. The detection of such ligands will almost certainly vary with disease state, namely the level of inflammation, the degree of fibrosis and so on. In this respect, our work provides a mere snapshot of ligand expression and indeed the involvement of NKG2D which we will certainly refine in future work.

Furthermore, the data we present in this work does provide evidence that NKG2D has a costimulatory role in chronic viral hepatitis. We could demonstrate the ability of NKG2D to increase CD8+ T cell degranulation of TCR stimulated T cells. This Thesis reports on how NKG2D and IL-15 act in concert to mediate this costimulatory effect and so undoubtedly contribute to liver damage in chronic viral hepatitis. I think this represents a significant line of investigation for the future as we know that therapeutic strategies may be able to focus on the blockage of the NKG2D receptor or even the IL-15 effect and so limit liver damage.

Finally, I conclude that NKG2D has a role in the liver damage associated with chronic viral hepatitis, but its precise level of involvement will need further evaluation. This work has laid the foundation for the further investigation of this important activating receptor, present on IHL of virally infected patients. I would like to take this work further to qualify the role of NKG2D in chronic viral hepatitis and its modulation of CD8+T cells.


Chien, R. N., Liaw, Y. F., and Atkins, M. (1999). Pretherapy alanine transaminase level as a determinant for hepatitis B e antigen seroconversion during lamivudine therapy in
patients with chronic hepatitis B. Asian Hepatitis Lamivudine Trial Group. Hepatology 30, 770-774.


lymphocytes expressing the HLA-E-specific natural killer receptor CD94 in celiac disease. Gastroenterology 118, 867-879.


TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. Immunity 21, 357-366.


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human immunodeficiency virus/hepatitis B virus-coinfected individuals for whom interferon-alpha and lamivudine therapy have failed. J Infect Dis 186, 1844-1847.


virus type 1 infection on the natural course of chronic parenterally acquired hepatitis C. Eur J Clin Microbiol Infect Dis 14, 949-953.


Tang, S., Collier, A. J., and Elliott, R. M. (1999). Alterations to both the primary and predicted secondary structure of stem-loop IIIc of the hepatitis C virus 1b 5' untranslated region (5'UTR) lead to mutants severely defective in translation which cannot be complemented in trans by the wild-type 5'UTR sequence. J Virol 73, 2359-2364.


# APPENDIX 1

## HCV Protein

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386. NS4B  GNHVSPTHYVPESDA  1926-1940
387. NS4B  PTHYVPESDAARVT  1931-1945
388. NS4B  PESDAARVTAILSS  1936-1950
389. NS4B  AARVTAILSSLTVTQ  1941-1955
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392. NS4B  LLRRHLGWISSECTT  1956-1970
393. NS4B/NS4A  HQWISSECTTPCGS  1961-1975
394. NS4B/NS4A  SECTTPCGSWLRTI  1966-1980
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