THE ROLE OF THE PROGRAMMED CELL DEATH (PD-1) PATHWAY IN THE IMMUNOPATHOGENESIS OF CHRONIC HEPATITIS B INFECTION

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Doctor of Philosophy (PhD)

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VIRAL HEPATITIS GROUP
UCL Institute of Hepatology
Dedication

To Ellora, Joseph, Mum & Dad

For supporting me and loving me in everything I do.
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Finally, I would like to thank all the patients who willingly participated in these studies and kindly provided their blood and tissues to make these studies possible.
**Abstract**

Chronic hepatitis B (CHB) results from a complex interaction between a replicating non-cytopathic virus and an impaired antiviral host immune response. The Programmed Cell Death (PD-1) pathway is an immunoinhibitory T-cell pathway implicated in virus-specific T-cell dysfunction in several chronic viral infections.

The role of the PD-1 pathway in the immunopathogenesis of chronic hepatitis B was investigated through several different approaches. Firstly, longitudinal changes in PD-1 expression in patients with CHB undergoing oral antiviral therapy was investigated. A direct correlation between viral load and PD-1 expression on virus-specific CD8+ T-cells was observed in this patient cohort, and treatment induced suppression of viraemia resulted in a significant decrease in PD-1 expression on virus-specific CD8+ T-cells with a decrease in HBV-DNA and improvement in virus specific T-cell reactivity.

Secondly, through the employment of a purposely-designed in vitro cell co-culture model of Hepatitis B virus infection the interactions between HBV-producing hepatoma cells (target cells) and HBV-specific CD8+ T-cells (effector cells) was investigated. This model provided evidence that both cytolytic and non-cytolytic CD8+ T-cell effector functions are important in effective control of viral replication, and blockade of the PD-1 pathway distorts the balance between these differential effector functions in vitro.
Finally through the transfection of a human hepatoma cell line with hepatitis B virus (HBV) and the analysis of hepatoma cell lines that differentially express HBV these studies demonstrate that the Hepatitis B virus itself upregulates PDL1 expression on infected hepatocytes in vitro and in doing so, are able to alter the balance between cytolytic and non-cytolytic CD8+ T-cell effector functions favouring chronicity of infection.

Manipulation of the PD-1 pathway may be a possible mechanism to improve virus-specific host immune responses and allow control of HBV infection. However, these immunotherapeutic strategies require careful application as there is a potential risk of immune-mediated host tissue damage.
Publications and presentations arising from this thesis.

PUBLICATIONS


PRESENTATIONS


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALT</td>
<td>Alanine Aminotransferase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric Bead Array</td>
</tr>
<tr>
<td>cccDNA</td>
<td>Covalently closed circular DNA</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>CHB</td>
<td>Chronic Hepatitis B</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA 4</td>
<td>Cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>EASL</td>
<td>European Association for the Study of Liver Disease</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>ELISpot</td>
<td>Enzyme linked immunospot assay</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scatter</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active anti-retroviral therapy</td>
</tr>
<tr>
<td>HBcAg</td>
<td>Hepatitis B core antigen</td>
</tr>
<tr>
<td>HBcAb</td>
<td>Hepatitis B core antibody</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HBeAg</td>
<td>Hepatitis B e antigen</td>
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<tr>
<td>HBeAb</td>
<td>Hepatitis B e antibody</td>
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<tr>
<td>HBsAg</td>
<td>Hepatitis B surface antigen</td>
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<tr>
<td>HBsAb</td>
<td>Hepatitis B surface antibody</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte antigen</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible co-stimulatory signal</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon Stimulated Gene</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine associated inhibitory motif</td>
</tr>
<tr>
<td>ITSM</td>
<td>Immunoreceptor tyrosine associated shock motif</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LIL</td>
<td>Liver infiltrating lymphocytes</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescent Intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural Killer cell</td>
</tr>
<tr>
<td>NKT cell</td>
<td>Natural Killer T-cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PD-1</td>
<td>Programmed Cell Death 1</td>
</tr>
<tr>
<td>PDL1</td>
<td>Programmed Cell Death Ligand 1</td>
</tr>
<tr>
<td>PDL2</td>
<td>Programmed Cell Death Ligand 2</td>
</tr>
<tr>
<td>PegIFN</td>
<td>Pegylated interferon</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>rIFN</td>
<td>Recombinant Interferon</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TP</td>
<td>Time point</td>
</tr>
<tr>
<td>T-regS</td>
<td>Regulatory T-cells</td>
</tr>
<tr>
<td>TW</td>
<td>Treatment week</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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Chapter 1

Introduction
1. INTRODUCTION

1.1. HEPATITIS B VIRUS BIOLOGY, EPIDEMIOLOGY AND NATURAL HISTORY

1.1.1. Discovery.

In 1965 Blumberg et al. identified an antibody in two haemophiliac patients who had previously undergone multiple blood transfusions. This antibody reacted with an antigen in a single serum sample from their panel, which came from an Australian Aborigine (Blumberg et al 1965). This “Australia Antigen” was subsequently found in patients with a viral hepatitis like illness, and was identified as the hepatitis B surface antigen (HBsAg). Blumberg was awarded the Nobel prize for this discovery in 1977. Further experimentation by London virologist David Dane led to the discovery of the complete hepatitis B virus, known as the Dane particle (Dane et al 1970) present in the blood of patients with hepatitis. The Dane particle is spherical with a diameter of 42nm and is the infectious particle responsible for transmission of infection.


HBV is a hepadnavirus with a 3.2kb partially double-stranded circular DNA representing the viral genome and replicates mainly in hepatocytes. The viral genome encodes several proteins from four overlapping open reading frames (ORF), which use the same DNA template to encode the major viral proteins including the precore and core proteins, the polymerase protein, large, medium and small envelope proteins and the transcriptional regulator (X-protein) (Scaglioni et al 1996).
The structure and organisation of the HBV virion and genome are illustrated in Figures 1 & 2 (Karayiannis 2003, Harrison 2006).

The preS-S ORF consists of three in-phase start codons and a common stop codon encoding three viral envelope proteins; preS1 (L or Large protein), PreS2 (M or medium protein) and S (small protein). The L envelope protein is predominantly found in complete virions, whilst the M and S envelope proteins are found in all forms of viral and subviral particles. The S envelope protein more commonly known as HBsAg is the most abundant of these surface proteins and its detection in serum is used as a marker in the diagnosis of an HBV infection (see Section 1.2. (Diagnosis)). The presence of HBsAg in the serum of a patient for > 6 months following acute infection, defines the presence of chronic hepatitis B infection (McQuillan et al. 1999).

The precore/core ORF is made up of 2 in-phase start codons. Translation from the precore start codon results in a precore polypeptide, which undergoes post-translational modification to produce a soluble protein, the Hepatitis B e antigen (HBeAg). This non-structural protein is conserved and yet is not a pre-requisite either for virus replication or infection. HBeAg’s precise function is unknown but it is thought to play a role in the induction of tolerance, shifting the immune response to an immunotolerant Th2 profile. (Milich et al 2003, Valsamakis. 2007). The presence or absence of HBeAg and antibodies to this protein (anti-HBe antibodies) defines the stage of chronic hepatitis B infection (see Chronic Hepatitis B virus Infection – Section 1.1.5.2.) and is important in the optimum management of these cases (see Treatment – Section 1.5.). Hepatitis B core antigen (HBcAg) is produced following
translation from the second start codon in the precore/core ORF. This forms the
inner core protein shell referred to as the core particle or nucleocapsid (Figure 1).

The polymerase ORF overlaps with the core, envelope and X ORFs. The
polymerase protein consists of a protein primer (or terminal protein (TP)), a spacer, a
reverse transcriptase/DNA polymerase, and an RNase H domain. The X protein is a
potent transcriptional transactivator of many promoters including HBV and cellular
oncogenes. It is not required for viral replication, and has been implicated in
hepatocarcinogenesis (Lupberger et al 2007).
Figure 1: The structure of the HBV virion

(Structure of HBV virion illustrating large, middle and small envelope proteins that are coded for by the pre-S/S ORF (pre-S1, pre-S2 and S regions respectively).)

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Figure 2: Hepatitis B virus genome organisation


Genomic organisation of the hepatitis B virus showing the partially double-stranded DNA and the positions of the direct repeats (DR) 1 and 2, and those of the enhancers 1 and 2 (EN). Also shown are the four open reading frames encoding the relevant viral proteins as indicated, as well as the various RNA transcripts which terminate at a common polyadenylation signal.
**Life Cycle of hepatitis B virus**

The HBV life cycle is illustrated in Figure 3. Following binding with an as yet unidentified cellular membrane receptor on the surface of the hepatocyte, the HBV particle fuses with the membrane and subsequently releases its nucleocapsid into the cytoplasm. During this process the viral envelope proteins are shed and the nucleocapsid migrates to the nucleus of the host cell.

Viral DNA enters the nucleus where it is transformed into a supercoiled covalently closed circular pro-viral DNA molecule (cccDNA) (Tuttleman et al 1986). This process results in the cleavage of the covalently attached viral polymerase from the negative strand and of the oligoribonucleotide sequence from the positive strand. Completion of the positive strand follows, with ligation of both strand extremities to form a covalently closed circular molecule, with subsequent supercoiling of the viral DNA in the host chromatin. The exact sequence of enzymatic processes involved in this process remains unknown.

Viral genome integration is not a pre-requisite for viral replication and the hepatitis B cccDNA is not incorporated into the host genome. The cccDNA has a long half life within infected hepatocytes and acts as a stable intermediate responsible for viral persistence and rebound viraemia when anti-viral therapy has been withdrawn, or following host immunosuppression (organ transplantation, HIV co-infection etc).

The cccDNA acts as a template for the transcription of pregenomic and subgenomic mRNAs which are subsequently translated into viral proteins in the cytoplasm. This transcription from cccDNA is performed by host RNA polymerase and is driven
Figure 3: The lifecycle of the hepatitis B virus

by the HBV core promoter in the nucleus of the host cell. The pregenomic RNA is then encapsidated together with the viral polymerase. Within this newly formed nucleocapsid, the negative DNA strand is synthesized using the pregenomic RNA as a template by the viral reverse transcriptase. Minus strand synthesis then proceeds and the viral pregenomic RNA is progressively degraded by the RnaseH domain of the polymerase. The positive strand is then synthesized from the negative strand (template) by the viral polymerase. Maturation of the nucleocapsid before positive strand synthesis is complete results in the characteristic, partially single-stranded genome. At this stage the capsid is mature and can be assembled with the viral envelope proteins present in the endoplasmic reticulum into a complete virion which can be released from infected cells into the circulation (Sheldon et al 2006).

During viral replication, a lack of proof reading together with a high daily virion production results in a significant error rate (Nowak et al 1996) with viral variants (with multiple base changes) existing within the same host at any given time. The genetically different viral strains concomitantly present in a single cell or individual are termed the viral quasispecies (Carman et al. 1993).

Selection pressures from both the host immune system (endogenous) and antiviral therapies (exogenous) will dictate the predominant HBV quasispecies that exist in an infected individual at any given time point. Viral fitness is determined both by at a cellular level (e.g. viruses which replicate most efficiently will predominate) and at an extra-cellular level (viruses that avoid immune elimination, or develop resistance to anti-viral drugs when a patient is on therapy, will become dominant) (Oldstone 1991).
1.1.3. Epidemiology, transmission and prevention of hepatitis B virus infection.

There are approximately 400 million people worldwide infected with chronic hepatitis B virus (HBV) (Maddrey et al 2000), the majority of whom reside in the developing world (Figure 4). Patients with chronic hepatitis B infection are at risk of developing progressive liver disease (including cirrhosis and hepatocellular carcinoma and it is estimated that worldwide over 200,000 chronic HBV carriers die each year from liver cirrhosis and over 300,000 die from hepatocellular carcinoma (HCC) (Perz et al 2006) making HBV the second commonest carcinogen worldwide after tobacco.

In the U.K. there are currently 180,000 patients with chronic hepatitis B, with 15,000 new cases reported annually. The majority (> 95%) of newly diagnosed cases result from immigration of persons from areas of high prevalence and further immigration patterns will have a significant impact on the prevalence of HBV in the UK (Hahne et al 2004). The lack of specific symptoms often accompanying infection with hepatitis B leads to concerns of an increasing pool of patients who remain undiagnosed with chronic HBV infection. Although efforts are underway with the construction of a national database of patients chronically infected with HBV (the Collaborative Hepatitis B in the UK Study Index – CUSHI-B study) currently there is a paucity of information regarding the true prevalence and genotypic distribution of HBV in the UK.
In areas of highest prevalence (e.g. China & the Far East, HBV is most commonly acquired, either during labour or perinatally (Vall Mayans et al. 1990, Mast et al 1993, Alter 1996) – (Vertical transmission). In areas of low prevalence the majority of transmissions are sexual, between both heterosexual and male homosexual partners (Arima et al 2003; Veldhuijzen et al 2005) (Horizontal transmission). Occupational exposure to contaminated blood and sharing of needles amongst intravenous drug users remains a significant mode of transmission. HBV has been identified in the blood, saliva, semen, vaginal secretions, menstrual blood, sweat, breast milk, tears
and urine of infected individuals (Boag et al 1991). However, many cases of acute HBV occur sporadically where the source of infection is not apparent.

Both active and passive immunisation strategies have been employed to limit viral transmission. Prior to the advent of vaccination, Hepatitis B immunoglobulin was used to prevent perinatal transmission of infection. By the mid-90’s, vaccination was preventing HBV transmission in up to 95% of those completing a course of 3 vaccinations. The current hepatitis B vaccine used in the U.K. contains one of the viral envelope proteins, hepatitis B surface antigen (HBsAg). A course of three of these vaccine injections are given with the second injection at least one month after the first dose and the third injection given six months after the first dose. To establish whether there has been an adequate immune response following vaccination, the levels of antibody to HBsAg (HBsAb) are measured in the blood. Levels above 100 mIU/ml are consistent with a satisfactory immune response and are achieved in approximately 85-90% of individuals. This antibody and concomitant immune system memory provide immunity to hepatitis B infection. The first vaccine became available in 1981.

Taiwan was the first country to employ a mass vaccination programme against HBV in 1984. As a result of this programme both perinatal and horizontal HBV transmission decreased, and the carrier rate was reduced by more than 10-fold (Chen 1996). A significant decrease in the incidence of childhood HCC was also subsequently observed some 10 years later. Now more than 140 countries
worldwide have added the hepatitis B vaccine to their national immunisation programmes and worldwide population-based studies show reductions from 8% or greater to less than 2% in immunised cohorts of children. For his work in initiating the Taiwan mass vaccination programme, D.S. Chen was awarded the International Recognition Award from the European Association for the study of the Liver (EASL) in 2009. In the U.K. infants are not currently routinely vaccinated against hepatitis B infection.

**Epidemiology of Hepatitis B in Africa**

The size and diversity of the African subcontinent, the lack of resources and the dispersion of much of its population across vast rural areas makes accurate determination of HBV carrier rates very difficult. However, epidemiological studies suggest that there are approximately at least 50 million people with chronic hepatitis B virus infection in Africa, with an associated 25% mortality. In sub-Saharan Africa, rates of infection range from 9-20%.(Kiire 1996).

Studies suggest that in contrast to the Far East where a high proportion of the population acquires infection perinatally, HBV infection of newborns is relatively uncommon in West Africa. Instead studies report that nearly all infections occur in childhood so that by the age of 10 years, 90% of children have become infected and 20% have become chronic carriers. (Whittle et al 1983, Hall et al 1988).
Important modes of transmission on this continent include percutaneous infection through saliva or traces of blood as well as through unsterile needles and tribal scarification.

1.1.4. Hepatitis B virus genotypes

The hepatitis B virus has been classified according to eight different HBV genotypes (A-H) that differ by 8% at the nucleotide level over the entire genome (Ganem 2001). These genotypes differ in their geographical distribution, with A distributed worldwide, B & C found predominantly in Asia, D mainly in Southern Europe, E mainly in Central Africa, F mainly in South America, G mainly in the USA and France, and H mainly in Central and South America (Lai et al 2003, McMahon et al 2004) (Figure 5). It is important to highlight that whilst these genotypes differ in their historical geographical distribution, migration significantly alters the actual genotypes that may be seen in clinical practice today. For example, more than 95% of new cases of chronic hepatitis B diagnosed in the UK are in immigrant populations, with significant numbers of patients from the Far East and Africa making up the diverse populations treated in the UK with all genotypes represented.

Viral genotype can impact on the replicative ability of the virus, the rate of progression of disease and the response to treatment. An association between HBV genotype and subsequent progression of liver disease has been reported in several studies, mainly in Asia. These studies provide evidence that HBV genotype B is associated with less active and more slowly progressive liver disease compared with

Large, multicentred trials conducted in Europe, Asia and North America have revealed a statistically significant association between viral genotype and response to pegylated interferon therapy ± Lamivudine. Importantly, the highest rates of HBeAg seroconversion was seen in patients infected with genotype A (47%), followed by B (44%), C (28%) and D (25%) (Janssen et al 2005). Further analysis demonstrated HBsAg clearance was also associated with genotype, with again the most favourable outcome seen in genotype A patients (14%), followed by B (9%), C (3%) and D (2%) (Flink et al 2006). Finally, re-evaluation of this cohort 3 years after treatment, revealed that genotype A patients had the most durable response, with 96% having sustained HBeAg-negativity and 58% having developed HBsAg negativity. The same endpoints were obtained in 86% and 14% of patients with genotype B, 67% and 0% of genotype C and 76% and 6% of genotype D patients (Buster et al 2008).

The reasons underlying these differences in virological response to treatment according to genotype remain unclear, but may reflect changes in viral sequences that occur during interferon therapy and their impact on host immune responses.
Figure 5: Worldwide distribution of Hepatitis B virus genotypes


(Whilst this figure demonstrates historical genotypic distribution, migration has profoundly altered the genotypes encountered in clinical practice – see text)
1.1.5. Natural History of hepatitis B virus infection

1.1.5.1. Acute Hepatitis B infection

Following exposure to HBV infection, individuals may mount a vigorous immune response and establish sustained control of infection with antibodies to HBsAg, or fail to achieve adequate control of viral replication with subsequent persistence of viral replication and the establishment of a chronic hepatitis B virus infection. The impact of immunological factors which can dictate whether chronicity of infection occurs are discussed in detail in subsequent chapters. The likelihood of developing chronic HBV is higher when exposed perinatally (90%) or during childhood (20-30%) compared with immunocompetent subjects infected during adulthood (1%) (Ganem et al 2004). The clinical presentation of acute hepatitis B in adults is typically that of an icteric disease, with the jaundice lasting 1-4 weeks. Rarely a fulminant hepatitis may develop (0.1-0.5%) with consequent massive immune-mediated lysis of infected hepatocytes (Berk et al 1970).
1.1.5.2. Chronic Hepatitis B infection

Chronic hepatitis B is defined as the presence of HBsAg in the serum of a patient for >6 months following exposure to the virus. Although the natural history of chronic HBV is complex and is not fully understood, 4 phases of chronic hepatitis B are now recognised (EASL consensus statement 2002, Lok et al 2007):

1) Immunotolerant – This phase is characterised by the presence in the serum of hepatitis B e antigen (HBeAg), high serum levels of HBV-DNA, but normal or minimally elevated serum alanine aminotransferase (ALT). These patients classically have minimal histological activity and scant fibrosis or normal histology on liver biopsy. This phase can last from 10-30 years in perinatally infected subjects, whereas it is short-lived (approx 5-10 years) in childhood-acquired infection and often absent in adult-acquired HBV infection.

2) Immunoactive – After a variable period of HBeAg positivity (immunotolerant phase), depending on the age at acquisition of HBV infection, immune tolerance to the virus is lost and the immune system attacks infected hepatocytes. This phase is characterised by an elevated ALT, fluctuating but in the main decreasing HBV-DNA levels and moderate to severe necroinflammation with variable amounts of fibrosis on liver biopsy (HBeAg+ve chronic hepatitis B).

3) Immunocompetent / Inactive carrier - This immunocompetent phase of infection results from seroconversion from HBeAg to HBeAb positivity,
marking a transition to a third phase of infection characterised by HBeAg negativity and anti-HBe Ab positivity, persistently normal ALT levels and inactive liver histology with very low or undetectable levels of HBV-DNA in the serum.

4) Reactivation phase – Reactivation may occur with wild type virus or much more commonly with replication-competent HBV variants that have mutated such that they do not express HBeAg, thereby avoiding the immune response. This phase is characterised by HBeAg negativity, anti-HBe antibody positivity, detectable, elevated HBV-DNA levels (>2000IU/mL), ALT elevation and significant ongoing necroinflammation of the liver with variable degrees of fibrosis on liver biopsy (HBeAg negative chronic hepatitis).

Inactive HBeAg carriers (phase 3) may over time lose HBsAg with the development of neutralising HBs antibodies. This HBsAg seroconversion results in long-term immune control of HBV, but in the event of immunosuppressive therapy (e.g. chemotherapy for malignancies or following organ transplantation), reactivation of hepatitis B with subsequent liver necroinflammation can occur.

The phase of chronic hepatitis B infection can therefore be categorised according to an individual patient’s serological status, ALT and HBV-DNA levels as shown in Table 1 & Figure 6 (Fattovich et al 2008 (i), Alawazi et al 2008).
Table 1: Recognised phases of chronic hepatitis B infection

<table>
<thead>
<tr>
<th>Phase</th>
<th>Serum ALT</th>
<th>HBeAg</th>
<th>HBeAb</th>
<th>Commonly observed HBV-DNA IU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>Positive</td>
<td>Negative</td>
<td>20 million – 20 billion</td>
</tr>
<tr>
<td>2</td>
<td>Elevated</td>
<td>Positive</td>
<td>Negative</td>
<td>200,000 – 2 billion</td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td>Negative</td>
<td>Positive</td>
<td>&lt;2000</td>
</tr>
<tr>
<td>4</td>
<td>Elevated</td>
<td>Negative</td>
<td>Positive</td>
<td>2000 – 20 million</td>
</tr>
</tbody>
</table>

(adapted from Fattovich G. Natural history and prognosis of hepatitis B. Semin Liver Dis. 2003 Feb; 23(1):47-58.)

Figure 6: Phases of infection in chronic hepatitis B

**HBeAg+ve chronic hepatitis B**

Adult patients with HBeAg positive chronic hepatitis usually present with the disease in the third or fourth decade of life and are more frequently males. The duration of HBeAg positive chronic hepatitis is variable, but can be prolonged and in some cases results in disease progression to liver cirrhosis. However, approximately 65% of patients with chronic hepatitis B infection will eventually undergo HBeAg seroconversion during the course of their infection. This is observed as a loss of HBeAg from the serum and the development of antibodies to this e-antigen (HBeAb positivity). This seroconversion is a favourable outcome and results in a reduction in HBV-DNA levels, normalisation of ALT and associated with this a decrease in the risk of disease progression (Fattovich et al 2008 (ii), Hsu et al 2002). HBeAg seroconversion occurs at an annual rate of 10-15% in adults with HBeAg+ve chronic hepatitis B and elevated ALT levels (Fattovich 2003). A longitudinal study in Italy has reported that 90% of Caucasian adults with chronic HBV will undergo HBeAg seroconversion within 10yrs of follow-up. (Fattovich et al 2008(ii)).

It is established that HBeAg seroconversion, has to be accompanied by normal ALT and HBV-DNA less than $10^4$ copies/ml if regression of fibrosis and inflammation is to occur. (Hui et al 2007).

Higher rates of HBeAg seroconversion are seen with older age, higher ALT levels, HBV genotype B (vs C) and A (vs D) and ethnicity with lower rates seen in the Asian population ( Fattovich 2003, Kao et al 2006, Lok et al 2007).
**HBeAg-ve chronic hepatitis B**

The natural selection pressure on hepatitis B virus following HBeAg seroconversion to anti-HBe can result in the eventual selection of pre-core mutant virions that do not produce HBeAg. In this way the virus evades effective immune control and continues to replicate. The most common mutation that prevents HBeAg production is a guanine (G) to adenine (A) change at nucleotide 1896 (G1896A) that creates a stop codon (at pre-core codon 28) that prematurely terminates synthesis of HBeAg. Other pre-core mutations and mutations in the basic core promoter region have been identified (Hadziyannis et al 2006). Epidemiological studies suggest that selection of these mutants can be influenced by viral genotype. HBeAg negative chronic hepatitis is more common in Southern Europe, where genotype D predominates, and in Asia where both B and C are common. The observed increased prevalence of HBeAg negative chronic hepatitis B probably reflects the aging of existing HBV carriers a proportion of which will undergo pre-core mutations with reactivation of HBeAg-ve chronic hepatitis B.

Patients with HBeAg-ve CHB are in general terms older than patients with HBeAg+ve CHB, have lower levels of HBV-DNA (2000-20 million IU/ml) than patients with HBeAg+ve chronic hepatitis B and have a poorer prognosis with many likely to have cirrhosis at the time of their first presentation (Zarski et al 2006, Hadziyannis et al 2006). They also have wide fluctuations in both HBV-DNA levels and ALT levels.
Inactive HBsAg carrier – Immunocompetent phase of infection

These patients have HBeAg negativity, low or undetectable HBV-DNA levels and normal ALT levels. Furthermore the relative risk of developing HCC is lower than in other phases of infection where a higher HBV-DNA level and necroinflammation of the liver is seen. However, long term longitudinal studies have revealed that 15-24% develop HBeAg-ve CHB and up to 17% have sustained reversion back to HBeAg positivity during the course of their infection (Chu et al 2004, Hsu et al, Fattovich et al (ii) 2008).

Spontaneous HBsAg clearance:

Spontaneous HBsAg loss is observed in approximately 1% per year of Caucasian carriers in the inactive carrier state. HBsAg loss is much less common in endemic areas where infection is acquired perinatally. (Lok et al 2007) Factors associated with HBsAg seroclearance include older age at diagnosis and sustained remission of hepatitis during follow up.

Disease Progression:

It has been observed that male sex, age and high circulating HBV viral loads are associated with increased incidence of cirrhosis and HCC (Fattovich et al 2008 (i), Iloeje et al 2006). The molecular mechanism underlying this observed increased risk in disease progression in men is unknown, but may relate to the anti-fibrogenic effect of oestrogen (Shimizu et al 2003). It has been demonstrated that CHB progresses
more rapidly in males than in females, and cirrhosis and HCC predominate in men and postmenopausal women. Premenopausal women have decreased production of proinflammatory cytokines, and hepatic steatosis has been reported in aromatase-deficient mice, and decreases in animals after estradiol treatment. Estradiol is a potent endogenous antioxidant which modulates induction of redox sensitive transcription factors, hepatocyte apoptosis and suppresses hepatic fibrosis in animal models, suggesting that greater progression of cirrhosis and HCC in men and postmenopausal women may in part be due to lower production of estradiol. (Shimizu et al 2007). A family history of HCC is also associated with an increased risk of HCC suggesting a genetic susceptibility to this disease. (Yu et al 2000).

Repeated severe acute exacerbations with failure to suppress HBV replication predict higher rates of cirrhosis (Liaw et al 1988). Delayed HBeAg seroconversion, indicating a long period of viral replication and necroinflammation are also associated with an increased rate of cirrhosis. Viral factors influencing disease progression include genotype (D more aggressive than A), certain HBV mutations and concurrent infection with HCV, HDV or HIV. Coinfection with HCV will result in liver inflammation and injury, resulting in faster progression of liver fibrosis. Most of the studies of HIV/HBV co-infection pre-date the use of HAART, but it seems that HIV co-infection may also result in more aggressive progression of disease. Heavy alcohol intake is also associated with an increased risk of progression to cirrhosis. Diabetes and obesity may also adversely affect disease progression.
The liver responds to immune-mediated injury through hepatocellular regeneration and the activation of reparative and fibrogenic processes in acute hepatitis B infection. However, in patients with chronic infection, the continuous cycle of low-level hepatocyte necroinflammation leads to fibrosis, cirrhosis and HCC. The pathogenesis of these secondary events involve both host and virus dependent factors that distort normal liver repair functions (Henderson et al 2008).

The rate of liver cirrhosis development depends on many factors including the phase or type of chronic hepatitis B suffered. In patients with HBeAg positive chronic hepatitis the rates are 1.6 and 3.8 per 100 patient years in East Asian and European countries respectively; the corresponding 5 year cumulative incidences of cirrhosis is 8% and 17% (Fattovich et al 2008 (i)). In patients with HBeAg negative chronic hepatitis the summary cirrhosis incidence rates are 2.8% and 9.7% per 100 person years in East Asian and European countries respectively; the corresponding 5-year cumulative incidence of cirrhosis is 13% and 38%. Inactive carriers have lower rates of cirrhosis development with less than 1 per 1000 patient years (Hsu et al 2002).

The incidence of hepatic decompensation with encephalopathy and/or variceal bleeding is 3-4 per 100 patient years in both European and Asian patients with early cirrhosis, with a 5yr cumulative incidence of 15% (Fattovich et al 2008 (i)). Once hepatic decompensation occurs, mortality rates increase to approximately 70% at 5yrs (Fattovich et al 2003).
The rate of development of HCC depends upon levels of HBV replication, the presence/degree of fibrosis/cirrhosis and the geographical area examined. This geographical factor probably relates to the age of infection (Higher frequency with perinatal infection in East Asia). The reported incidence of HCC is illustrated in Table 2.

Table 2: Rates of HCC development according to Phase of Infection and Geographical Area

<table>
<thead>
<tr>
<th>Phase of HBV infection</th>
<th>Geographical Area</th>
<th>HCC incidence per 100 person years</th>
<th>5yr HCC cumulative incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactive Carriers</td>
<td>East Asia</td>
<td>0.2</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>Europe &amp; U.S</td>
<td>0.02</td>
<td>0.1%</td>
</tr>
<tr>
<td>Chronic Hepatitis B without cirrhosis</td>
<td>East Asia</td>
<td>0.6</td>
<td>3%</td>
</tr>
<tr>
<td></td>
<td>Europe &amp; U.S</td>
<td>0.3</td>
<td>1%</td>
</tr>
<tr>
<td>Chronic Hepatitis B with compensated cirrhosis</td>
<td>East Asia</td>
<td>3.7</td>
<td>17%</td>
</tr>
<tr>
<td></td>
<td>Europe &amp; U.S</td>
<td>2.2</td>
<td>10%</td>
</tr>
</tbody>
</table>

(Reproduced from Fattovich G. Natural history and prognosis of hepatitis B. Semin Liver Dis. 2003 Feb; 23(1):47-58.)
Disease Progression and rates of HCC in Africa – the role of aflatoxin:

In the 1960s it was noted following a survey of cancer incidence in the capital city of Mozambique, that there was an exceptionally high rate of H.C.C. even in relation to HBV prevalence (Prates & Torres 1965). Further work demonstrated the hepatocarcinogenic properties of aflatoxin (Newberne & Butler 1969, Carnaghan 1967). Following on from these studies a relationship has been observed between HBV infection and aflatoxin in the development of H.C.C. in sub-Saharan Africa, with mean aflatoxin dietary intake values significantly associated with HCC rates (see Table 3 - Van Rensburg et al 1985). HBV interferes with the ability of hepatocytes to metabolise aflatoxins, therefore an aflatoxin M1-DNA conjugate exists for a longer period of time in the liver, increasing the probability of damage to tumour suppressor genes such as p53. This effect is synergistic with the resulting damage far greater than just the sum of aflatoxin and HBV (Williams et al 2004).
Table 3: Summarised Results of studies measuring crude rates of HCC (male & female) and aflatoxin intake (ng kg\(^{-1}\) body wt day\(^{-1}\)) with cooked food

<table>
<thead>
<tr>
<th>Locale</th>
<th>HCC rate (10(^5)/yr)</th>
<th>Aflatoxin B1 intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kenya High Altitude</td>
<td>1.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Thailand Songkhla</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Swaziland Highveld</td>
<td>2.2</td>
<td>5.1</td>
</tr>
<tr>
<td>Kenya Middle Altitude</td>
<td>2.5</td>
<td>5.9</td>
</tr>
<tr>
<td>Swaziland Middleveld</td>
<td>3.8</td>
<td>8.9</td>
</tr>
<tr>
<td>Kenya Low Altitude</td>
<td>4.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Swaziland Lebombo</td>
<td>4.3</td>
<td>15.4</td>
</tr>
<tr>
<td>Thailand Ratburi</td>
<td>6.0</td>
<td>45.0</td>
</tr>
<tr>
<td>Transkei Four Districts</td>
<td>6.9</td>
<td>16.5</td>
</tr>
<tr>
<td>Mozambique Manhica-Magude</td>
<td>5.9</td>
<td>20.3</td>
</tr>
<tr>
<td>Swaziland Lowveld</td>
<td>9.2</td>
<td>43.1</td>
</tr>
<tr>
<td>Mozambique Massinga</td>
<td>5.0</td>
<td>38.6</td>
</tr>
<tr>
<td>Mozambique Inharrime</td>
<td>9.0</td>
<td>86.9</td>
</tr>
<tr>
<td>Mozambique Inhambane</td>
<td>12.1</td>
<td>77.7</td>
</tr>
<tr>
<td>Mozambique Morrumbene</td>
<td>15.5</td>
<td>87.7</td>
</tr>
<tr>
<td>Mozambique Homoine-Maxixe</td>
<td>17.7</td>
<td>131.4</td>
</tr>
<tr>
<td>Mozambique Zavala</td>
<td>14.0</td>
<td>183.7</td>
</tr>
</tbody>
</table>

HCC incidence vs aflatoxin intake: r=0.8792, p<0.001

(Reproduced from Van Rensburg et al 1985 Br.J.Cancer 51: 713-26)
1.2. DIAGNOSIS

1.2.1. Hepatitis B virus serology

There are three distinct antigen-antibody systems that are significant during HBV infection. Hepatitis B surface antigen (HBsAg) is produced in great excess during viral replication (100-1000 times more than HBV virions, Hollinger et al. 2001). This viral protein is associated with the viral surface coat. Its presence in serum is usually the first evidence of acute hepatitis B infection appearing during the incubation period 1-6 weeks before clinical symptoms develop. The corresponding antibody (HBsAb) appears weeks or months later and persists, thus its detection in the serum indicates past HBV infection and consequent immunity. Failure to clear the virus results in persistence of HBsAg and chronic hepatitis B infection.

Current vaccination strategies for HBV use a surface antigen and therefore patients who have undergone a successful vaccination will have HBsAb detectable in their serum.

Hepatitis B core antigen (HBcAg) is the viral nucleocapsid. It can be found within infected hepatocytes but not routinely in serum. Antibodies to HBcAg (HBcAb) appear in the serum after the onset of clinical symptoms. The presence of HBcAb and HBsAb indicate previous infection, with the presence of IgM anti-HBcAb suggestive of recent infection.

Hepatitis B e antigen (HBeAg) is a secretory protein that results from post-translational modification of the precore protein and is detectable in the serum.
Whilst not required for viral replication or assembly, HBeAg is conserved in HBV wild type virions and is thought to play a role in promoting immunotolerance of infection (Milich et al 1990) – (see Section 1.3.3.1). Over time, tolerance to the HBV infection breaks down and host immune responses lead to reduced virus replication, HBeAg and HBV-DNA serum levels. Ultimately HBeAg may become undetectable with seroconversion to antibody (HBeAb), signalling an inactive carrier phase of infection (see section 1.1.5.2) (Harrison et al 2006). The selection pressure of HBeAb can result in selection of pre-core & core mutant virions that do not produce HBeAg with subsequent escape from immune control and liver injury (see Section 1.1.5.2).

1.2.2. Molecular assays for HBV-DNA quantitation, genotyping and resistance testing.

In addition to the established serological markers of antigen and antibodies described above, molecular quantitation of HBV-DNA levels, viral genotyping and resistance sequencing have improved the assessment of hepatitis B viral infection, allowing more accurate assessment during the diagnosis, treatment and outcome of chronic hepatitis B infection.

**HBV-DNA quantitation**

It is well established that the level of circulating HBV (viraemia) measured by HBV-DNA levels, is a risk factor for the development of cirrhosis and hepatocellular carcinoma. (Chen C. et al 2006, Iloeje et al 2006, Chen G. et al 2006). It is also used
to define phase of infection and also response to treatment (Pawlotsky et al. 2008) (see Chronic hepatitis B infection (Section 1.1.5.2.) & Treatment (Section 1.5.).

Historically there was no internationally accepted assays for HBV-DNA quantification, and assays varied widely from centre to centre, with different sensitivities and also a difference in the units used to describe HBV levels (e.g. copies/ml, megaequivalents/ml and the WHO’s international units (IU/ml). The IU/ml is now the internationally accepted standard both for clinical trials and clinical practice, with 1IU equivalent to 5.82 copies. HBV-DNA levels in this study were quantified using a real-time polymerase chain reaction (Garson et al 2005).

**HBV genotyping**

Hepatitis B virus genotyping is not widespread in clinical practice. However, HBV genotyping can provide valuable information for clinicians and patients in deciding optimum treatment strategies (e.g. in HBeAg positive patients who are considering a course of pegylated interferon therapy). Clinical trials have demonstrated that genotype A & B have a greater than 40% chance of undergoing HBeAg seroconversion following a 48 week course of pegylated interferon, compared with much lower rates observed for genotypes C and D (Janssen et al 2005).

A variety of methods have been used to assess HBV genotype including whole or partial genome sequencing, restriction fragment length polymorphism (RFLP),
genotype-specific PCR amplification and PCR plus hybridisation. At the Health Protection Agency (HPA) which performs all HBV genotyping for University College Hospital (UCH), they use an in-house protocol to amplify the entire HBsAg region and this encompasses domain A to E of the overlapping polymerase region. They then sequence across the region and carry out phylogenetic analysis comparing the generated sequence against known genotypes. On the basis of clustering, it is therefore possible to identify the genotype.

**Antiviral Resistance testing**

With the growing armamentarium of anti-virals and cross-resistance amongst drugs of the same structural class (e.g. lamivudine and entecavir), identification of the mutation conferring drug resistance is an increasingly important part of the management of anti-viral resistance (Lok et al 2007). Resistance can be documented by both phenotypic analysis and/or through methods to detect genotypic resistant mutations. Phenotypic analysis entails assessment of mutant replication in the presence of drug and requires some form of genetic engineering (either site-directed mutagenesis of wild-type sequence or construction of full-length mutant clones expressed in baculovirus models) followed by expression in cell culture systems (Shaw et al 2006). This approach is the most effective means of ascertaining whether a complex set of mutations confers antiviral resistance. However, it is far too cumbersome for standard clinical molecular laboratories and is usually limited to specialized laboratories with a specific interest in antiviral resistance (Chotiyaputtha et al 2009).
In the assessment of genotypic mutations, direct sequencing can identify known and new mutations that potentially confer resistance. However, this method will only detect mutations in the dominant HBV strain and is not sufficiently sensitive for the detection of emerging, resistant mutants that are present in low concentrations (<20% of total HBV quasispecies) (Lok et al 2007). Although these minor populations can be identified by large-scale cloning and sequencing protocols this is beyond the capacity of clinical laboratories. In comparison, hybridization-based methods are more sensitive and less labour-intensive. However only known mutations can be identified with this technique, and individual probes are required to detect each mutation. Furthermore single-nucleotide polymorphisms that have no effect on phenotype can impair probe binding and produce false-negative results (Lok et al 2002). A small number of sequence determination assays are commercially available, including hybridization (biotinylated amplicons hybridized to membrane-bound oligonucleotides specific for each mutation) and direct-sequencing formats.
1.3. IMMUNOPATHOGENESIS

The immune determinants of successful clearance of hepatitis B are complex and as yet remain to be fully elucidated. It is clear however that both cellular and humoral responses play important roles (Rehermann et al 1995, Penna et al 1997, Webster et al 2000). As previously described, chronic infection with Hepatitis B virus can result in persistent necroinflammation of the liver with progression to cirrhosis and liver cancer. However, it is well established that HBV is a non-cytopathic virus, and liver injury with subsequent disease sequelae are largely immune mediated. There is therefore a complex, variable and dynamic interaction between the host immune defences, which attempt to prevent and eradicate infection with minimal host collateral damage, and the hepatitis B virus, which tries to remain undetected and/or to overcome or escape any immunological response directed against it. This interface occurs during the initial clearance of the virus, the long-term persistence of HBV and the pathogenesis of HBV-related liver disease (Bertoletti et al 2003, Rehermann & Nascimbeni 2005, Chang et al 2007).
Figure 7: Immune responses to HBV infection and the effects of chronic hepatitis B infection

The study of the immunopathogenic mechanisms involved in Hepatitis B virus infection has been limited by a lack of available animal models and cell lines that support HBV infection. HBV can infect chimpanzees, but they only acquire a self-limiting acute hepatitis. More recent developments of transgenic mouse models of HBV infection have allowed for a better understanding of the various components of the immune response in the clearance of HBV infection (See Figure 7).

There is a paucity of data surrounding early intrahepatic events following human infection with HBV, but available animal model data suggests that clearance of HBV-DNA is largely mediated by antiviral cytokines produced by cells of the innate and adaptive immune response. Type 1 Interferons (IFN) α/β, Tumour Necrosis Factor (TNF) and IFNg trigger pathways that in turn inhibit viral replication without direct destruction of target cells (infected hepatocytes) (Guidotti et al 1996). Evidence for the relative roles of components of the innate and adaptive immune system in dictating the outcome of acute hepatitis B virus infection are discussed below.
1.3.1. Innate immune response to hepatitis B virus

The innate immune system represents the first line of host defence against viral pathogens. In response to acute infection with hepatitis B virus, it is postulated that hepatocytes produce Type I interferons (IFN α/β). This is thought to be via recognition of highly conserved viral signatures known as pathogen associated molecular patterns (PAMPs) by toll-like receptors on the surface of dendritic cells, with subsequent activation of intracellular pathways such as the RIG-1 pathway, leading to an increase in the transcription of Type 1 interferons (Pichlmair et al 2007). These in turn upregulates several interferon stimulated genes (ISG). The upregulation of these genes has both autocrine and paracrine effects, inducing an anti-viral state in both the infected hepatocyte and the surrounding liver parenchyma. These genes have a variety of functions; in a transgenic mouse model of HBV infection, production of IFN α/β is associated with a 10-fold reduction of viral capsids containing HBV pregenomic RNA and the activation of double-stranded dependent protein kinase activity (PKR), which inhibits HBV protein synthesis (Wieland et al 2000). Type 1 interferons also recruit and mediate the activities of antigen-presenting cells (APCs), in particular Kupffer cells and dendritic cells, which in turn produce IL-18 and CCL3. These cytokines induce natural killer (NK) and natural killer T-cell (NKT) activity.

NK and NKT cells have two main effector mechanisms for the control of HBV infection. They can directly kill infected hepatocytes and they inhibit HBV replication via robust production of anti-viral cytokines e.g. IFNγ / TNFα as observed in
transgenic mouse models of HBV infection (Kimura et al 2002 (i) & (ii), Kakima et al 2000).

These cytokines destabilise viral capsids via NFκB pathway, increase proteosome activity, degrade viral protein via nitric oxide activity and prompt post-transcriptional degradation of HBV-RNA (Visvanathan et al. 2006). Studies from experimentally infected chimpanzees have demonstrated that control of HBV replication is mediated by the early burst of intrahepatic IFNγ production by NK and NKT cells and this is correlated with the development of effective downstream adaptive immune responses. (Guidotti et al 1999).

Kupffer cells are thought to play a major role in both the early innate and adaptive immune response to infection. These cells are known to coordinate recruitment and maturation of HBV-specific T-cells by the synthesis of chemokines and cytokines such as IFNγ, CXCL9 and CXCL10 (Kakimi et al 2001). The activation of these cells by other infections can lead to a cytokine response which effectively will control an ongoing HBV infection – Following infection of HBV transgenic mice with a liver specific malaria strain, Kupffer cells produce cytokines that reduce both malaria and HBV levels (Pasquetto et al 2000).

There is considerable evidence therefore that cells of the innate immune response play an important part in the anti-HBV response that precedes upregulation of HLA
class 1 expression and activation of the adaptive immune response. However, genomic analysis of the host immune response to hepatitis B virus infection in acutely infected chimpanzees demonstrated that viral infection and replication did not result in the upregulation of 2′5′ oligoadenylate synthetase (2′5′OAS) mRNA, which is a protein involved in the innate immune response to viral infection and known to be induced by Type 1 interferons (Wieland et al 2004). As Type 1 interferons are produced as part of the innate immune response, primarily by infected hepatocytes and plasmacytoid dendritic cells, it is postulated that, in contrast to HCV, HBV may act as a stealth virus early in infection, remaining undetected and spreading until the onset of the adaptive immune response with infiltration of the liver parenchyma by virus specific CD3(+) T-cells several weeks later. Wieland and co-authors also demonstrated in a chimpanzee model of acute HBV infection, there was no activation of IFNγ-stimulated genes early in infection, which is primarily produced by NK and NKT cells during the innate immune response. The authors conclude that virus-specific T-cells produce IFNγ, and infection is controlled through both cytolytic and non-cytolytic effector mechanisms of the adaptive immune response. Whether results from these animal models of HBV infection can be translated into man is unclear; one obvious difference is that the chimpanzee model does not develop chronic hepatitis B. Other studies using a different animal model of HBV infection (Woodchucks infected with hepatitis B) did reveal early activation of NK and NKT cells occurring 48-72 hrs after infection (Clifford et al 2008). Furthermore a recent report (Fisicaro et al 2009) demonstrated prompt activation of NK and NKT cells with IFNγ production prior to maximum HBV DNA elevation in 2 patients with newly acquired hepatitis B infection.
Given the T-cell priming and subsequent shaping of the adaptive immune response by the innate immune response, it seems likely that the innate immune response is important in determining the outcome of an acute hepatitis B virus infection.
1.3.2. Adaptive Cellular Immunity

The adaptive immune response is thought to play a crucial role in determining whether chronicity of infection occurs following acute exposure to the hepatitis B virus. It is mediated by a complex interplay between many different cells. Antigen presenting cells (dendritic cells, kupfer cells) orchestrate the virus-specific T-cell responses through presentation of viral antigens, in the context of HLA class I and II to CD8+ and CD4+ T-cells respectively. These antigen presenting cells also produce cytokines which are necessary for maturation, differentiation and activation of virus-specific T-cells.

1.3.2.1. CD8+ T-lymphocyte response

Virus-specific CD8+ T-cells and CD4+ T-cells play key effector and regulatory roles in antiviral immunity. However, virus-specific CD8+ T-cells have also been implicated in the immunopathogenesis of HBV infection. For example, the onset of liver injury in a chimpanzee model of acute HBV infection coincides with the entry of virus-specific CD8+ T-cells into the liver (Guidotti et al 1999, Thimme et al 2003). Furthermore the depletion of these cells (but not CD4+ T-cells) delays the onset of biochemical, histological and clinical evidence of hepatitis (Thimme et al 2003). It has also been observed that when virus-specific CD8+ T-cells are adoptively transferred into immunologically tolerant HBV transgenic mice, the resulting liver injury histologically closely resembles acute viral infection in humans (Ando et al 1994, Guidotti et al 1996).
A robust, virus-specific CD8+ T-cell response targeting multiple epitopes during an acute hepatitis B infection is associated with a more severe degree of hepatitis and liver injury, but is also associated with successful resolution of infection. Conversely a weak CD8+ T-cell response with a narrow repertoire, whilst associated with less liver injury during the acute infection, is also associated with failure to clear HBV infection, with subsequent chronicity of infection (Ferrari et al 1990, Chisari et al 1995, Rehermann et al 1995, Thimme et al 2003). The importance of the CD8+ T-cell response in dictating the outcome of acute hepatitis B infection was elegantly demonstrated by Thimme et al 2003, in studies where depletion of CD8+ T-cells following acute HBV infection of chimpanzees led to failure of viral clearance and subsequent persistence of HBV infection. It has also been observed that patients with chronic HBV infection have a significantly diminished HBV-specific CD4+ and CD8+ T-cell response (Chang et al 2005, Maini et al 2000) to cognate antigens. Patients with HBeAg+ chronic hepatitis and high viral loads, have almost undetectable core epitope (c18-27) specific CD8+ T-cell responses. It has also been demonstrated that individuals with chronic hepatitis B infection, with a high ALT and HBV-DNA levels have HBV-specific CD8+ T-cells with decreased proliferative capacity compared with individuals with low ALT and low HBV-DNA levels (Maini et al 2000).

Virus-specific CD8+ T-cells are primed by viral antigens which have been processed and subsequently expressed bound to MHC class 1 molecules on the surface of professional antigen presenting cells (APCs) e.g. dendritic cells, in the lymphoid tissues (Steinman et al 1999, Sallusto et al 1999). In contrast, if priming occurs
within the liver then it is more likely to result in T-cell inactivation with tolerance and apoptosis of antigen specific CD8 T-cells. This is likely to have evolved to cope with the unique architecture and micro-environment in the liver, where the liver is exposed to many food antigens that are absorbed from the GI tract and travel via the portal system to the liver.

Virus-specific CD8+ T-cells display dual effector functions in vivo; cytolysis of infected cells mediated through FasL and perforin, and the production of anti-viral cytokines (eg. IFNγ, TNFα) which purge HBV from infected hepatocytes (Guidotti et al 1996). The balance between these cytolytic and non-cytolytic CD8+ T-cell functions is vital in the successful clearance of HBV infection without concurrent overwhelming immunopathogenesis and subsequent fulminant hepatitis. Transferring of HBV-specific CD8+ T-cell clones that are genetically deficient in perforin or FasL into transgenic mice has shown that both the perforin and FasL death pathways must be simultaneously activated for CD8+ T-cell mediated killing of infected hepatocytes (Nakamoto et al 1997).

Evidence for the importance of cytolytic mechanisms in the successful clearance of HBV infection was reported by (Nair et al 2001) who observed that in a group of patients undergoing treatment for chronic hepatitis B infection, a severe flare in ALT levels (a surrogate marker of immune-mediated cytolysis of hepatocytes) was the most powerful predictor of a sustained loss of HBV-DNA. It is also recognised that a flare in ALT often accompanies HBeAg seroconversion; a favourable outcome and
an established treatment goal resulting in patients entering an immunocompetent phase of infection, characterised by low/undetectable HBV-DNA levels and minimal necroinflammation of the liver (Liaw et al 2003).

In murine and chimpanzee models of chronic hepatitis B infection, HBV-specific CD8+ T-cells can be shown to kill a minority of the infected hepatocytes, and also to downregulate expression and replication of HBV by all hepatocytes in the absence of cell lysis (Guidotti et al 1996, Guidotti et al 1999). This non-cytolytic mechanism was elegantly demonstrated following adoptive transfer of virus-specific CTLs from perforin deficient and Fas-ligand deficient mouse models into a HBV transgenic mouse model with an observed abolition of HBV replication in the absence of liver disease. (Guidotti et al. 1994 & 1996, Nakamoto et al.1997). Non-cytolytic inhibition of HBV replication is mediated by several independent mechanisms and has been shown to be induced in the main by IFNγ and TNFα. In transgenic models of HBV infection, administration of anti-IFNγ and anti-TNFα antibodies resulted in failure of the CD8+ T-cells to clear HBV-RNA intermediates and nucleocapsid protein (HBcAg) demonstrating the importance of these cytokines and the non-cytolytic clearance of HBV infections (Guidotti et al 1994 & 1996, Wieland et al 2004, Cavanaugh et al 1998). Furthermore, patients with chronic hepatitis B treated with recombinant IL-12 had an increase in IFNγ production and a dose-dependent reduction in HBV-DNA levels without a concurrent increase in ALT levels. (Rigopoulou et al 2005).
As described, both curative and destructive CD8+ T-cell functions are crucial both in dictating the outcome of infection (resolution vs chronicity) and in limiting extensive immunopathogenesis. The mechanisms which determine the balance between these cytolytic (destructive) and non-cytolytic (curative) virus-specific-CD8+ T-cell functions and dictate which of these effector functions are dominant during resolution / chronicity of infection are, however, poorly understood. The mechanisms which dictate the degree of virus-specific CD8+ T-cell activation are also not clear. The recent identification of many co-stimulatory and co-inhibitory T-cell pathways (e.g. CTLA-4, PD-1 etc) suggest that T-cell activity is regulated by a balance between inhibitory and stimulatory T-cell signalling. These immunoinhibitory pathways and in particular the PD-1/PDL1/L2 pathway are discussed below.

1.3.2.2. CD4+ T-lymphocyte response

CD4+ T “helper” cells have a central role in the regulation of immune response to viral infection. As well as the production of anti-viral cytokines (eg IFNγ), CD4+ T-cells facilitate the induction and maintenance of virus-specific CD8+ T-cells. A lack of CD4+ “help” can impair CD8+ T-cell activity and antibody production (Sun et al 2003) culminating in viral persistence. Conversely a robust CD4+ T-cell response is always accompanied by a significant CD8+ T-cell response in the resolution of acute hepatitis B infection (Chisari et al 1995). Although previous in vitro studies have suggested that CD4+ T-cells demonstrate cytolytic activity, when these cells are depleted in a chimpanzee model of acute HBV infection, there was no significant decrease in the level of liver cell injury observed (Thimme et al 2003), suggesting that the main role of the virus-specific CD4+ T-cell in HBV infection is
immunoregulatory with CD8+ T-cell induction and the formation of a memory cell pool. The importance of CD4+ T-cells in CHB infection is confirmed by the observation of a generalised CD4+ hyporesponsiveness in individuals with chronic HBV infection (Boni et al 2001). This observed CD4+ T-cell hyporesponsiveness may be related in turn to impairment of dendritic cells, which have a reduced frequency, lower expression of co-stimulatory molecules and produce less antiviral cytokines, associated with reduced T-cell activity and poor viral clearance (Duan et al 2005, Beckebaum et al 2003). This deficiency has been demonstrated to be reversible, with adoptive transfer of HBcAg-specific CD4+ T-cells in chronic HBV carriers resulting in HBsAg clearance and resolution of infection (Lau et al 2002).

CD4+ T-cells also drive the production of antibodies to components of the HBV virus by B cells (See Humoral response - Section 1.3.2.4.).

**1.3.2.3. Regulatory T-cell response**

The concept of T-cell mediated immune suppression was initially postulated in the early 1970s by Gershon and colleagues who coined the phrase “suppressor T-cells”. Although this concept fell out of favour over the proceeding 2 decades, there has been a recent flurry of activity in revisiting this phenomenon. A subset of specialised T-cells known as regulatory T-cells (T-regs) that suppress auto-reactive cells, maintaining immunological tolerance and inhibiting autoimmunity has recently been reported. (Sakaguchi 2005).
These regulatory T-cells make up a heterogenous population including the classical CD4+CD25+Foxp3+ T-cells, CD4 T-cells which secrete IL-10 or TGF-β, CD8 T-cells, double negative T-cells and γδ T-cells (Alatrakchi et al 2009).

There is a growing body of evidence that these regulatory T-cells play an important role in the suppression of anti-viral T-cell responses in the acute and chronic phases of hepatitis B infection. Most of the studies of these regulatory T-cells focus on the classical CD4+CD25+ population. One report suggest a higher frequency of these T-regulatory cells in the peripheral blood and liver of patients with chronic hepatitis B than in patients with resolved infection or healthy controls together with an association between increased frequency of T-regulatory cells and blood titres of HBeAg (Yang et al 2007). In another study, depletion of CD4+CD25+ T-regulatory cells in vitro enhanced proliferation of HBV specific effector cells (Franzese et al 2005).

It is postulated that these T regulatory cells have evolved to prevent excessive immunopathogenesis following acute viral infection and that, as a consequence of their immunosuppressive actions, chronicity of infection is favoured (Belkaid 2007).

1.3.2.4. Humoral Immunity

The exact role of neutralising antibodies in the context of acute hepatitis B infection remains poorly understood. The fact that neutralising antibodies tend to appear relatively late on following exposure to HBV suggests that they do not play a role in determining the outcome of an acute infection. Rather it is likely that these antibodies
prevent the re-emergence of hepatitis B virus in patients who have successfully resolved an acute infection. Evidence for this is observed in the chimpanzee model of HBV infection, and in the success of the protective vaccination used in humans, where individuals with high titres of antibodies to HBsAg are in the main protected from subsequent infection with HBV. The humoral response is also important not only in protecting from further exposure to hepatitis B virus, but also in the long-term clearance of the virus. Following successful resolution of acute HBV infection, CD4+ T-cells induce B cell production of antibodies to components of the HBV virus (HBsAb, HBcAb, and HBeAb).

Neutralising antibodies can also enhance the removal of virions from the blood through the interaction between the Fc portion of virus-bound antibody complexes, with the Fc receptors found on the surface of phagocytic cells (Cooper et al 1984). These Fc-dependent interactions also occur on APCs and may facilitate the uptake and presentation of viral antigens to T and B cells in the lymphoid tissues (Bachmann et al 1997).
1.3.3. Viral and host factors which attenuate the immune response to HBV infection

1.3.3.1. Viral Factors

Although it is clear that a robust immune response to infection results in successful resolution of acute hepatitis B infection, there is an increasing body of evidence to suggest a role for various viral factors that impact on the adaptive immune response to hepatitis B infection. That of HBeAg - a truncated form of the HBV core protein (HBcAg) that is secreted in hepatitis B virus infection. The role of this viral protein remains incompletely understood. Despite its preserved production, HBeAg is not required for viral assembly, replication or infection (Chang et al 1987, Tong et al 1991, Chen et al 1992); indeed HBeAg negative chronic hepatitis B, which occurs following a mutation in the pre-core / core region, is a recognised phase of chronic infection with ongoing viral replication and necroinflammation of the liver in the absence of HBeAg production. Interestingly, there is some evidence to suggest that HBeAg may be important in the induction of immune tolerance to HBV with subsequent chronicity of infection. HBeAg can cross the placenta, and furthermore induces neonatal tolerance to HBV infection in transgenic mice (Milich et al 1990). HBeAg has also been shown to inhibit induction of the T-cell response to a cross reactive epitope in HBcAg in adult T cell receptor transgenic mice (Chen et al 2004). Furthermore, recent work on Toll-like receptors (TLRs) which play an important role in the innate response to viral infections demonstrated that HBeAg leads to the reduction of TLR2 expression on monocytes (Riordan et al 2006). This in turn can result in inefficient triggering of a cascade of responses including the release of inflammatory cytokine TNFα. A recent study by Wu et al (2010) in the HepG2
hepatoma cell line, demonstrated that HBeAg downregulated transcription of the inflammatory cytokines TNF, IL-6, IL-8, IL-12A, IFN-α1, and IFN-β compared to HBeAg-negative HepG2 cells. Real-time RT-PCR-based cytokine-related gene arrays also demonstrated downregulation of both cytokine and IFN production. Inhibition of the activation of NF-κB- and IFN-β-promoter in HBeAg-positive HepG2, as well as inhibition of IFN and IL-6 production in HBeAg-positive HepG2 cell culture fluids was also seen. These results suggest that HBeAg may modify disease progression by inhibiting inflammatory cytokine and IFN gene expression, while simultaneously suppressing NF-κB-signaling- and IFNβ-promoter activation.

There is also evidence to suggest that HBsAg acts as a tolerogen at higher doses. HBsAg levels correlate closely with HBV-DNA levels and change according to the phase of infection with lower levels seen in the immunocompetent phase of infection (Nguyen et al 2009, Jaroszewicz et al 2009). Furthermore it has been demonstrated that baseline HBsAg levels can predict HBsAg loss in patients with chronic hepatitis B undergoing treatment with pegylated interferon and adefovir (Takkenberg et al 2009, Lau et al 2009). It has also been reported that the HBsAg-specific CD8+ T-cell response is blunted and exhibits altered HLA/peptide tetramer-binding properties in patients with chronic hepatitis B who have high serum HBsAg titres (Reignat et al 2002).

Finally the HBV X protein can reduce proteosome activity in vitro (Hu et al 1999). If this phenomenon is present during infection, then the X protein may decrease the
visibility of infected hepatocytes to the adaptive immune response by inhibiting antigen processing and presentation.

1.3.3.2. Host Factors

Chronic hepatitis B does result from acute infection in a small minority of immunocompetent adults (<5%), and it is thought that there are several possible explanations as to why these patients fail to mount an adequate cellular response:

1) Virus-induced deficiencies in antigen presentation. (see above)

2) Genetically determined restriction of HBV-specific T-cell repertoire.

3) Antigen overload during immunological priming.

4) Induction of exhaustion /anergy of an initially vigorous virus-specific T-cell response (either through the effects of T regulatory cells (described above) or through the delivery of co-inhibitory T-cell signals e.g. via the PD-1/PDL1 pathway (described below).
1.3.4. T-cell co-stimulation in activation/inhibition of T-cell responses

The immune system has evolved to recognise and eliminate infectious organisms (non-self) whilst tolerating host tissues, leaving them intact. This highly selective immune response is achieved through tight regulation of the activation of cells of the immune system. In recent years, significant advances have been made in the identification of co-stimulatory T-cell signalling pathways that can activate or inhibit T-cell effector functions with the PD-1/L1 pathway increasingly recognised as important in the regulation of immune responses to potentially chronic viral infections.

The model by which naïve T-cells require two-signals for activation was originally proposed by Lafferty and colleagues in 1975. The first signal is provided by the interaction of the T-cell receptor with the antigenic peptide-MHC complex and this specifies the immune response (Th1 vs Th2) with T-cell activation via an intracellular signalling pathway involving an immunoreceptor tyrosine-based activation motif. The second signal is in fact antigen independent and is delivered by antigen-promoting cells to promote T-cell proliferation, cytokine production and effector functions. In the absence of this second signal, antigen specific T-cells are functionally inactivated, or anergic, and fail to activate in response to subsequent antigen exposure (Jenkins et al. 1987). The recognition that CD28 on T-cells interacted with its ligands B7-1 (CD80) and B7-2 (CD86) demonstrated the molecular basis for this costimulatory signal. CD28 signalling positively influences T-cell proliferation, IL-2 production and promotes cell survival through the induction of anti-apoptotic genes (e.g. BCLXL, clonal expansion and differentiation (Boise et al 1995, reviewed by Sharpe and
Freeman 2002), it has also been reported that antibodies to B7-1 and B7-2 can inhibit T-cell activation \textit{in vitro},\cite{Saloman et al 2001} and CD4+ T-cell proliferation is severely impaired in CD28-deficient mice \cite{Green et al 1994}.

Additional members of the B7:CD28 family, including the PD-1 receptor and its ligands PDL1/L2, have subsequently been identified and recognition of a spectrum of costimulatory pathways that provide a balance of positive and negative secondary signals to antigen experienced effector T-cells has evolved (Figure 8).
**Figure 8: Structures of the B7-1/B7-2-CD28/CTLA-4 superfamily members**

![Diagram of B7-1/B7-2-CD28/CTLA-4 superfamily members]

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### 1.3.4.1. Cytotoxic T-lymphocyte antigen 4 (CTLA-4)

Cytotoxic T Lymphocyte-associated antigen 4 (CTLA-4) is a member of the CD28-like receptor family and has been shown to have an important inhibitory role in T-cell signalling. Following activation of T-cells, CTLA-4 is rapidly expressed on the cell surface of T-cells and is highly upregulated by CD28 engagement (Walanus et al 1994). CD28 engagement therefore not only activates T-cells, but also initiates a
negative feedback loop with upregulation of CTLA-4 that ultimately allows regulation of T-cell activation. CTLA-4 shares the ligands B7-1 and B7-2 with the positive costimulatory molecule CD28, adding to the complex biology of CTLA-4. (Salomon et al 2001 (i))

On a cellular level, CTLA-4 engagement antagonizes early T-cell activation, leading to decreased IL-2 production, inhibition of cell cycle progression, decreased cyclin expression, and modulation of TCR signalling (Fife et al 2008). In addition, CTLA-4 binds to B7 proteins and therefore as a result of direct ligand competition CD28 cannot physically bind and mediate the positive signals required for efficient activation. The signalling molecules involved in CTLA-4 (and CD28) function are illustrated below(Figure 9).

It has been reported that T-cells with the highest affinity for a given antigen have the highest level of CTLA-4 expression; therefore these cells will be preferentially inhibited, allowing the lower affinity clones to respond and participate in the immune response (Allison et al 1998). It is postulated that this CD28/CTLA-4 sliding threshold acts to ensure that the body mounts a diverse T-cell response and this increases the potential to clear invading pathogens.
The CTLA4 deficient mouse strain rapidly develops lymphoproliferative disease with multiorgan lymphocytic infiltration and tissue destruction, which is invariably fatal at 3-4wks (Tivol et al 1995), demonstrating the importance of CTLA-4-mediated inhibition of T-cell responses in immune homeostasis. It is therefore postulated that CTLA-4 is critical in early tolerance induction, when T-cells are generated and rapidly expand to fill the lymphatic environment. (Fife et al 2008).
CTLA-4 has also been implicated in susceptibility for several autoimmune diseases including diabetes, multiple sclerosis and rheumatoid arthritis (Greenwald et al 2005) and there is therefore currently a great deal of interest in targeting this pathway as a therapeutic approach to treat autoimmunity and prevent organ transplant rejection.

1.3.4.2. Inducible Costimulatory Signal (ICOS)/ICOS-Ligand Pathway.

As its name implies, ICOS is not constitutively expressed but induced in CD4+ and CD8+ T-cells upon TCR engagement and T-cell activation (Hutloff et al 1999). In contrast to CD28 and CTLA-4, blockade of ICOS has no significant effect on IL-2 production (Yoshinaga et al 1999). However, ICOS expression significantly impacts on the production of several other cytokines in activated T-cells, with production of IL-4, IL-5, IL-10, IL-13 and IFNγ being highly ICOS dependent (Hutloff et al 1999). These cytokines follow a Th2 milieu and it is therefore postulated that ICOS is important in Th2 cell differentiation and effector functions.

Studies using pathway antagonists, transgenic mice and knockout mice have revealed that ICOS also plays a crucial role in B-cell differentiation, germinal centre formation, memory B cell development and immunoglobulin class switching (reviewed in Greenwald et al 2005).
1.4. THE PROGRAMMED CELL DEATH (PD-1) PATHWAY

1.4.1. Introduction

The Programmed Cell Death (PD-1) receptor was discovered in 1992 on a T-cell hybridoma undergoing cell death (Ishida et al 1992). The autoimmune prone phenotype of a PD-1/− mice first suggested an important negative regulatory function for PD-1 in 1999 (Nishimura et al 1999 & 2001). Since identification of the ligands for PD-1 (PDL1 & PD-L2) in 2000 and 2001 (Freeman et al 2000, Latchman et al 2001) there is an increasing body of evidence suggesting that PD-1 and its ligands play an important role in regulating immune defences against microbes that cause acute and chronic infections, regulating the delicate balance between effective antimicrobial immune defences and immune-mediated tissue damage. There is also currently considerable interest in the therapeutic potential of manipulating this pathway.

1.4.2. PD-1 pathway in infectious disease and microbial pathogenesis

In a murine model of chronic lymphocytic choriomeningitis virus infection, Barber et al. (2006) using a genome wide microarray in exhausted vs functional virus-specific CD8+ T-cells, demonstrated a significant up-regulation of PD-1 expression on exhausted virus-specific CD8+T-cells, compared with functional LCMV-specific CD8+ T-cells. Furthermore, blockade of PD-1 expression on these exhausted CD8+ T-cells resulted in restoration of CD8+ T-cell functions, with increased proliferation, cytotoxicity and cytokine production and a decrease in viraemia. A similar relationship between PD-1 expression and virus-specific T-cell reactivity was subsequently observed in HIV infection and PD-1 expression on HIV-specific CD8+...
T-cells was shown to directly correlate with viral load (Trautmann et al 2006, Day et al 2006, Colle et al 2006). This has important implications as it is known that viral load directly correlates with disease progression in HIV infection. Blockade of PD-1/PDL1 engagement results in enhancement of the proliferative and survival capacity of HIV-specific CD4+ and CD8+ T cells with increased production of cytokines and cytotoxic molecules in response to cognate antigen.

PD-1 expression was also found to affect T-cell reactivity and consequently may contribute to the outcome of acute hepatitis B or C infection, (i.e. resolution of infection or viral persistence), (Urbani et al 2006, Boettler et al 2006).

There is emerging evidence to suggest a role for the PD-1 pathway in maintaining the balance between immune defences and immune-mediated tissue damage. This is demonstrated by the observation that adenovirus infected PD-1−/− mice, clear an adenoviral infection more rapidly, but develop more severe hepatocellular injury than wild-type mice (Iwai et al 2003). PD-1−/− knockout mice also succumb early to extensive immunopathogenesis in a mouse model of chronic LCMV infection (Barber et al 2006). Furthermore with the use of monoclonal antibodies which block PD-1/PDL1 ligation, it has been demonstrated in murine transgenic models of herpes stromal keratitis that blockade of this pathway resulted in significant exacerbation of the keratitis (Jun et al 2005). PD-1 blockade in a further transgenic mouse model which expresses OVA as a self-antigen throughout the small bowel resulted in a
highly specific CD8+ T-cell mediated fatal auto-immune enteritis (Reynoso et al 2009).

It is unclear as to whether PD-1 has a global impact in inhibiting all aspects of CD8+ T-cell effector functions (both cytolytic – through perforin and FasL-mediated target cell apoptosis and non-cytolytic – through the production of anti-viral cytokines e.g. IFNγ and TNFα) or whether there is a “skewing” of the delicate balance between CD8+ T-cell effector functions, in favour of non-cytolytic mediated viral clearance. The temporal relationship between PD-1 mediated inhibition and the suppression of differential effector functions is different in acute versus chronic infection states.

The expression of PDL1 has been shown to be up-regulated on hepatocytes, stellate cells and Kupffer cells in response to infection with adenovirus or exposure to activated T cells and/or Type 1 interferons (Mulbauer et al 2006). Whether this phenomena is also observed following infection with chronic viral infections, such as hepatitis B/C is currently unknown. The mechanism by which viral infections may up-regulate PD-1 expression also remains to be fully elucidated.

Impairment and skewing of T cell maturation and altered T cell differentiation is another important factor that may contribute to the persistence of chronic viral infections. It has been established that there are subgroups of virus-specific CD4 and CD8+ T-cells that differ in function and phenotypic appearance (Harari et al 2006). The relative proportions of central memory (CM), effector memory (EM) and
effector (E) CD8+ T cells is thought to play a central role in immune modulation of chronic viral hepatitis. It has been demonstrated in HIV that there is skewed maturation of memory HIV-specific CD8+ T-cells and treatment with HAART therapy can favourably alter the balance of these cell lines as the immune system reconstitutes (Day et al 2006, Trautmann et al 2006). The relationship between PD-1 expression on virus specific T-cells and memory phenotype in chronic hepatitis B is currently unknown.

In chronic HBV infection, virus-specific T-cells are functionally impaired, but the relative role of viremia (HBV-DNA levels) and/or HBeAg in the impairment of T-cell reactivity has not been defined. Given the role of PD-1 as an important inhibitory pathway in T-cell function, the relative expression of PD-1 before and after seroconversion in the absence of changes in HBV-DNA may provide useful information as to the mechanism of tolerance induced by the Hepatitis B e antigen.
1.4.3. Expression of PD-1 and its ligands

PD-1 is not expressed on naïve T-cells, but is found on activated T-cells, B-cells, NK T-cells, activated monocytes and dendritic cells. PDL1 is expressed on a wide range of non-haematopoietic cells including hepatocytes, as well as on cells of the immune system. PDL1 expression is upregulated by Type 1 and 2 interferons (Eppihimer et al 2002, Schreiner et al 2004).

Analysis of the human PDL1 promoter demonstrated that both constitutive and inducible PDL1 expression are dependent on two IFN regulatory factor-1 (IRF-1) binding sites that are between 200 and 320bp upstream of the transcriptional start site. (Freeman et al 2000)

In contrast, PD-L2 expression is much narrower, with inducible expression seen on dendritic cells, macrophages and bone-marrow derived mast cells (Zhong et al 2007). PD-L2 can also be induced by IFNγ and this induction is partially dependent on NF-κB (Liang et al 2003).
1.4.4. The PD-1 signalling pathway

The signalling pathway for PD-1 is illustrated below (Figure 10). Upon ligand engagement, PD-1 is phosphorylated on its two intracellular tyrosines, and subsequently binds phosphatases that downregulate antigen receptor signalling through direct dephosphorylation of signalling intermediates.

SHP-1 & SHP-2 (SH2-domain containing tyrosine phosphatase 1 and 2), bind to the ITIM (immunoreceptor tyrosine associated inhibitory motif) and ITSM (immunoreceptor tyrosine associated shock motif) of PD-1 (Okasaki et al 2001). PD-1 inhibitory function is lost when the ITSM is mutated, demonstrating that this tyrosine plays the primary functional role of PD-1 mediated inhibition. The proximity of PD-1 to the antigen receptor appears to be important in PD-1 mediated inhibition; PD-1 ligation only inhibits antigen receptor signalling in cis and not in trans.
In contrast to CTLA-4 which inhibits Akt activation, PD-1 ligation inhibits PI3K activity, indicating these co-inhibitory pathways function through distinct mechanisms. PD-1 ligation inhibits phosphorylation of CD3ζ, ZAP70 & PKCθ (Parry et al 2005).
1.5. TREATMENT

Introduction

There are currently 7 drugs that are licensed for the treatment of chronic hepatitis B infection in the UK including interferon (both standard and pegylated forms), and oral antiviral therapies which target the HBV polymerase enzyme. These oral antiviral agents can be divided into L-nucleoside analogues (lamivudine & telbivudine), acyclic phosphonates or nucleotide analogues (adefovir dipivoxil and tenofovir disoproxil fumarate) and cyclopentanes (entecavir).

The primary long-term goal of therapy is to improve the quality of life as well as patient survival by preventing progression of liver disease to cirrhosis, decompensated cirrhosis, end-stage liver disease, hepatocellular carcinoma and death (EASL guidelines 2009). These therapeutic goals can be achieved with a long term sustained suppression of viral replication (Chen CJ et al 2006, Chen G et al 2006, Iloeje et al 2006) through the use of potent oral anti-virals that directly inhibit viral replication, and/or through the induction of robust anti-viral immune responses through immuno-modulatory (interferon-alpha based) therapeutic strategies. However, HBV infection cannot be eradicated entirely due to the persistence of covalently closed circular DNA (cccDNA) in the nucleus of infected hepatocytes.

A further important treatment goal in the management of chronic hepatitis B infection is HBeAg and/or HBsAg seroconversion (Liaw YF. 2009). Reports have demonstrated that following HBeAg seroconversion, >75% of patients will have low/undetectable HBV-DNA levels and normal ALT levels indicating effective
immune control of viral replication off therapy (Hadziyannis et al 2001). A minority of these patients will however develop HBeAg negative chronic hepatitis B (see Section 1.1.5.2). Whilst HBsAg seroconversion, with the development of anti-HBsAg antibodies and the loss of HBsAg from the serum is overwhelmingly associated with indefinite control of viral replication, in the event of patients subsequently being exposed to immunosuppressive therapy, reactivation of hepatitis B virus with re-emergence of HBsAg and subsequent liver damage has been reported (Palmore et al 2009).

1.5.1. Definitions and Assessment of treatment response

The definition of a response to antiviral therapy varies according to whether interferon alpha or a nucleos(t)ide analogue is used (EASL Clinical Practice Guidelines 2009):

On interferon alpha-based therapy:

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary non response</td>
<td>&lt;1log$_{10}$ IU/ml decrease in HBV-DNA level from baseline at 3 months of therapy.</td>
</tr>
<tr>
<td>Virological response</td>
<td>HBV-DNA concentration of less than 2000IU/ml at 24 weeks of therapy.</td>
</tr>
<tr>
<td>Serological response</td>
<td>HBeAg seroconversion in patients with HBeAg positive chronic hepatitis B virus infection.</td>
</tr>
</tbody>
</table>

(adapted from EASL Clinical Practice Guidelines 2009)
On nucleos(t)ide analogue therapy:

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary non response</strong></td>
<td>$&lt;\log_{10}10$ IU/ml decrease in HBV-DNA level from baseline at 3 months of therapy</td>
</tr>
<tr>
<td><strong>Virological response</strong></td>
<td>Undetectable HBV-DNA by real-time PCR assay within 48 weeks of therapy.</td>
</tr>
<tr>
<td><strong>Partial Virological response</strong></td>
<td>Decrease in HBV-DNA by $&gt;\log_{10}10$ IU/ml but detectable HBV-DNA by real-time PCR assay. This should be assessed at 24 weeks in drugs with low genetic barrier to resistance (e.g. lamivudine) and at 48 weeks for drugs with high genetic barrier to resistance (e.g. entecavir).</td>
</tr>
<tr>
<td><strong>Virological Breakthrough</strong></td>
<td>Confirmed increase in HBV-DNA level of more than $1\log_{10}10$ IU/ml compared to the nadir (lowest value) HBV-DNA level on therapy; it usually will precede a <em>biochemical breakthrough</em>, characterised by an increase in ALT levels. Virological breakthrough arises either from the selection of drug-resistant HBV variants, or through poor compliance with therapy.</td>
</tr>
</tbody>
</table>

(adapted from EASL Clinical Practice Guideines 2009)

A sustained HBV-DNA reduction to undetectable levels is necessary in NUC therapy to reduce the risks of HBV resistant strains emerging. Undetectable HBV-DNA also increases the chances of HBeAg seroconversion in HBeAg-positive chronic hepatitis B virus infection. The management of resistant strains of HBV are discussed in a subsequent section.
1.5.2. Interferon Therapy

1.5.2.1. Mechanism of action

Type I interferons (including interferon alpha) are naturally occurring cellular proteins that act through a variety of mechanisms to induce a non-specific antiviral state in both infected and uninfected target cells thereby reducing the spread of infection.

Following a pegylated subcutaneous injection, IFNα, binds to high affinity receptors on the surface of target cells triggering a cascade of intracellular reactions that activate numerous IFN-inducible genes which in turn mediate the antiviral effects of IFNα (Katze et al 2002).

In Hepatitis C virus infection, IL-28B has been found to predict the response to treatment with interferon, but this has not been established in hepatitis B virus infection.

As well as induction of this non-virus-specific anti-viral state which results in direct inhibition of viral replication, IFNα also has immunomodulatory effects that enhance the host’s specific antiviral immune responses and may accelerate the death of infected cells (Sen et al 1993).
1.5.2.2. Pegylated Interferons

Pegylated interferons are formed by covalent binding of an inert polyethylene glycol moiety to the interferon molecule resulting in a higher molecular weight, with a subsequently reduced renal clearance and altered metabolism, prolonging the half-life of the peg-IFN molecule (Reddy et al 2001). This allows for a steadier serum level of interferon and also allows patients to undergo subcutaneous injection once a week, compared to the 3 times a week necessary with standard interferon. For these reasons, pegylated interferons have almost entirely replaced standard interferon preparations in the treatment of chronic hepatitis B infection. There are 2 main preparations that are currently used in clinical practice, Viraferon™ (Schering-Plough) and Pegasys™ (Roche). In a large, multicentred randomized controlled trial, pegylated interferon alpha 2a demonstrated superior efficacy over lamivudine on the basis of HBeAg seroconversion, HBsAg seroconversion and HBV-DNA suppression. (Lau et al 2005). Further randomized prospective clinical trials have confirmed the efficacy of pegylated interferons in the management of chronic hepatitis B infection (Marcellin et al 2004, Janssen et al 2005).

Pegylated interferons are most effective in the treatment of chronic hepatitis B infection, when administered for a finite period of time (e.g. 48 weeks), when there is an active immune-mediated inflammatory response to infection that can be augmented through the administration of IFNα. Given the immunomodulatory mechanisms of action, it is perhaps unsurprising that IFNα therapy is associated with relatively high rates of HBeAg seroconversion (Figure 11).
Figure 11: Rates of HBeAg seroconversion following one year of therapy

(These numbers come from different randomized clinical trials, and were not from a head-to-head study.) *(Adapted from EASL Clinical Practice Guidelines: Management of chronic hepatitis B. 2009)*

Large, multicentred trials conducted in Europe, Asia and North America revealed a statistically significant association between viral genotype and response to pegylated interferon therapy ± Lamivudine (see Section 1.1.4).

Multi-variate analysis has suggested that as well as genotype, baseline ALT, baselines HBV-DNA levels (<10^9 copies/mL) and low concentrations of pre-treatment HBeAg are also predictive of HBeAg seroconversion.

The main disadvantages of treatment with pegylated interferons are the inconvenient mode of administration (subcutaneous injections weekly) and the frequent and on
occasion severe side effects of interferon therapy. The comonest adverse effect is a flu-like syndrome characterised by fever, chills, arthralgia, fatigue and malaise (Schellekens et al 1984, Gota et al 2003). It is recognised that 3-14% of patients on interferon therapy will develop anti-thyroid antibodies resulting in thyroiditis, hypothyroidism or hyperthyroidism (Ward et al 2001). Other AI diseases associated with interferon therapy include SLE, RA, dermatomyositis & polymyositis, psoriasis, vitiligo and many others (Burdick et al 2009). Significant neuropsychiatric side effects have also been reported with interferon treatment, including irritability, depression, memory loss and rarely psychosis. In patients with pre-existing neuropsychiatric problems, treatment with interferon alpha must be in conjunction with ongoing specialist psychiatric care (Schaeffer et al 2007). Interferon-alpha therapy may also result in bone marrow suppression with subsequent neutropenia, thrombocytopenia and anaemia.

These disadvantages prompt many patients and clinicians to favour nucleos(t)ide analogues in the treatment of chronic hepatitis B infection. In the main pegylated interferon use is restricted to patients with HBeAg-positive chronic hepatitis B infection. In this patient group, genotyping of the virus, together with the assessment of other parameters including HBV-DNA levels and baseline ALT should be assessed, before a frank discussion with individual patients about the benefits and risks of a course of therapy.

Interferon therapy can lead to an immune mediated flare in ALT, which in the context of cirrhosis with borderline liver failure, can result in hepatic decompensation.
Patients with pre-existing cirrhosis should therefore be carefully monitored for this potentially serious complication. Interferon is also contraindicated in pregnancy.

1.5.3. Nucleos(t)ide Analogues

1.5.3.1. Mechanisms of action

All of the currently licensed direct oral anti-viral therapies are competitive inhibitors of the HBV polymerase enzyme, competing with the incorporation of endogenous intracellular nucleotides in nascent viral DNA. This inhibition targets the priming of reverse transcription, viral minus strand DNA synthesis (i.e. RNA dependent DNA polymerase activity or reverse transcription), or plus strand DNA synthesis (i.e. DNA dependent DNA polymerase activity of the viral enzyme) (Zoulim et al 2004).

In contrast to pegylated interferons these oral anti-virals are therefore given for many years (sometimes indefinitely) to maintain suppression of viral replication.

Lamivudine acts predominantly as an inhibitor of the reverse transcriptase activity of the HBV polymerase enzyme. Adefovir and tenofovir are active on the priming of the reverse transcription as well as on elongation of viral minus strand DNA (Delaney et al 2006, Seigneres et al 2001). Telbivudine inhibits the priming of reverse transcription, as well as viral minus and plus strand DNA synthesis (Standring et al 2001). Entecavir inhibits both minus and plus strand DNA synthesis (Seifer et al.1998).
Once incorporated these nucleos(t)ide analogues can also terminate DNA synthesis by preventing the incorporation of subsequent nucleotides in the viral DNA strand. By inhibiting the HBV polymerase enzyme, there is decreased production of infectious viral particles, which limits the spread of virus to uninfected hepatocytes.

Although some in-vitro studies suggest an additive effect for combinations of nucleos(t)ide analogues (Delaney et al 2004, Seigneres et al 2003), clinical trials have shown no additional benefit, with the most potent anti-viral drug in any combination dictating anti-viral efficacy (Lai et al 2005, Sung et al 2008).

1.5.3.2. Oral anti-viral drug resistance and viral breakthrough

A major drawback of the long-term use of oral anti-viral therapies is the emergence of resistant HBV strains which can result in viral breakthrough with an increase in necroinflammation of the liver, hepatic decompensation, increased Child-Pugh scores in cirrhotic patients and ultimately death (Lok et al 2003). As described above, resistance rates with lamivudine or adefovir monotherapy, which until recently have represented the standard of care for chronic HBV patients are disquieting with 5 year viral resistance rates of >50% and 20% respectively. However newer agents such as Tenofovir and Entecavir, with a higher genetic barrier to resistance and greater anti-viral potency have thus far demonstrated very low resistance rates, even when used as a monotherapy (Tenofovir – 0% at 3 years, Entecavir 1.2% at 6 years).
1.5.3.3. Managing Anti-viral Resistance

The major challenge currently facing hepatologists is how best to avoid the development of these multi-drug resistant strains of hepatitis B virus infection. There is however, much controversy over the most effective way to achieve this. Some clinicians advocate single agent therapy with either the substitution or addition of a second agent should resistance occur. Experience with lamivudine and adefovir have demonstrated that the addition of a second agent is a superior strategy to substitution in preventing the development of multi-drug resistant HBV to both of these agents (Lampertico et al 2007, Lee et al 2006). The much lower rates of resistance observed with newer more potent oral anti-virals e.g. entecavir (1.2% at 5 years) & tenofovir (0% at 3yrs) have led to a monotherapeutic approach to care being widely re-adopted. Whether in the long-term these agents will also select resistant strains of hepatitis B virus is disputed.

When antiviral resistance does emerge to a particular agent then it is important to consider the issue of cross-resistance when selecting a rescue agent to add in.

Table 4 illustrates cross-resistance data for the most frequent resistant HBV variants.
Table 4: Cross resistance data for the most frequently occurring HBV resistant strains

<table>
<thead>
<tr>
<th>HBV Variant</th>
<th>Level of Susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lamivudine</td>
</tr>
<tr>
<td>Wild type</td>
<td>S</td>
</tr>
<tr>
<td>M204I</td>
<td>R</td>
</tr>
<tr>
<td>L180M + M204V</td>
<td>R</td>
</tr>
<tr>
<td>A181T/V</td>
<td>I</td>
</tr>
<tr>
<td>N236T</td>
<td>S</td>
</tr>
<tr>
<td>L180M + M204V ±I169T±V173L±M250V</td>
<td>R</td>
</tr>
<tr>
<td>L180M + M204V ±T184G±S202I/G</td>
<td>R</td>
</tr>
</tbody>
</table>

S = sensitive       I = intermediate/reduced susceptibility       R = resistant

1.5.3.4. Impact of antiviral therapy on host immune responses

HBeAg seroconversion with subsequent transition to an immunocompetent phase of infection characterised by low HBV-DNA levels and normal ALTs is defined as an endpoint of treatment. Although a proportion of patients will undergo HBeAg seroconversion spontaneously, Antiviral treatment with pegylated interferon or an oral nucleoside analogue can accelerate this transition with seroconversion rates of 20-30% following 1 year of therapy (Wong et al 1993, Lai et al 1998, Marcellin et al 2003, Krogsgaard et al 1994). As previously described a robust, multi-specific host immune response is associated with long-term control of viraemia and the ultimate goal of newer therapeutic approaches must be the restoration of immune responses with subsequent control of viraemia.

The five currently licensed oral anti-viral agents (Lamivudine, Adefovir, Telbivudine, Tenofovir and Entecavir) have been shown to be efficacious in suppression of viral load through inhibition of the HBV polymerase. Immunological studies have reported that high viral loads directly contribute to T-cell hyporesponsiveness, and the frequency of intrahepatic virus-specific T-cells is inversely proportional to the level of HBV replication (Webster et al 2004). It is therefore reasonable to hypothesize that viral load reduction due to the direct anti-viral action of these nucleos(t)ide analogues is responsible for any subsequent improvements in immune responses to HBV which allow control of viral replication and “T-cell driven” HBeAg seroconversion.

However, further studies suggest a wide degree of heterogeneity in patients with chronic hepatitis B infection, with viral suppression not always temporally associated with restoration of anti-HBV immunity, and often with only a transient increase was observed in a proportion of treated patients (Marinos et al 1996, Boni et al 1998,
Boni et al 2003). Analysis of T-cell responses during lamivudine therapy in HBeAg+ve chronic hepatitis B infection in patients who had failed previous interferon therapy showed no significant improvement of T-cell reactivity to HBV and an early relapse of HBV replication after stopping Lamivudine (Marinos et al 1996). However, in another study, lamivudine monotherapy in a group of patients with treatment naïve HBeAg positive chronic hepatitis B led to a marked enhancement of HBcAg-specific reactivity for both CD4+ and CD8+ T-cells as early as 2-4 weeks after starting treatment although importantly this enhancement was observed to be only transient (Boni et al 1998, Boni et al 2003).

A further study in 2004 examined cellular immune responses to the hepatitis B virus polymerase during a course of oral antiviral treatment with Lamivudine (Mizokoshi et al 2004). They reported that antiviral therapy enhanced HBV-specific T-cell responses during the first year of treatment, but thereafter, responses decreased such that after 3 years of treatment responses were no different to those observed in untreated patients. They observed that the decrease in T-cell responsiveness during prolonged therapy was associated with an increased prevalence of lamivudine-resistant HBV mutants and increased HBV titres.

A prospective, longitudinal, placebo-controlled trial demonstrated that suppression of HBV replication with adefovir dipivoxil significantly enhances virus-specific CD4+ T-cell reactivity in a proportion on patients in whom greater suppression of HBV-DNA replication, and higher HBeAg seroconversion rates were observed (Cooksley et al. 2008).

Finally the functional characteristics and memory phenotype of CD4+ and CD8+ T-cells were assessed in a subgroup of patients receiving either telbivudine or
lamivudine as part of the Phase 3 GLOBE trial (Riva et al 2007). This prospective study demonstrated that pharmacological suppression of HBV replication was associated with increased breadth of T-cell reactivity in patients infected with genotype A or D. Crucially however, no increase in frequency and central memory phenotypes were observed, which may explain why individual T-cell subsets fail to establish host-immune control during pharmacological suppression of HBV replication, and thus the need for long-term maintenance therapy in the management of chronic hepatitis B infection with oral nucleos(t)ide analogue therapies.
Chapter 2

Hypothesis to be tested
HYPOTHESIS TO BE TESTED

The Programmed Cell Death (PD-1) immunoinhibitory T-cell pathway plays a central role in the impaired adaptive immune responses characteristically observed in chronic hepatitis B.

OBJECTIVES:

1. To characterize the relationship between the PD-1 pathway, virus-specific CD8+ T-cell responses and control of viraemia in patients with chronic hepatitis B.

2. To assess the impact of the PD1 pathway on differential virus-specific CD8+ T-cells effector functions in hepatitis B virus infection.

3. To define whether the Hepatitis B virus itself exploits the PD-1 pathway to favour chronicity of infection.
Chapter 3

Materials & Methods
3. MATERIALS AND METHODS

3.1. Patients

Patients with chronic hepatitis B virus (HBV) infection were referred from primary care to the specialist viral hepatitis clinics at University College London Hospitals, for assessment and management. At initial assessment, confirmation of chronic HBV infection was performed with HBV serology and HBV-DNA determination. Patients were considered for anti-viral therapy and where appropriate underwent percutaneous liver biopsy.

Written informed consent was obtained from each patient and all study protocols were approved by the Ethics Committee of University College London Hospitals.

3.2. Liver Histology

Diagnostic liver biopsies were carried out using a Menghini suction needle to obtain a tissue core. All histological samples contained ≥ 5 portal tracts for optimum assessment of the necroinflammatory activity based on the Ishak modification of the Histological Activity Index of Knodell (Ishak et al 1995).
3.3. Reagents used for T-cell reactivity and cell culture assays

sRPMI

RPMI 1640 (Gibco BRL, Life Technologies, Paisley, UK) is a neutral nutrient media used for cell cultures. It is supplemented with 11.5ml of 1M HEPES (Sigma, Poole, Dorset, UK) as a buffer, 4ml of 10M NaOH to maintain neutral pH, 0.5ml glutamine to allow for any breakdown of this essential amino acid during transport and storage, and 5ml of penicillin / streptomycin (Sigma, Poole, Dorset, UK) to prevent bacterial contamination of medium. These solutions were prepared and used within 30 days, stored at 4°C and filtered using a 0.22 micron filter (Acrodisc, Gelman Sciences, Ann Arbor, MI, USA) to ensure sterility.

DMEM (Dulbecco/Vogt modified Eagle’s minimal essential medium)

DMEM is a cell culture medium used to maintain cells in tissue culture. It is a modified form of the cell culture medium originally produced by Harry Eagle. DMEM / F12 is DMEM supplemented with Ham’s F12.

Foetal Calf Serum

Foetal calf serum (F.C.S.) or foetal bovine serum (F.B.S.) is commonly used as a supplement to growth media in cell culture. Foetal bovine serum is obtained from foetuses harvested in abattoirs from healthy dams fit for human consumption. Occasionally, there may be use of other bovine sera, such as newborn calf serum or donor bovine serum. In cell culture, serum provides a wide variety of macromolecular
proteins, low molecular weight nutrients, carrier proteins for water – insoluble components, and other compounds necessary for in vitro growth of cells, such as hormones and attachment factors. Serum also adds buffering capacity to the medium and binds or neutralizes toxic components. Attempts to replace serum entirely with serum-free medium have met only with limited success.

**Human AB serum**

This serum was used to supplement culture media with essential nutrients allowing cell proliferation and growth. Foetal calf serum (FCS) was also used depending on the cell culture being performed. In cell cultures involving primary human lymphocytes (CD8+ T-lymphocytes) growth media was supplemented with human AB serum as FCS can cause non-specific activation of T-lymphocytes.

**Lymphoprep (Nycomed, Nyegaard, Norway)**

Lymphoprep allows the separation of peripheral blood mononuclear cells from other blood constituents according to their density upon centrifugation (see PBMC separation).

**Hanks Balanced Salt solution:**

This is a balanced salt solution used for a wide variety of tissue culture applications.
Counting Solutions:

For Freshly Isolated Cells: This was composed of 450ml of distilled water, 50ml of acetic acid and 5 drops of tryptan blue. This solution lyses red blood cells from a PBMC suspension so only lymphomonocytes are seen and counted under the microscope.

For Cryopreserved Cells:

Trypan Blue Exclusion

Diluted trypan blue was prepared (160µl of sRPMI + 40µl of trypan blue(Sigma, Dorset, UK)). 10µl of the cell suspension was then added to 190µl of diluted trypan blue. 10µl of this mix were loaded into the haemocytometer. To assess the concentration of PBMCs, the live cells (white cells) were counted in two diagonal quadrants and multiplied by $10^5$ to give the number of cells per ml of cell suspension. The number of blue cells (dead cells) was also counted in two diagonal quadrants. The % viability was calculated by the following equation:

Viability (%) = 100 x number of white cells / number of total cells (white and blue)

A viability of >90% was achieved for all samples used to analyse T-cell reactivity.
Figure 12: Counting number of cells using a haemocytometer

Cells were counted in 2 diagonal quadrants
3.4. Peripheral Blood Mononuclear Cell (PBMC) preparation

3.4.1. PBMC isolation

PBMCs were isolated from heparinised blood by standard density gradient centrifugation.

1. 40ml of blood was venesected from each patient into a vacutainer containing 30IU (0.03ml) of sodium heparin.

2. The blood was subsequently diluted in a ratio of 1:1 with 0.9% sterile saline.

3. 20ml of the blood/saline mixture was carefully layered over 10ml of Lymphoprep (Axis-­Shield, Oslo, Norway) in a universal container ensuring that a distinct interface between the two layers was maintained (Figure 13).

4. The blood was then centrifuged at 750g for 30 minutes at 20°c, with no brake applied, to ensure separation of the PBMCs from the denser Ficoll/erythrocyte layer below, and the less dense dilute plasma layer above (Figure 13).

5. The top plasma level was siphoned off and discarded. The peripheral blood mononuclear cell layer was then gently removed with a sterile pipette and placed in a fresh universal container.

6. The PBMCs were further washed twice with sRPMI and centrifuged at 1000g for 10 minutes with brake applied.

7. The cell pellet was finally resuspended in 3-­4mls of sRPMI/10% human AB serum.
8. The freshly isolated PBMCs were counted by adding 25µl of cell suspension to 475µl of counting solution. 10µl of this counting mix was loaded in a haemocytometer (Neubauer).

9. Cells were counted in all 4 quadrants. The concentration of cells (x10^6/ml) was calculated by dividing the number of cells counted by 20 (dilution factor). The cell concentration was adjusted to 5-10 x 10^6 cells/ml with sRPMI/10% AB serum.

Figure 13: PBMC separation by Ficoll Density Gradient Centrifugation

(adapted from Lan et al 2007)
3.4.2. PBMC cryopreservation

1. Freezing mix was prepared by adding 3 volumes of s-RPMI to 2 volumes of dimethyl sulfoxide (DMSO), and cooled at room temperature for 20 minutes. (This reaction is exothermic and therefore freezing mix must be prepared at least 20 minutes before being added to cells).

2. The cell suspension from the PBMC separation was pelleted by centrifugation at 1000g for 10 minutes.

3. The pellet was then resuspended in 750µl heat-inactivated foetal calf serum per 5x10⁶ PBMC, and 250µl of pre-prepared freezing mix was added.

4. This 1ml mixture was placed into a cryovial, and deposited into a Nalgene cryocontainer (Mr Frosty, Merck BDH, Leicestershire, U.K.) with isopentane to freeze to -70°C at a cooling rate of 1°C per minute.

5. Vials were transferred to liquid nitrogen or a -80°C freezer after 24 hours.
3.4.3. Defrosting of cryopreserved PBMCs

1. Vials of PBMCs were transferred from liquid nitrogen or from -80°C freezer on dry ice for sequential defrosting.

2. 50mls of sRPMI / 20% FCS was prepared and warmed in a water bath.

3. Each vial was defrosted in a water bath until the cell pellet became mobile. Once mobile, the pellet was transferred into a fresh 30ml universal container and 1ml of warmed media was added.

4. PBMCs were then defrosted using a “step-by-step” method, with the addition of 3 drops of media, followed by 20 seconds of gentle swirling of the universal until a total volume of 5mls was reached. 1ml of media was subsequently added followed by 20 seconds of gentle swirling until a total volume of 10mls was reached.

5. The defrosted cells were centrifuged at 1600 rpm for 10 minutes with brake applied, the supernatant discarded and the cell pellet resuspended in 1ml of sRPMI / 10% AB serum.

6. Cells were then counted by trypan blue exclusion.
Fresh vs Frozen Peripheral Blood Mononuclear Cells

Although there has been some data suggesting that cryopreservation of PBMCs resulted in attenuation of their functionality, we observed significantly lower levels of variability both within experimental duplicates and from one experiment to another by batch testing frozen PBMCs rather than by processing fresh PBMCs as they were obtained from patients. There is also data suggesting that CD4$^+$ and CD8$^+$ cells in cryopreserved human PBMC maintain full functionality in cytokine ELISPOT assays (Kreher et al 2003).
3.5. Hepatoma Cell lines

The cell lines that were used comprised Huh-7 cells, HepG2.2.15 cells, HepG2 cells, AD38 cells and Alexander cells. The Huh-7 cell line is a commercially available human hepatoma cell line that was derived from a 57 year old Japanese male patient with well differentiated hepatocellular carcinoma. Hepatoma tissue was removed and minced and cultivated in RPMI 1640 supplemented with 20%FCS and 0.4% LAH. An epithelial cell colony isolated from primary culture on Day 28 was designated as Huh-7 (Nakabayashi et al 1982).

The HepG2.2.15 cell line is derived from the parent HepG2 line and is stably transfected with the full HBV genome supporting the production and secretion of infectious virions (Sells et al 1988). HepG2 cells are HLA-A2-positive and express cognate HLA-A2. This cell line was originally derived from a 15yr old male (Aden et al 1979).

It was confirmed that HepG2.2.15 cells are HLA-A2-positive by flow cytometry using FITC-labelled antibody (Serotec, Oxford, UK). The AD38 cell line is also derived from the parent cell lines HepG2. The AD38 cell line contains a complete HBV genome under the control of a tetracycline-off promoter region (Ladner et al 1997). By the addition or removal of tetracycline from the growth media, this cell line can have HBV production switched on/off. This allowed the study of the impact of HBV replication in this cell line on cell surface expression of PDL1. Both HepG2.2.15 and AD38 cell lines were grown in containment level 3 laboratory.
3.5.1. Hepatoma cell line thawing and propagation

Growth media (DMEM + 10%FCS + Penicillin/Streptomycin + L-glutamine) was warmed to 37°C. For HepG2.2.15 cell lines, growth media (GM) was supplemented with Geneticin (0.4mg/ml) to maintain the 2.2.15 cell line during propagation. For the AD38 cell line the following GM was used: DMEM/F12 + 20%FCS (500ml) + Penecillin/Streptomycin (5ml) + L-Glutamine (5ml) + Geneticin (4.4ml of 50mg/ml) + Gentamicin (1.1ml of 10mg/ml) +/- Tetracycline (66.6ul at 2.5mg/ml)). For “switching on” of HBV production this GM was used without the tetracycline.

1. Cells were removed from -80°C freezer and thawed in a 37°C water bath until the cell pellet was mobile. The pellet was then placed into a 30 ml sterile universal and 1ml of warmed GM was added.

2. Hepatocytes were then defrosted using a “step-by-step” method, with the addition of 3 drops of media, followed by 20 seconds of gentle swirling of the universal until a total volume of 5mls was reached. 1ml of media was subsequently added followed by 20 seconds of gentle swirling until a total volume of 10mls was reached.

N.B. (This “step-by-step” method resulted in an improved cell viability and total number of cells recovered following thawing compared with the immediate addition of 10mls of growth media and is now standard operating procedure within the laboratory.)
3. The defrosted cells were then centrifuged at 1400 rpm (1000g) for 10 minutes with a brake applied.

4. The supernatant was discarded and the cell pellet resuspended in 1ml of growth media.

5. A further 9ml of media was added to the cells and a cell count was performed by tryptan blue exclusion (see previous section).

6. 10ml of media was then added to a 75cm$^2$ tissue culture flask.

7. The hepatocytes were subsequently seeded into a tissue culture flask and incubated at 37$^\circ$C / 5%CO$_2$ until the cells were confluent, changing the media every 2-3 days.
3.5.2. Cell Line Propagation

1) Acutase (cell dissociation solution) – (eBioscience, Hatfield, UK) GM and Hanks BSS (Sigma, Dorset, UK) were warmed to 37°C in a water bath.

2) The tissue culture flasks were removed from the incubator and the supernatants were discarded.

3) The cell monolayer was then washed with Hanks BSS, with 5ml of Hanks BSS added to the flask, and gently rocked back and forth over the cell monolayer before being aspirated and discarded.

4) 3ml of Acutase was then added to cover monolayer, and the cells were then incubated at 37°C / 5%CO₂ for 10 minutes to allow cell detachment. Cell detachment was visually monitored, and the sides of the flasks were tapped to facilitate this process as necessary.

5) The cells were then aspirated and transferred to a universal tube. 3ml of warmed media was added to the flask and rocked back and forth to collect any cells remaining in the tissue flask. This was then aspirated and added to the universal tube containing the cells.

6) This cell suspension was then centrifuged at 1400rpm for 10 minutes with brake applied.

7) The supernatant was discarded and the cells were resuspended in 1 ml of GM.

8) For cell line propagation, a further 9ml of GM was added and the cells were subsequently seeded into tissue culture flasks.
8i) For plating of hepatocytes, following resuspension of cells in 1ml of GM, a cell count was performed with tryptan blue exclusion (see previous section). Cells were then plated in 2-3 ml of medium in each well. The number of cells per plate was dependent on cell type and based on cell confluence at 24-48 hours:

1) HepG2/G2215/AD38 – 800,000 cells/well
2) Huh7 – 500,000 cells/well

9) For freezing of hepatocytes, once a cell count has been performed, cells were frozen with up to 7 million cells / cryovial with 1ml of DMEM 20%FCS, 10% DMSO and kept at -80⁰c for up to 2 years without loss of viability or recovery.
3.6. Transient transfection of Human hepatoma cell line

3.6.1. Principle of Transfection

Transfection is the general process of bringing foreign DNA into cells and monitoring protein expression. DNA transfection is essential for the study of gene function and regulation. Common transfection techniques include calcium phosphate coprecipitation, electroporation, and the use of viral vectors. Fugene 6 Transfection Reagent (Roche, Indianapolis, USA) is a multi-component lipid-based transfection reagent that complexes with and transports DNA into the cell during transfection.

3.6.2. Transient transfection of Huh7 cells

1) Huh-7 cells were thawed and plated as described in a previous section.

2) Huh-7 cells were grown into a confluent monolayer in a 150mm flask. This confluent monolayer was then washed with Hanks Balanced Salt Solution (HBSS).

3) 2-3ml of trypsin was added ensuring complete coverage of cell monolayer and the cells were incubated for 5 minutes at 37°C to allow cell detachment.

4) The cells were then transferred to a red topped vacutainer and 10ml of growth medium were added (DMEM + 10%FCS + glutamine + penicillin/streptomycin).

5) The cells were centrifuged at 1400rpm for 10 minutes. The supernatant was discarded and cells were resuspended in growth medium without penicillin/streptomycin (all growth medium used from this point onwards is without penicillin/streptomycin).
6) The cells were then passed several times through a 1ml tip of a pipette to ensure a cell suspension with no cell clumping, and then a further 9ml of growth medium was added.

7) The cells were then counted and the concentration adjusted to 1.5-3 x 10^6/ml.

8) 0.9ml of growth medium was pipetted into individual wells of a 12 well plate.

9) 5 x 10^5 cells were added to each well and the plates were incubated for 16-18 hours at 37°C.

N.B. (For optimum transfection rates cells were between 80-90% confluent at this stage; therefore if cells were <60% confluent further time was allowed for the cells to grow before transfection. Alternatively if the cells were already 100% confluent, they were re-seeded to achieve confluency rates of 80-90% before proceeding with transfection.)

10) The plasmid DNA (pCMV-HBV) - pSM2, (containing an HBV head-to-tail dimer DNA of subtype ayw cloned via the EcoRI site with a CMV promoter (Galibert et al 1979) and Fugene 6 transfection reagent was thawed.

11) 100µl of DMEM was aliquoted into a red-topped vacutainer. 4µl of Fugene6 was added directly to the DMEM. These were mixed gently and incubated at room temperature for 5 minutes.

12) 1µg of total plasmid DNA was added to the Fugene/DMEM and mixed gently before being incubated at room temperature for 15-20 minutes.

13) The growth medium was removed from the cells (in the 12 well plate) and 0.9ml of fresh growth medium was added.
14) 100-110µl of the DMEM/Fugene6/DNA mix was added drop-by-drop to the cells whilst gently rocking the plate.

15) The plates were then incubated at 37°C for a further 6 hours.

N.B. (In practice when the same plasmid was used for an experiment (in triplicate wells), a mastermix of DMEM/Fugene/DNA was prepared to the required volume in the above ratios.)

This mode of transfection was repeated with an empty (mock) plasmid, in order to evaluate the impact of non-specific transfection reagents on cell surface expression of PDL1 by hepatocytes.

The efficiency of transfection was estimated by performing transfection with the plasmid containing LacZ (a reporter gene that codes for beta galactosidase).

Following transfection, cells were subsequently stained with X-gal solution (0.2% X-gal, 2mM MgCl₂, 5mM K₄Fe(CN)₆.H₂O, 5mM K₃Fe(CN)₆ in PBS. Cells which had undergone successful transfection with LacZ stained blue on microscopy. Transfection rates achieved were 30-50%. The subsequent production of HBV by transfected Huh7 cells (determined by HBV-DNA quantification with PCR) further confirmed successful transfection.
3.7. T cell clone generation

HBC\textsubscript{18-27} -specific CD8+ T-cell clones were generated from an HLA-A2 positive patient who resolved acute HBV infection as described by Gehring et al 2007. This T-cell clone was kindly donated for this work by Prof. Antonio Bertoletti (Singapore Institute for Clinical Sciences, Singapore.) The CD8+ T-cell clone was >90% specific for the HBC\textsubscript{18-27} epitope (see Figure 14).

Figure 14: Specificity of T-cell clone

3.8. Co-culture of HepG2.2.15 cell line (Target cells) with HBV-specific CD8+ T-cell clone (Effector cells)

The HepG2.2.15 cells (target cells) were seeded in 24-well plates and once confluent (after 3 days); the CD8+T-cells (effectors cells) were added. Two models were investigated in parallel: 1) Direct Effector:Target (E:T) contact where CD8+T-cells were stimulated by recognising the viral peptides expressed on the surface of HepG2.2.15 cells. 2) Indirect E:T contact where the effector and target cells were
separated by a 0.4μm membrane (Marathon, London, UK) which allows the passage of soluble factors only. In this second model, CD8+ T-cells placed in the insert were stimulated with the corresponding HBV core 18-27 peptide at 1μM together with EBV-B-cells used as antigen presenting cells (Figure 15). The CD8+ T-cells and HepG2.2.15 cells were co-cultured for 24hrs and 48hrs at E:T ratios: 1:60 and 1:6. As controls, HepG2.2.15 cells were cultured alone, and CD8+ T-cells were cultured in the insert with EBV-B cells but without HBV peptide. These direct and indirect co-culture models allowed the study of both cytolytic and non-cytolytic virus-specific CD8+ T-cell pathways respectively.

These direct and indirect co-cultures were performed in the presence/absence of neutralising monoclonal antibodies to IFNγ, TNFα and PD-1 (R&D systems, Abingdon, UK), at neutralization doses of 36μg/mL, 0.9 μg/mL and 10μg/mL respectively. Hepatoma cell lines HepG2 and 2.2.15 cells were also treated with recombinant IFNγ (rIFNγ) and/or TNFα (rTNFα). The concentration of rIFNγ and rTNFα used were determined by measuring the concentrations of these cytokines produced by the CD8+ T-cell clone, in the supernatant of the direct and indirect cell co-culture models described above. The cytokines concentration was assessed using cytokine bead array (CBA) (BD Biosciences, Oxford) described below. Confluent HepG2 and 2.2.15 cell lines were incubated with 4 concentrations of rIFNγ (100,1000,5000 and 10,000pg/ml) and/or 4 concentrations of rTNFα (100,300,700 and 1000pg/ml)(R&D Systems, Abingdon, UK) for 24 hrs.
Figure 15: Schematic representation demonstrating direct and indirect co-culture models

(Co-cultures were performed for 24 and 48 hours, in the presence/absence of neutralising antibodies to IFNγ, TNFα and PD-1. Virus-specific CD8+ T-cells in the indirect co-culture were stimulated with HBC18-27 peptide in the presence of EBV-B-cells for antigen presentation. Representative flow cytometric dot plots illustrate T-cell lineage (CD8+) and >90% HBC18-27 specificity as assessed by pentamer staining.)
3.9. RNA extraction from PBMCs and hepatocytes

For quantitation of PD-1/PDL1/PDL2 at an mRNA level, total cellular RNA was isolated from cryopreserved PBMCs or snap frozen hepatocytes. Contamination with RNases was minimised by the use of certified RNase-free tips and regular glove changing, as well as using RNAse/DNAse free water.

5 x 10^5 PBMCs and 3 x 10^5 hepatocytes were used for total RNA extraction.

1. Cryopreserved PBMCs / Snap frozen hepatocytes were taken from -80^0c freezer, and kept on dry-ice. The pellets were quickly thawed in 37^0c water bath until mobile within eppendorf, and were then resuspended in 500µl of Trireagent (Ambion, Applied Biosystems, Warrington, UK) to preserve RNA whilst breaking down cellular components.

2. The suspension was homogenised by passing the lysate 10 times through a 20G sterile needle fitted to an RNAse-free syringe.

3. The suspension was then allowed to stand at room temperature for 5 minutes to ensure complete homogenisation.

4. 100µl of chloroform was added to the suspension, vortexed for 15-20 seconds and allowed to stand for 15 minutes.

5. The suspension was then centrifuged for 15 minutes at 4^o c at 13,000 rpm.

6. The aqueous phase of this suspension was transferred to new RNAse free tubes.
7. 250µl of isopropranol and 1µl of glycoblue was then added to this aqueous phase and vortexed for 15-20 seconds, before resting for 10 minutes.

8. The suspension was subsequently centrifuged for 8 minutes at 4°C at 13,000 rpm.

9. The supernatant was carefully removed and discarded.

10. 500µl of 75% Ethanol was added, and the suspension was further centrifuged at 4°C at 13,000 rpm for 5 minutes.

11. The Ethanol was carefully removed and the RNA pellet was dried at room temperature for 4-5 minutes.

12. The RNA was then re-suspended in 12µl of RNase free water and quantified using a NanoDrop spectrophotometer (Labtech, International, Sussex, UK).

13. Extracted RNA was stored at -80°C.

3.10. Reverse transcription of extracted total cellular RNA

Extracted RNA was reverse transcribed using a Quantitect reverse transcription kit (Qiagen, Hilden, Germany).

1. 0.5µg of extracted RNA was added to 2µl of gDNA wipeout solution (Qiagen, Hilden, Germany) and the total volume was then made up to 14µl with RNase free water.

2. Samples were kept on ice once prepared.
3. A mastermix was subsequently prepared, with 1µl/sample of Quantitect Reverse Transcriptase (Qiagen, Hilden, Germany), 4µl/sample of Quantitect Buffer solution (Qiagen, Hilden, Germany) and 1µl/sample of R.T. primer mix (Qiagen, Hilden, Germany).

4. Samples were then placed on a standard PCR machine and heated to 42°c for 2 minutes.

5. 6µl of master mix was added to each sample, and they were then heated to 42°c for 15 minutes.

6. Finally samples were heated to 95°c for 3 minutes.

7. cDNA was subsequently quantified using a NanoDrop spectrophotometer (Labtech, International, Sussex, UK) (see pg. 105).
3.11. Quantitation of PD-1/PDL1 mRNA by real time PCR

cDNA from reverse transcription of extracted total cellular RNA was used to quantitate PD-1/PDL1/PDL2 mRNA through real time PCR. Real time PCR was performed with SYBR green using the Quantitect Two-Step RT-PCR according to manufacturer’s instructions on an ABI 7500 Real-time PCR machine (Applied Biosystems, Foster City, USA).

For each sample, 1µl of cDNA was added to 12.5µl of SYBR green – a fluorescent dye which binds to all double-stranded DNA molecules emitting a fluorescent signal on binding (Qiagen, Hilden, Germany), 2.5µl of QT primer assay (Qiagen Hilden, Germany) and 9µl of RNAse free water. Samples were placed in a 96 well optical reaction plate (Applied Biosystems).

In order to standardise PD-1/PDL1 quantitation results, cDNA was normalised to β-actin – a widely used control housekeeping gene present in all cells. The presence and stable expression of β-actin, was confirmed in the hepatoma cell lines and PBMCs in which subsequent assessment of PD-1/PDL1 expression was anticipated. β-actin was stably expressed in these cells, and the expression did not significantly change following cell culture (up to 72hrs), or during a “starvation experiment”, where cells were incubated in media in the absence of FCS for 14 hours followed by the addition of 10% FCS. β-actin expression was then evaluated (Figure 16).
Figure 16: β-actin expression following 14 hours “starvation”

β-actin expression remains constant in HepG.2.2.15 cells following 14 hours starvation. β-actin expression is normalised to 0hrs, when 10% FCS was added to HepG2.2.15 cell culture following 14hrs with no FCS in growth media.

The QT primers used were commercially available for PD-1/PDL1 (PDCD1_1_SG, CD274_1_SG) and for β-actin as the house-keeping gene (HB-ACTB_1_SG) (Qiagen, Sussex, UK).

The cycling conditions for two-step PCR are illustrated in Table 5. All plates incorporated a negative control (no template control) to ensure there was no contamination of samples.

Following RT-PCR the results of each plate were analysed with AB 7500 software.
Table 5: Cycling conditions for two-step RT-PCR

<table>
<thead>
<tr>
<th>Stage</th>
<th>TEMP</th>
<th>DURATION</th>
<th>No. of cycles</th>
<th>Additional comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>50°C</td>
<td>2 minutes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td>95°C</td>
<td>15 minutes</td>
<td>1</td>
<td>Activates HotStarTaq DNA polymerase</td>
</tr>
<tr>
<td>Stage 3</td>
<td>94°C</td>
<td>15 secs</td>
<td>40</td>
<td>Denaturation</td>
</tr>
<tr>
<td></td>
<td>55°C</td>
<td>30 secs</td>
<td></td>
<td>Annealing</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>34 secs</td>
<td></td>
<td>Extension</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Fluorescence data collection)</td>
</tr>
<tr>
<td>Stage 4</td>
<td>95°C</td>
<td>15 secs</td>
<td>1</td>
<td>Dissociation curve</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>1 minute</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.12. Purification of HBV-DNA

3.12.1. Purification of HBV-DNA from supernatants

HBV-DNA was extracted and quantitated from the supernatants of cell cultures performed using an in-house protocol which was developed and is described below.

1. 400µl of supernatant was placed in a 1.5mL RNAse/DNAse free sterile eppendorf tube and centrifuged at 15,000rpm for 5 minutes to remove any cell debris.

2. The supernatant from this centrifugation was carefully aspirated and transferred to a new 1.5mL eppendorf tube.

3. 2µl of DNAse I (Sigma, Dorset, UK) (10mg/mL) and 2µl of MgCl₂ (1M) was subsequently added.

4. The supernatant was then vortexed and heated for 1 hour at 37°C.

5. 40µL of proteinase K at 10mg/ml, 10µl of Tris base pH8 at 2M and 27µl of 15% SDS(Sigma, Dorset, UK) were then added sequentially, and the suspension vortexed and heated for 30 minutes at 70°C.

6. The samples were removed from the heating block and phenol:chloroform was added at a 1:1 ratio.

7. MaXtract High Density columns (Qiagen, Sussex, UK) were pre-spun at 15,000rpm for 30s in a microcentrifuge.

8. The samples were then added to the pre-spun columns and centrifuged at 15,000rpm for 5 minutes.
9. The upper aqueous layer was carefully removed and transferred to a clean 1.5ml eppendorf tube.

10. Sodium Acetate (NaAc) at a concentration of 3M was added at a ratio of 1/10 (40µl).

11. 800µl of 100% ethanol was subsequently added to each tube.

12. Samples were then incubated for 1hr at -80°C.

13. Following this incubation, the samples were centrifuged at 15,000rpm for 10 minutes at 4°C.

14. A white pellet was visible at the bottom of the eppendorf following this centrifugation.

15. The supernatant was carefully aspirated and discarded, 0.5ml of 70% ethanol was added to these samples and they were incubated at room temperature for 5 minutes, before a further centrifugation at 15,000rpm for 5 minutes at 4°C.

16. The supernatant was again discarded. The tubes containing the samples were then placed in a speed vac in the fume hood with open lids.

17. Samples were spun for 30 minutes at 45°C to evaporate any remaining liquid.

18. Finally the samples (dried DNA pellet) were resuspended in 30µl of low TE buffer (1mM Tris.cl/0.01mM EDTA pH 8) and digested with 0.3µl RNAse A at 10mg/ml.
19. The quantity and purity of the DNA was then assessed on the nanodrop (see pg. 132).

20. The extracted DNA was subsequently stored at -20°C.
**3.12.2. Purification of intracellular HBV-DNA from cells**

Intracellular HBV-DNA was also isolated and quantified from cryopreserved PBMCs or snap frozen hepatocytes using an in-house technique described below.

1. Cell pellets were resuspended in lysis buffer (50mM Tric HCL pH 7.5; 150mM NaCl; 1% NP40 (now called Ipgal); 1mM EDTA) and incubated for 10 minutes at 37°C in 1.5ml eppendorfs.

2. The resulting suspensions were vortexed for 15-20 seconds, and then centrifuged at 15,000rpm for 2 minutes.

3. The supernatants were transferred to clean 1.5ml tubes and 3µl of 1M MgCl₂ was added to each tube.

4. Subsequently 10µl of DNAsel solution (10mg/ml) and 5µl RNAse A solution at 10mg/ml was added, and the tubes were vortexed and incubated at 37°C for 2 hours.

5. Following this digestion, the remaining lysate was centrifuged at 15,000rpm for 1 minute, and the supernatant was collected.

6. The following were then successively added to these supernatants: 23µl of 0.25M EDTA, 39µl of 15% SDS, 12µl of 5M NaCl and 12µl of Proteinase K solution (10mg/ml).

7. These samples were then vortexed and incubated at 55°C for 1 hour.

8. Purification was completed by continuing with protocol 3.13.1. from step 6 (the addition of phenol:chloroform)
3.13. Nanodrop Spectrophotometer

The NanoDrop ND-1000 (Labtech, Sussex, UK) is a full-spectrum (220-750nm) spectrophotometer that measures 1µl samples with high accuracy and reproducibility. A sample is pipetted onto the end of a fibre optic cable (the receiving fibre). A second fibre optic cable (the source fibre) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fibre optic ends (see Figure 17). A pulsed xenon flash lamp provides the light source and a spectrometer utilizing linear CCD array is used to analyse the light after passing through the sample.

Figure 17: The Nanodrop spectrophotometer

The ratio of absorbance at 260nm and 280nm is measured to assess the purity of DNA and RNA. A 260/280 ratio of ~1.8 is generally accepted as “pure” for DNA; a ratio of ~2.0 is generally accepted as “pure” for RNA. The quantity of RNA / DNA is also assessed in ng/ul based on absorbance at 260 nm.
3.14. HBV-DNA quantitation with Polymerase Chain Reaction (PCR)

Extracted HBV-DNA from supernatants and cells was quantitated using RT-PCR. The standards were prepared (Table 6) from an HBV plasmid donated by Dr John Taylor (University of Auckland, New Zealand). This plasmid had 1.3 copies of genome / molecule. The HBV genome was again under a CMV promoter. The stock solution provided was $7.5 \times 10^8$ copies /ml. The standard curve was validated using the WHO HBV international standard 97/746 (National Institute for Biological Reference Standards, Potters Bar, UK). HBV DNA quantitation was normalized using human β-actin, which was quantitated with a commercial human β-actin kit (Eurogentec Ltd., Hampshire, UK).
Table 6: Preparation of standard for HBV quantification

<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>HBV plasmid</th>
<th>Volume of RNase/DNase free H₂O added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>9uL neat</td>
<td>36uL</td>
</tr>
<tr>
<td>1:10</td>
<td>4.5uL neat</td>
<td>40.5uL</td>
</tr>
<tr>
<td>1:100</td>
<td>4.5uL 1/10</td>
<td>40.5uL</td>
</tr>
<tr>
<td>1:1000</td>
<td>4.5uL 1/100</td>
<td>40.5uL</td>
</tr>
<tr>
<td>1:1x10⁴</td>
<td>4.5uL 1/1000</td>
<td>40.5uL</td>
</tr>
<tr>
<td>1:1x10⁵</td>
<td>4.5uL 1/1x10⁴</td>
<td>40.5uL</td>
</tr>
</tbody>
</table>

Amplification was performed in 25 µl reactions (in triplicate for all samples) containing 12.5µl of 2×TaqMan Universal PCR mix, 0.6 µM (0.225µl) forward and reverse primers (HBV core 2253-2274, HBV core 2422-2405), 0.15 µM probe (0.05µl) (HBVcprobe:2279-2304-JOE), 7µl of RNAse free water and 5 µl of template.

A mastermix containing the primers, probe and universal PCR mix was prepared and 20µl of this was pipette into designated wells in a 96-well optical reaction plate (Applied Biosystems, Warrington, UK). 5µl of sample was then added to respective wells. The plate was then covered with an adhesive cover and centrifuged at 3000rpm for 2 minutes. The plate was then loaded into a 7500 ABI real-time PCR.
machine (Applied Biosystems, Warrington, UK). Table 7 below illustrates the PCR-set up for HBV-DNA absolute quantitation:

Table 7: Cycling conditions for absolute quantitation of HBV-DNA

<table>
<thead>
<tr>
<th>Stage</th>
<th>TEMP</th>
<th>DURATION</th>
<th>No. of cycles</th>
<th>Additional comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>50°C</td>
<td>2 minutes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td>95°C</td>
<td>10 minutes</td>
<td>1</td>
<td>Activates HotStarTaq DNA polymerase</td>
</tr>
<tr>
<td>Stage 3</td>
<td>94°C</td>
<td>15 secs</td>
<td>40</td>
<td>Denaturation</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>1 minute</td>
<td></td>
<td>Annealing &amp; Extension</td>
</tr>
</tbody>
</table>
3.15. Enumeration of HBV-specific, IFNγ-producing T-cells by Elispot assays

3.15.1. Principle

The Elispot (enzyme-linked immunospot) assay is used for the detection and quantitation of individual cells secreting specific cytokines in response to an antigenic stimulus. The Elispot assays are reproducible and sensitive and accredited to GCLP standards in our laboratory.

This section describes the procedure for the detection of HBcAg-specific IFNγ producing cells. The detection of other cells secreting other cytokines (e.g. IL-4, IL-5, IL-10) can also be performed using appropriate antibody kits.

3.15.2. Materials

Equipment:

1. Class II laminar flow cabinet.
2. 37⁰C / 5% CO₂ incubator.
3. Stereomicroscope or automated Elispot reader system.
4. Multi-channel pipette.

Reagents:

2. Phytohaemagglutinin (Sigma, Poole, UK) – mitogen used as positive control.
3. 96-well round-bottomed tissue culture plates
4. Tetanus toxoid – positive control (recall antigen) – (Connaught Int. Laboratories, Ontario, Canada).

5. Elispot kit for human interferon-γ (Mabtech, Nacka, Sweden).

6. PVDF (polyvinylidenedifluoride) – backed microplates (Millipore, MA, USA)

7. Human AB serum

8. Buffered RPMI 1640 media (see prev)

9. BCIP (5-Bromo-4-chloro-3-indoyl phosphate / NBT (Nitrotetrazolium blue chloride) tablets (Roche, Lewis, UK).

10. Buffers:
   a. 70% ethanol
   b. Phosphate buffered saline (PBS)
   c. PBS/0.05% Tween
   d. PBS/1% bovine serum albumin

3.15.3. Method

1. PBMCs are prepared as described in previous section (3.4.1).

2. The protocol was split over 3 days required to perform the assay (see Figure 18 & 19).
Figure 18: Schematic illustrating Elispot methodology

(adapted from: Generalized Steps of the ELISPOT Assay Procedure – Sigma-Aldrich).
Figure 19: Representative plates showing PBMCs activated with PMA / Ionomycin in a human IFNγ ELISPOT assay

Day 1: Stimulation of cells

1. PBMCs concentration was adjusted to $2 \times 10^6$ PBMCs/ml.

2. Antigens were prepared at working concentrations in buffered RPMI / 10% human AB serum as shown in Table 8 below:
### Table 8: Preparation of antigens for ELISPOT

<table>
<thead>
<tr>
<th>Well</th>
<th>Antigen</th>
<th>Working concentration</th>
<th>Final Concentration in well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No antigen</td>
<td>Medium only</td>
<td>Medium only</td>
</tr>
<tr>
<td>2</td>
<td>HBcAg</td>
<td>4µg/ml</td>
<td>2µg/ml</td>
</tr>
<tr>
<td>3</td>
<td>Tetanus Toxoid</td>
<td>1µg/ml</td>
<td>0.5µg/ml</td>
</tr>
<tr>
<td>4</td>
<td>PHA</td>
<td>2µg/ml</td>
<td>1µg/ml</td>
</tr>
</tbody>
</table>

3. 100µl of each antigen was added per well in triplicate to a round bottom 96 well tissue culture plate.

4. 100µl of PBMC suspension at $2 \times 10^6$/ml was added to each experimental well.

5. Tissue culture plates were incubated for 24 hours at 37°C in a CO₂ incubator.

### Day 2: Preparation of Elispot plate

1. Add 100µl of 70% Ethanol to the required wells of the Elispot 96 well plate.

2. Incubate the plate for 15 minutes at room temperature with the prepared captured antibody (supplied in kit) 100µl of 10µg/ml concentration per well.
3. Add 200µl of sterile PBS to each well using a multichannel pipette. Flick off PBS, blot plate on tissue thoroughly to remove all PBS. Repeat this wash step a further 5 times.

4. Add 100µl of capture antibody to each experimental well.

5. Incubate at 4°C for 6 hours in the dark.

6. Wash each well with 200µl of sterile PBS using a multichannel pipette. Perform 6 washes.

7. Add 100µl of buffered RPMI/10% AB serum to block the membrane and incubate plate(s) for 1 hour at 37°C in a CO₂ incubator.

8. Flick off buffered RPMI/10% AB serum and blot plate.

9. Transfer PBMCs prepared on day 1 to corresponding wells on the Elispot plate.

10. Incubate plate(s) for 20 hours at 37°C in a CO₂ incubator.

Day 3: Development

1. Flick cells off and wash Elispot plate with PBS/0.05% Tween.

2. Wash twice with PBS.

3. Wash twice with distilled water.

4. Prepare 1µg/ml concentration of biotinylated anti-IFNγ antibody in PBS 1% bovine serum albumin.
5. Add 100µl of antibody to each experiment well of the Elispot plate.

6. Incubate for 2 hours at room temperature.

7. Wash 5 x with PBS/0.05% Tween.

8. Wash 2 x with PBS.

9. Prepare 1:1000 dilution of the streptavidin-alkaline phosphatase conjugate in PBS 1% bovine serum albumin.

10. Add 100µl of streptavidin solution to each experiment well of the Elispot plate.

11. Incubate for 1.5 hours at room temperature.

12. Wash 6 x with PBS.

13. Wash 1 x with distilled water.

14. Prepare the BCIP (5-Bromo-4-chloro-3-indoyl phosphate / NBT (Nitrotetrazolium blue chloride)(Roche, Lewis, UK) solution by adding 1 tablet to 10ml of distilled water.

15. Add 100µl of BCIP/NBT solution to each experiment well of the Elispot plate.

16. Allow colour to develop for 15 minutes. At this point flick off BCIP/NBT solution and immerse in tray of tap water.

17. Flick off water and rinse thoroughly under running water. Ensure each well is filled and emptied at least 5 times.

18. Blot dry, remove plastic base and dry inverted.

Notes on Elispot assay:

- Ensure strict adherence to aseptic technique on Day 1 and 2 of the protocol, after which the assay can be performed on the bench.

- AB serum and recombinant HBcAg must be batch tested as prone to inherent variability.

- If performed on cryopreserved cells, the assay is not reliable if the viability of the cells falls below 95%.

- Always run assay with a minimum of 3 replicates to reduce variability.

- Each spot represents an antigen-specific interferon-\(\gamma\) producing cell. Results are usually expressed as the number of spot forming cells per million PBMCs. As there are 200,000 PBMCs in this assay the number of SFC is multiplied by 5 to get the SFC/\(10^6\) PBMCs.
3.16. Flow Cytometry

3.16.1. Principle

Flow cytometry is a technique for counting and examining individual cells suspended in a stream of fluid. This technique allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of individual cells flowing through an optical, electronic detection system.

A beam of light of a single wavelength is directed onto a hydrodynamically-focused stream of fluid. A number of detectors are aimed at a point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors). Each cell passing through the beam scatters light in some way, and fluorescent markers present within the cells, or attached to the surface of the cells (e.g. bound to a cell surface receptor), can be excited and emit light at a different wavelength than the light source. By detecting the combination of light scatter and fluorescent light detected, it is possible to derive information about the physical and chemical structure of each particle (Figure 20). For example FSC correlates with the cell volume, whilst SSC is influenced by the inner complexity of the cell (e.g. shape of nucleus etc).
Figure 20: Principle of Flow Cytometry
The data from flow cytometric analysis is usually represented in 2 dimensional representative dot plots. The regions of these plots can be separated by creating a series of subset extractions, termed “gates” (Figure 21).

**Figure 21: Representative dot and plot illustration of flow cytometric data and how “gates” are applied to study cell subsets**

(Representative dot plot illustrating cell gating strategy for the selection of CD3+CD8+cell from PBMCs)

This technique can be utilised with fluorescent tagged antibodies that will bind to specific antigens on the target cells and fluoresce following appropriate excitation.
The following commercially available fluorescent antibodies were used for PBMC and hepatocyte staining throughout the course of these experiments:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD8/APC-Cy7</td>
<td>Anti-CD3/PE-Cy7</td>
</tr>
<tr>
<td>Anti-PD-1/PE</td>
<td>Isotype control/PE</td>
</tr>
<tr>
<td>Anti-CD45RA/PECy7</td>
<td>Anti-CD127/PE</td>
</tr>
<tr>
<td>Anti-CD62L/PE</td>
<td>Anti-CD4/APCCy7</td>
</tr>
<tr>
<td>Anti-PDL1/PeCy7</td>
<td></td>
</tr>
</tbody>
</table>

(All antibodies were obtained from BD Biosciences, Oxford, UK).

Virus-specific T-cells were also identified by using Class I - peptide tetrameric and pentameric complexes. These tetramers/pentamers are produced using MHC-peptide complexes that mimic the situation on the surface of an infected cell.

These pentamers are limited in use to cells from patients who are HLA-A2 positive, as this is the basis of the MHC component of these tetramers.

HBV-specific CD8+T-cells were identified with HLA-A2 pentamers containing HBcAg 18-27 peptide (FLPSDFFPSV) and HBsAg 183-191 peptide (FLLTRILTI), which
were labelled with the fluorochrome Allophycocyanin (APC) (Proimmune, Oxford, UK). CMV-specific CD8+ T-cells were identified with an APC-labelled HLA-A2 pentamer containing CMV pp65 495-504 (NLVPMVATV). According to published guidelines (Rehermann et al 2007) background PBMC staining with pentamers was established using PBMCs from 10 HLA-A2 negative patients, and a cut-off of 0.02% was identified.

3.16.2. Staining Protocol of PBMCs for Flow Cytometric Analysis

1) PBMCs were thawed using the method previously described.

2) The PBMC’s were then resuspended in 1ml of PBS/1%FCS.

3) The cell number and viability were assessed with tryptan blue as previously described.

4) A further 9ml of PBS/1%FCS was added, and the cells were centrifuged at 1400rpm for 10 minutes.

5) The supernatant was discarded and the cell concentration was adjusted to 2.5 million PBMCs/100µl of PBS/1%FCS.

6) 5 x 10^6 cells/200µl was aliquoted into wells in a 96 well U-bottomed plate.

7) HBV and CMV specific pentamers were then briefly centrifuged (few seconds at 15,000rpm) and then 10µl was aliquoted into each well.

8) The cells were incubated at room temperature in the dark for 15 minutes to allow pentamer staining.
9) 100µl of PBS/1%FCS was then added, and the cells were centrifuged at 1500rpm for 5 minutes.

10) The supernatant was discarded and the cells resuspended in 50µl/well of PBS/50% mouse serum. The cells were rested for 5 minutes to allow non-specific binding.

11) The fluorescent tagged antibodies were then added to stain cell surface markers of interest: αCD3(PECy7) 3µl; αCD8(APC-Cy7) 10µl; αPD-1(PE) 10µl, isotype control (PE) (for PD-1) 10µl; αCD45RA(PECy7) 10µl; αCD127(PE) 10µl; αCD62L(PE) 10µl; αCD4(APCCy7) 10µl.

12) Cells were then rested for 30 minutes at 4°C to allow staining to occur.

13) Following this staining, 100µl of PBS/50% mouse serum was added to each well and the cells were centrifuged at 1500rpm for 5 minutes.

14) The supernatants were again discarded, and cells were resuspended in 200µl/well of PBS / 50% mouse serum and again centrifuged for 5 minutes at 1500rpm.

15) Finally the cells were resuspended in 200µl/well of PBS / 1%FCS and acquired on a Becton Dickinson FACS Array and analysed with BD FACS-Canto software.

Lymphocytes were gated according to their physical parameters (forward and side scatter) and CD3+CD8+ lymphocytes were then selected. Virus-specific CD8+T-cells were examined as a percentage of the total CD8+T-cell population, as well as the Mean Fluorescent Intensity (MFI) on the pentamer positive cells.
The memory phenotype of CD4+ T-cells was determined by staining with fluorochrome-labelled CD45RA and CD62L antibodies. Four subsets of memory cells were identified: naive cells (N: CD45RA+/CD62L+), central memory cells (CM: CD45RA-/CD62L+), effector memory cells (EM: CD45RA-/CD62L-) and effector cells (E: CD45RA+/CD62L-).

The memory phenotype of CD8+ T-cells was determined by staining with fluorochrome-labelled CD45RA and CD127 antibodies. Four subsets of memory cells were identified: naive cells (N: CD45RA+/CD127+), central memory cells (CM: CD45RA-/CD127+), effector memory cells (EM: CD45RA-/CD127-) and effector cells (E: CD45RA+/CD127-). The frequency of each memory subsets was evaluated within the total CD4+ or CD8+ populations. For detection of HLA-A2 positive cases, PBMC were labelled with a mouse anti-human HLA-A2 (OneLambda, Inc., San Diego, CA), followed by a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody (Sigma, Dorset, UK).

3.16.3. Staining of hepatocytes and virus-specific CD8+ T-cell clone from cell co-cultures for Flow Cytometric Analysis

Hepatocytes were gently harvested using a cell dissociation solution (Accutase - eBioscience, Hatfield, UK) from cell culture experiments and resuspended in PBS/1% FCS at a concentration of 3 x 10^5 cells/100µl. Staining was then carried out on the hepatocytes or T-cell clone, as described above with the fluorescent labelled antibody of interest: The following antibodies were used to stain T-cells: Anti-
CD8(APC-Cy7) 10µl; Anti-PD-1(PE) 10µl; Isotype control(PE) 10µl (BD Biosciences, Oxford, UK). The following antibodies were used to stain hepatocytes: Anti-PDL1(PeCy7) 10µl (BD Biosciences, Oxford, UK). The specificity of the T-cell clone to HBcAg 18-27 was confirmed by FACS analysis after staining with HLA-A2 pentamers containing HBcAg 18-27 peptide (FLPSDFFPSV) labelled with the fluorochrome Allophycocyanin(APC) (Proimmune, Oxford, UK). After washing the stained cells were re-suspended in PBS/1%FCS and acquired on a Becton-Dickinson FACS Canto II and analysed. Subsequent analysis was performed using FACSDiva software.

3.16.4. Assessment of cytotoxicity by Annexin V/PI staining

The proportion of apoptotic 2.2.15 cells was assessed using the TACS™ Annexin V-FITC Apoptosis detection kit (R&D Systems, Abingdon, UK). Cells were stained with Annexin V and PI to assess the proportion of cells undergoing apoptosis. Propidium iodide (PI) intercalates into double-stranded nucleic acids. It is excluded by viable cells but can penetrate cell membranes of dying or dead cells, Thus cells which are dead or in the late apoptotic stage of death will stain positive for PI. Cells undergoing apoptosis also express phosphatidylserine on their cell surface. Annexin A5 is a protein that binds to phosphatidylserine containing membrane surfaces. Thus cells in the early and late phase of apoptosis will stain positive for Annexin V (see Fig. 22). By combining PI and Annexin V staining it is therefore possible to distinguish viable cells from those in the early and late phases of apoptosis.
3.16.5. Assessment of cytotoxicity through ALT measurements

The proportion of HepG2.2.15 cells undergoing cytolysis was also evaluated through the assessment of ALT (an enzyme released from hepatocytes undergoing cytolysis), in the supernatants of the cell co-culture experiments. 200µl of supernatant was centrifuged on a microcentrifuge at 13000rpm for 5 minutes and then directly processed through an automated clinical chemistry analyzer (COBAS Integra 400, Roche Diagnostics, Ltd, Sussex, UK). This analyser directly measures ALT in samples processed comparing them against a standard curve generated from stock solutions.
3.17. Cytometric Bead Array (CBA)

3.17.1. Principle

Cytometric bead array (CBA) utilises the principles of flow cytometry and employs particles with discrete fluorescent intensities to detect soluble analytes. This test uses single bead populations with distinct fluorescence intensity coated with a capture antibody specific for a soluble protein (e.g. IL-10). Each bead population (flex set) is given an alphanumerical position designation indicating its position relative to other bead populations.

3.17.2. Cytometer Optimisation

Cytometer Set-up and Tracking (CST) beads (BD Biosciences, Oxford, UK) allow the software to automatically characterise, track and report measurements of supporting BD digital flow cytometers. Each vial of CST beads contains equal concentrations of beads of 3 fluorescent emission intensities. The beads are used to define a cytometer baseline. Median fluorescent intensity (MFI) and robust CV (rCV) are measured for each bead in all fluorescent detectors. The software then calculates the fluorescence detection efficiency (Qr), relative background (Br), the standard deviation of electronic noise, and cytometer settings can subsequently be adjusted for maximising population resolution in each detector.
3.17.3. Preparation of CBA Human Soluble Protein Flex Set Standards

For each multiplex assay a standard curve must first be prepared. One lyophilized standard vial from each Flex Set to be tested was opened and pooled together into one tube, labelled “Top Standard”. These standards were then reconstituted with 4.0 ml of Assay Diluent and allowed to stand for 15 minutes. Tubes for the standard dilutions were subsequently prepared (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256), and 500 µl of assay diluent was added to each of the remaining tubes. A serial dilution was then performed, transferring 500 µl from the “top standard” to the 1:2 dilution tube and so forth.

3.17.4. Preparation of CBA Human Soluble Protein Flex Set Capture Beads and PE detection reagents

Each capture bead stock (BD Biosciences, Oxford, UK) was vortexed for at least 15 seconds to resuspend the beads thoroughly. The total volume of diluted beads needed was calculated by the number of tests to be performed multiplied by 50 µl (volume of diluted beads needed for each test). The volume of capture beads and capture bead diluents (BD Biosciences, Oxford, UK) was then calculated and combined in a tube labelled “Mixed Capture Beads”.

The PE detection reagents provided with each Flex set were mixed and diluted with Detection Reagent Diluent (BD Biosciences, Oxford, UK) to their optimal volume per test (50 µl/test). The Detection Reagents and Detectin Reagent Diluent was then combined in a tube labelled “Mixed PE Detection Reagents”. The PE detection
reagents should be protected from exposure to direct light as they can become photobleached and lose fluorescent integrity.

3.17.5. CBA Assay Procedure

1) Mixed capture beads were vortexed for 5 seconds and 50µl was added to each assay tube.

2) 50µl of the standard or the sample to be tested was then added to each assay tube.

3) The assay tubes were then incubated for 1 hour at room temperature.

4) 50µl of Mixed PE Detection Reagent was added to each assay tube.

5) The assay tubes were then incubated for 2 hours at room temperature.

6) 1mL of Wash Buffer was added to each assay tube and the tubes were centrifuged at 13,000rpm for 5 minutes.

7) The supernatant was aspirated and discarded from each tube.

8) 300µl of Wash buffer was added to each assay tube.

9) Finally, the assay tube was briefly vortexed to resuspend the beads and the samples were acquired and analysed on a B.D. FACS Canto II.

10) The concentration of all cytokines was calculated using BD FCAP Array software (BD Biosciences, Oxford, UK).
3.18. Statistical Analysis

Changes in PD-1/PDL1/L2 expression at different time points/different experimental conditions were analysed by Student’s t-test and Mann-Whitney U test. Correlation between HBV-DNA levels over time and PD-1/PDL1 expression by FACS was assessed by Pearson’s correlation analysis. Correlations between HBV-DNA levels, PD-1 expression, memory phenotype and the frequency of virus-specific IFNγ & IL-10 producing T-cells were assessed by Pearson’s correlation analysis.
Chapter 4

PD-1 Expression During Antiviral Treatment of Chronic Hepatitis B: Impact of HBeAg Seroconversion
4. PD-1 EXPRESSION DURING ANTIVIRAL TREATMENT OF CHRONIC HEPATITIS B: IMPACT OF HBeAg SEROCONVERSION

4.1. Background to study

Virus-specific T-cells are functionally impaired, but the relative role of viraemia (HBV-DNA levels) and/or HBeAg in the impairment of T-cell reactivity have not been defined. The biological function of HBeAg is not fully understood, as it is not required for virus assembly, infection or replication (Milich et al 2003). HBeAg is secreted from hepatocytes and is thought to have a central role as a tolerogen during HBV infection and during vertical transmission appears to be fundamental to the induction of immunological tolerance in utero (Milich et al 1990). It is also well established that HBeAg seroconversion is a T-cell driven phenomenon and therefore it is likely that expression of PD-1, an immune-inhibitory T-cell pathway may be important in HBeAg seroconversion. The relative expression of PD-1 before and after HBeAg seroconversion, in the absence of changes in HBV-DNA, may provide useful information as to the mechanism of tolerance induced by the Hepatitis B e antigen.

To gain further understanding of the role of PD-1 in chronic HBV infection the relationship between PD-1 expression, viral load and HBeAg in patients with chronic hepatitis B undergoing treatment with oral antiviral agents was investigated longitudinally. The impact of changes in viral load and PD-1 levels on the frequency of virus-specific T-cells producing IFN-γ and IL-10 was also examined. In addition, the memory phenotype of the T-cells during treatment, particularly before and after HBeAg seroconversion was characterised.
4.2. Materials and Methods

Patients

Eighteen treatment-naïve patients with chronic hepatitis B infection, attending the Hepatitis Clinic at University College Hospital, London, were enrolled into the study (Table 9). All patients were seropositive for HBsAg, hepatitis B e-antigen (HBeAg), with HBV-DNA levels >10^6 IU/ml. All patients were negative for anti-hepatitis D virus, anti-hepatitis C virus, anti-human immunodeficiency virus (HIV1/2), and autoantibodies. Twelve of the patients were male and the mean age of the patients was 38.9±9.9yrs. A liver biopsy was performed in all patients as part of a routine diagnostic evaluation and the inflammation grade and fibrosis stage were scored according to established criteria (Ishak et al 1995). Patients were followed serially with protocol visits for a median period of 18 months (range 12-25 months) during a course of antiviral treatment with nucleoside analogues (Telbivudine or Lamivudine). Written informed consent was obtained from each patient and the study protocol was approved by the Ethics Committee of University College London Hospitals. During treatment 6 patients seroconverted to anti-HBe (Group 1) after a median period of 9 months (range 6-10 months), while 12 patients (Group 2) remained HBeAg positive throughout the monitoring period – median 18 months (range 12-25 months).
### Table 9: Baseline characteristics of patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>HBV DNA Log_{10} copies/ml</th>
<th>ALT IU/ml</th>
<th>Ishak Fibrosis score</th>
<th>HBeAg status</th>
<th>% pent* (+) on total CD8+ T-cells</th>
<th>% of PD-1(+) on pent*(+) CD8+ T-cells</th>
<th>% of PD-1(+) on total CD8+ T-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.52</td>
<td>206</td>
<td>1</td>
<td>+ve</td>
<td>0.41</td>
<td>3.72</td>
<td>9.17</td>
</tr>
<tr>
<td>2</td>
<td>9.78</td>
<td>233</td>
<td>1</td>
<td>+ve</td>
<td>1.29</td>
<td>4.73</td>
<td>3.34</td>
</tr>
<tr>
<td>3</td>
<td>9.11</td>
<td>436</td>
<td>1</td>
<td>+ve</td>
<td>0.91</td>
<td>2.69</td>
<td>5.67</td>
</tr>
<tr>
<td>4</td>
<td>8.48</td>
<td>81</td>
<td>4</td>
<td>+ve</td>
<td>1.34</td>
<td>1.47</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>8.59</td>
<td>363</td>
<td>3</td>
<td>+ve</td>
<td>0.89</td>
<td>1.57</td>
<td>2.95</td>
</tr>
<tr>
<td>6</td>
<td>9.08</td>
<td>221</td>
<td>1</td>
<td>+ve</td>
<td>0.60</td>
<td>4.77</td>
<td>5.12</td>
</tr>
<tr>
<td>7</td>
<td>8.05</td>
<td>48</td>
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<td>+ve</td>
<td>1.23</td>
<td>7.19</td>
<td>9.75</td>
</tr>
<tr>
<td>8</td>
<td>7.76</td>
<td>82</td>
<td>3</td>
<td>+ve</td>
<td>0.76</td>
<td>nd</td>
<td>0.05</td>
</tr>
<tr>
<td>9</td>
<td>9.25</td>
<td>113</td>
<td>4</td>
<td>+ve</td>
<td>1.04</td>
<td>nd</td>
<td>3.95</td>
</tr>
<tr>
<td>10</td>
<td>8.19</td>
<td>252</td>
<td>2</td>
<td>+ve</td>
<td>0.45</td>
<td>nd</td>
<td>Nd</td>
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<tr>
<td>11</td>
<td>7.37</td>
<td>59</td>
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<td>+ve</td>
<td>0.99</td>
<td>3.21</td>
<td>6.04</td>
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<tr>
<td>12</td>
<td>7.93</td>
<td>35</td>
<td>5</td>
<td>+ve</td>
<td>0.76</td>
<td>nd</td>
<td>Nd</td>
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<tr>
<td>13</td>
<td>6.92</td>
<td>107</td>
<td>1</td>
<td>+ve</td>
<td>0.59</td>
<td>nd</td>
<td>Nd</td>
</tr>
<tr>
<td>14</td>
<td>10.07</td>
<td>153</td>
<td>2</td>
<td>+ve</td>
<td>0.90</td>
<td>nd</td>
<td>1.37</td>
</tr>
<tr>
<td>15</td>
<td>7.06</td>
<td>99</td>
<td>1</td>
<td>+ve</td>
<td>1.98</td>
<td>0.07</td>
<td>Nd</td>
</tr>
<tr>
<td>16</td>
<td>8.78</td>
<td>56</td>
<td>3</td>
<td>+ve</td>
<td>1.46</td>
<td>6.27</td>
<td>7.75</td>
</tr>
<tr>
<td>17</td>
<td>7.75</td>
<td>139</td>
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<td>+ve</td>
<td>0.63</td>
<td>nd</td>
<td>1.16</td>
</tr>
<tr>
<td>18</td>
<td>8.56</td>
<td>150</td>
<td>2</td>
<td>+ve</td>
<td>0.54</td>
<td>1.47</td>
<td>2.25</td>
</tr>
</tbody>
</table>

*Combined frequency detected with pentamers for both HBcAg and HBsAg.*
Investigations were focused on three time points with heparinised blood for separation of peripheral blood mononuclear cells (PBMC) obtained serially from all patients (Table 10).

**Table 10: Timepoints when peripheral blood mononuclear cells were obtained from heparinised blood for analysis**

<table>
<thead>
<tr>
<th>Timepoints</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time point 1(T1)</strong></td>
<td>Baseline - high viral load, HBeAg positive</td>
</tr>
<tr>
<td><strong>Time point 2(T2)</strong></td>
<td>Early on antiviral treatment (treatment week TW12-16) - reduced viremia, all patients HBeAg positive;</td>
</tr>
<tr>
<td><strong>Time point 3(T3)</strong></td>
<td>Late on antiviral treatment (TW32-56) - undetectable or very low serum HBV-DNA (&lt;10^3 copies/ml) with or without HBeAg loss and anti-HBe positivity in Group 1 and 2 respectively.</td>
</tr>
</tbody>
</table>
Hepatitis Serology

HBsAg, HBeAg, anti-HBe, anti-human immunodeficiency virus(1&2) were determined by commercial immunoassays (Abbott Laboratories, Maidenhead, UK). Anti-HCV was detected using Ortho HCV 3.0 ELISA (OrthoDiagnostics, High Wycombe, UK). Serum HBV-DNA was quantified by a sensitive real time PCR technique with a lower limit of quantitation of 300 copies/ml (Garson et al 2005).

Flow Cytometry

Flow cytometric assessment was performed according to methods previously described (see Chapter 3). Due to limitations in the number of cells available and the relatively low frequency of virus-specific T-cells, staining was carried out simultaneously with pentamers to both HBsAg and HBcAg performed.

Quantitation of PD-1 mRNA by real time PCR

Total RNA was extracted from 5x10^5 PBMCs obtained from the same time points as described above. RNA was extracted, quantitated and reverse transcribed according to techniques previously described (see Chapter 3).
Enumeration of HBV-specific, IFNγ and IL-10 producing T-cells by Elispot assays

The Elispot assays were performed with freshly isolated PBMCs from the time points described above. Enumeration of HBV-specific, IFNγ and IL-10 producing T-cells by Elispot assays was performed according to previously described methods (see Chapter 3).
4.3. Results

4.3.1. Clinical Outcomes

Six of 18 patients enrolled lost HBeAg and became anti-HBe(+) (Group 1), while 12 patients remained HBeAg+ (Group 2). At baseline, serum HBV-DNA levels were comparable between the two groups, while patients in Group 1 had significantly higher ALT levels (p<0.01, Table 10). The profound suppression of HBV replication early after starting antiviral treatment (T1 to T2) was paralleled by a decrease in serum ALT levels with no significant differences in the magnitude of HBV-DNA or ALT reductions between the two groups (Table 11). Fifteen of 18 patients had undetectable serum HBV-DNA levels by time point 3 (T3).
Table 11: Changes in serum HBV-DNA and ALT levels over course of antiviral treatment

<table>
<thead>
<tr>
<th></th>
<th>HBV-DNA (log₁₀ copies/ml)</th>
<th>Δ serum HBV DNA (log₁₀ copies/ml)</th>
<th>ALT baseline (IU/ml)</th>
<th>Δ serum ALT levels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 1</strong></td>
<td>9.1±0.5</td>
<td>5.1±1.1</td>
<td>256±125</td>
<td>97±128</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>133±91</td>
</tr>
<tr>
<td></td>
<td>(♦)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td>8.1±0.9</td>
<td>4.2±1.2</td>
<td>107±60.5</td>
<td>23±78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>46±70</td>
</tr>
<tr>
<td></td>
<td>(♦)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(The baseline values are reported as mean±standard deviation. The differences (Δ) in HBV DNA or ALT levels between the time points (T1-T2 and T2-T3)) are shown as mean±standard deviation. (♦) indicates a significant difference between Group 1 and 2 (p<0.01).
4.3.2. Longitudinal Analysis of PD-1 expression by flow cytometry

In all patients the frequency of HBV-specific CD8+ T-cells (combination of pentamers including HBcAg and HBsAg epitopes) ranged between 0.4 and 1.98% of the total CD8+ T-cells and did not change between the three time points of the study (Table 8). In contrast, PD-1 expression significantly decreased both on total CD8+ and HBV-specific CD8+ T cells in all patients (Figures 23 & 24). During anti-viral treatment between T1 and T3, the proportion of PD-1 positive cells within the total CD8+ T-cells decreased from 4.98±0.94 to 1.71±0.86, respectively (p=0.002) and within HBV-specific CD8+ T-cells from 3.28±1.05 to 0.95±0.75, (p=0.01).
Figure 23: PD-1 expression on total CD8+ T-cells, CMV-specific CD8+ T-cells and cell gating strategy

(A) Representative dot plot illustrating cell gating strategy for the selection of CD3+CD8+PD-1+ cells from PBMCs.

(B) Representative dot plots for PBMCs from 2 patients with chronic HBV infection stained with anti-CD3, anti-CD8 and anti-PD-1, at baseline (T1) and following a course of anti-viral therapy (T3). A reduction in PD-1 expression on CD8+ T-cells over time is illustrated in 2 representative patients.

(C) Representative dot plots for PBMCs illustrating staining with anti-CD3, anti-CD8, anti-PD-1 and CMV-specific pentamers. Levels of PD-1+ CMV-specific CD8 T-cells were low and did not significantly change from T1 to T3.
Figure 24: PD-1 expression on total CD8+ and HBV-specific CD8+ T-cells during a course of anti-viral therapy

(Decrease in PD-1 expression seen both in terms of percentage of cells staining positive for PD-1 (Panel A & B) and Mean Fluorescent Intensity (MFI), (Panel C & D), from baseline(T1) through a course of anti-viral treatment. This decrease was observed in both total CD8+ T-cells (Panel A & C) and in virus-specific CD8+ populations (Panel B & D). Each line represents changes in an individual patients’ PD-1 expression from baseline(T1) to T3. Changes in HBV-DNA levels from T1 to T3 are shown as bar graphs.)
The levels of PD-1 expression, as assessed by MFI, were also reduced between the same time points: total CD8 subset T1:721±206.59, T3:383.59±71.27 (p=0.03) and HBV-specific subset T1:762.38±280.26, T3:528±170.3 (p=0.05).

In contrast to HBV-specific CD8+T-cells, CMV-specific CD8+T-cells exhibited low levels of PD-1 positivity which did not change over time (p>0.1) (Figure 23C).

There was no correlation between PD-1 expression on HBV-specific CD8+T-cells and baseline serum ALT levels. Although serum ALT levels were significantly higher in patients who seroconverted on treatment, there was no difference between PD-1 expression between Group 1 and 2 at baseline (p>0.1).
PD-1 expression on CD3+ve/CD8-ve T-cells was also assessed over the three time points described. A significant decrease in both the percentage of CD3+ve/CD8-ve T-cells staining positive for PD-1 (p=0.014) and the MFI (p=0.014) from baseline (T1) to T3 was observed.

HBeAg seroconversion, which occurred in patients in Group 1 between T2 and T3, was associated with a trend towards a decrease in the frequency of PD-1 expressing total CD8+T-cells: T2:3.27±0.86, T3:1.41±0.64 (p=0.068), despite no significant decrease in viral load. It is possible that the sample size (6 patients, Group 1) precluded statistical significance in this observation. In contrast, there was no decrease in PD-1 expression between T2 and T3 in the absence of seroconversion Group 2 (p>0.1). Furthermore, the magnitude of PD-1 changes from T2 to T3 differed between the two groups. A 51% decrease in the frequency of PD-1-expressing HBV-specific CD8+T-cells from T2 to T3 associated with HBeAg seroconversion (Group 1) compared with only a 3% decrease in the absence of seroconversion (Group 2) (p=0.057) was observed.

Three of 18 patients did not achieve undetectable HBV-DNA levels at T3 but also showed a reduction in PD-1 expression between T1 and T3, which mirrored the decrease in serum HBV-DNA levels. In a representative patient, MFI on total CD8 decreased (T1 to T3) from 1509 to 565 for a 3-log drop in HBV-DNA levels. None of these patients had seroconverted, and no significant difference in PD-1 expression was observed between T2 and T3.
During antiviral treatment the frequency of PD-1-expressing virus-specific CD8+T-cells correlated closely with HBV-DNA levels ($r=0.998$, $p=0.036$). The strength of PD-1 expression on CD8+T-cells also decreased in all patients from baseline to T3. This decrease directly correlated with a decrease in HBV-DNA levels ($r=0.996$, $p=0.054$), (Figure 25).
Figure 25: Correlations between PD-1 expression and HBV-DNA levels

(Reduction in PD-1 expression on HBV-specific and total CD8+ T-cells correlates with reduction in HBV-DNA levels. The average HBV-DNA and level of PD-1 expression for each timepoint (T1-T3) are represented (♦). Error bars illustrate standard errors. The four panels demonstrate changes in PD-1 expression on total CD8+ (A & C) and virus specific CD8+ T-cells (B & D), both as changes in MFI (C & D) and changes in percentage of positive cells (A & B).
4.3.3. Longitudinal Analysis of PD-1 mRNA expression

PD-1 mRNA levels decreased significantly during antiviral treatment between T1 and T3 (p=0.001, Figure 26). There was a correlation between PD-1 mRNA and HBV-DNA levels, but this was not statistically significant (r=0.977, p=0.1). A further analysis was performed to assess whether there was a direct correlation between PD-1 expression on the cell surface (as assessed by flow cytometry) and PD-1 mRNA levels, as quantitated by real time PCR. A direct correlation between PD-1 expression on HBV-specific CD8+T-cells in terms of MFI and relative gene expression as assessed by RT-PCR (r=0.994, p=0.07) was observed.
Figure 26: Relative expression of PD-1 at the mRNA level as assessed by Real Time quantitative PCR from baseline to time point 3

(PD-1 expression at the mRNA level is displayed from high to low viral load (T1 – T3). The y-axis represents the relative PD-1 gene expression for each patient from T1 to T3. Each line represents changes in an individual patients’ relative PD-1 expression from T1 to T3. A significant decrease in PD-1 expression was seen (p=0.001)).
4.3.4. Correlation between PD-1 expression, HBV-DNA levels, and frequency of IFNγ and IL-10 producing T-cells.

In 14 of 18 patients the frequency of HBV-specific T-cells producing IFNγ was assessed at the three time points specified. There was a significant increase in the frequency of IFNγ producing CD4+T-cells between T1 and T3 in response to both HBV-core (p=0.009) and surface (p=0.002) antigens (Figure 27A). This inversely correlated with the decrease in PD-1 expression and HBV-DNA levels (r=−0.994, p=0.067). In contrast, the frequency of HBV-specific CD4+T-cells producing IL-10 decreased markedly between T1 and T3 in response to surface antigen (p=0.02) and a similar trend was observed in response to HBV-core antigen (p=0.08).

In 6 of 18 patients the frequency of HBV-specific CD8+T-cells producing IFNγ was assessed at the three time points specified. The frequency of Influenza-specific CD8+T-cells was also assessed as a control (Figure 27B). There was a significant increase in the frequency of IFNγ producing HBV-specific CD8+T-cells between T1 and T3 (p=0.03), whereas there was no significant change in the frequency of IFNγ producing influenza-specific CD8+T-cells (p>0.1).
Figure 27: Frequency of HBV-specific CD4+ and CD8+T-cells and Influenza-specific CD8+T-cells producing IFNγ and PD-1 expression over time

(An increase in the mean frequency of IFNγ producing HBV-specific CD4+ (Fig 5A) and CD8+T-cells (Fig 5B) was observed from T1 to T3. In contrast there was no significant change in the frequency of Influenza-specific CD8+T-cells producing IFNγ from T1 to T3 (Fig 5B). In parallel there was a decrease in mean PD-1 expression on total CD8+ T-cells (MFI) in the subgroup of patients analysed. Error bars represent standard error of the mean.)
4.3.5. Correlation between PD-1 expression, HBV-DNA levels and memory phenotypes

Four of 6 patients who seroconverted to anti-HBe (Group 1) were investigated to determine whether reduction in serum HBV-DNA levels or HBeAg loss lead to changes in the four memory phenotypes. At baseline (T1), there was a significant direct correlation between frequency of HBc-specific central memory phenotype and PD-1 expression as assessed either by MFI on total CD8+ cell population (r=0.988, p=0.012) or by the combined frequency of HBc and HBs specific pentamer positive PD-1+T-cells (r=0.985, p=0.0015) (Figure 28A). There was also a direct correlation, at baseline, between effector memory phenotype and PD-1 expression as assessed by MFI on total CD8+ cell population (r=0.995, p=0.005) (Figure 28B). In parallel, there was a strong inverse correlation between HBcAg-specific effector phenotype and PD-1 expression at baseline (r=−0.999, p=0.0005; Figure 28C). A similar, but not significant relationship, was seen in HBsAg-specific cells.
Figure 28: Correlations between PD-1 expression and memory phenotypes at baseline

(A) A significant direct correlation was observed between frequency of HBc-specific central memory phenotype (CM) and PD-1 expression as assessed either by MFI on total CD8+ cell population \( (r=0.988, p=0.012) \) or frequency of HBc & HBs-specific pentamer positive cells \( (r=0.985, p=0.0015) \) at baseline.

(B) A direct correlation was observed between effector memory phenotype (EM) and PD-1 expression as assessed by MFI on total CD8+ cell population \( (r=0.995, p=0.005) \) at baseline.

(C) An inverse correlation between HBcAg-specific effector phenotype (E) and PD-1 expression was observed at baseline \( (r=-0.999, p=0.0005) \).
Any change in the frequency of subsets of various memory phenotypes in this subgroup of patients over the three defined time points was also examined. The frequency of differential memory phenotypes did not significantly change during anti-viral therapy over time (see Figure 29).
In a subgroup of patients analysed, there was no significant change observed in the frequency of central memory, effector memory and effector subsets in: A) Total CD4+ population, B) Total CD8+ population, C) HBcAg-specific CD8+ cell population, D) HBsAg-specific CD8+ cell population; over a course of anti-viral therapy (T1-T3).
4.4. Summary of Results:

The present study demonstrates that viremia levels directly correlate with PD-1 expression on total CD8+, virus-specific CD8+ and CD3+ve/CD8-ve (CD4+) T-cells in chronic HBV infection and that treatment-induced suppression of viral replication, manifested by a marked reduction in serum HBV-DNA levels, results in a significant decrease in PD-1 expression on the T-cell surface, as well as PD-1 mRNA transcription.

The significant reduction in PD-1 expression on antiviral treatment is accompanied by improved virus-specific T-cell reactivity with increased IFNγ production. In addition, these results show that serum HBeAg loss is associated with a decrease in PD-1 expression that is independent of viral load, and is accompanied by a further improvement in virus specific T-cell reactivity.

There was no observed relationship between baseline ALT and PD-1 expression in this cohort of patients.

There was no significant relationship between frequency of different memory phenotypes of CD4+ and CD8+ T-cells expressed at different time points, and HBV-DNA levels, HBeAg status and PD-1 expression. However, at baseline elevated PD-1 levels are associated with a decrease in effector cells and an increase in central memory and effector memory phenotypes in HBcAg-specific T-cells.
In conclusion, treatment-induced suppression of HBV replication resulted in significant reduction of PD-1 expression on the T-cell surface and PD-1 transcription, thus reducing its negative impact on T-cell activation and function. However, these results indicate that to completely restore anti-viral T-cell function a combination of potent suppression of HBV replication plus the use of an immunotherapeutic strategy that amplifies virus-specific T-cell reactivity may result in sustained control of HBV replication and resolution of liver disease (Bertoletti et al 2003).
Chapter 5

The role of the PD-1 pathway in defining the differential effector function commitments of virus-specific CD8+ T-cells to cytolytic and non-cytolytic mechanisms
5. The role of the PD-1 pathway in defining the differential effector function commitments of virus-specific CD8+ T-cells to cytolytic and non-cytolytic mechanisms.

5.1. Background to study

Chronic viral hepatitis is primarily the result of a complex interaction between a replicating non-cytopathic virus and an impaired antiviral host immune response. The importance of a vigorous CD8+ T-cell host response directed against multiple epitopes in the successful clearance of acute hepatitis B infection is well established (Rehermann et al 1995, 1996 & 2005, Ferrari et al 1990, Webster et al 2004). These virus-specific CD8+ T-cells display dual effector functions in vivo; cytolysis of infected cells mediated through FasL and perforin, and the production of anti-viral cytokines (e.g. IFNγ, TNFα) which purge HBV from infected hepatocytes through antiviral intracellular mechanisms (Guidotti et al 1996).

The successful control of HBV replication in immunocompetent adults exposed to hepatitis B virus occurs, in the majority of cases, in the absence of overwhelming immune-mediated cytolysis and fulminant hepatitis. Whilst both cytolytic (destructive) and non-cytolytic (curative) CD8+ T-cell effector functions have been shown to be critical in the control of infection in animal models of hepatitis B virus infection (Guidotti et al 1996 & 1999), the relative importance of these different effector functions are poorly understood.
Moreover, the mechanisms which determine the balance between these cytolytic (destructive) and non-cytolytic (curative) virus-specific-CD8+T-cell functions and dictate which of these effector functions are dominant during resolution/chronicity of infection are poorly understood. Of possible importance in the maintenance of this balance is the “Programmed Death” pathway (PD-1 pathway) which has recently been identified as an immuno-inhibitory pathway, belonging to the B7 family of immune regulators, that is involved in the development of T-cell tolerance and has been implicated in the persistence of a variety of chronic viral infections including LCMV, HIV, HBV and HCV (Barber et al 2006, Trautmann et al 2006, Velu et al 2009, Day et al 2006, Golden-Mason et al 2007, Urbani et al 2006, Penna et al 2007, Boni et al 2007, Evans et al 2008).
5.2. Materials and Methods

**Cell lines and T cell clone generation and co-cultures.** HepG2.2.15 cells and HBV-specific CD8+ T-cells were propagated and co-cultured as described in chapter 2 (see Figure 15). This cell co-culture model was used to specifically dissect and evaluate the cytolytic and non-cytolytic effector virus-specific CD8 T-cell and target cell interactions.

**Real-Time PCR.** HBV replication in 2.2.15 cells was assessed by quantitation of core-associated HBV-DNA (cytoplasmic DNA) and HBV-DNA from the viral particles secreted in the supernatants of these co-culture experiments (secreted DNA) as previously described (see Chapter 3).

**Flow Cytometry of cell co-cultures**

Flow Cytometry was performed as previously described (see Chapter 3). In all samples containing both T-cells and hepatocytes, these different cell populations were easily distinguishable through physical characteristics using FSC and SSC properties, allowing gating and analysis of each cell population individually.
5.3. Results

5.3.1 Both cytolytic and non-cytolytic CD8+ T-cell effector functions are important in the control of HBV replication

To evaluate the relative role of cytolytic and non-cytolytic CD8+ T-cell effector functions in the control of HBV replication, core-associated HBV-DNA (cytoplasmic) and HBV-DNA in the supernatant from the secreted viral particles was quantitated in the direct and indirect cell co-culture systems (Figure 15, Chapter 3). The addition of effector virus-specific CD8+ T-cells to target 2.2.15 cells resulted in a significant decrease in both cytoplasmic (p=0.01, p=0.02) and secreted HBV-DNA (p=0.03, p=0.03) in both direct and indirect co-culture models respectively (Figure 30). This data confirms for the first time in a model of human hepatitis B virus infection, the importance of non-cytolytic control of infection through the production of anti-viral cytokines by virus-specific CD8+ effector T-cells, complementing recent data confirming the importance of non-cytolytic control of viral replication in chronic hepatitis C infection in a similar model of human HCV infection (Jo et al 2009). To establish whether antiviral cytokines IFNγ and TNFα, which are central for the control of HBV replication in animal models (Guidotti et al 1996 & 1999), are also important in this model of human infection virus-specific CD8+ T-cells were co-cultured with target hepatocytes in the presence / absence of neutralising antibodies to IFNγ and/or TNFα. The addition of neutralising antibodies to IFNγ and TNFα abrogated this non-cytolytic control of viral replication (p=0.05) (Figure 30). This confirms an important role in non-cytolytic “purging” of infected hepatocytes for these anti-viral cytokines in addition to the established cytolytic mediated mechanisms of viral eradication. There was a lower level of HBV-DNA present in the indirect cell co-
culture model compared to the direct co-culture model, and this represents leakage of HBV-DNA into the supernatant from cytolysis of infected cells in the direct model. This observation was further supported by dilutional experiments with decreased number of effector T-cells (5,000 vs 50,000). No increase was observed in the rate of cells undergoing apoptosis in the indirect cell co-cultures, suggesting that these anti-viral cytokines do not induce apoptosis of infected cells as a means of control of viral replication (see Chapter 6).
Figure 30: Intracellular HBV-DNA levels in target HepG2.2.15 cells

Following co-culture experiments in the presence/absence of neutralising antibodies to anti-viral cytokines, HBV-DNA levels were quantitated using PCR techniques.

A = 2215 cells cultured alone.
B = 2215 cells + HBV-specific CD8+ T-cells in a direct co-culture.
C = 2215 cells + HBV-specific CD8+ T-cells in an indirect co-culture model.
D = C + neutralising antibodies to IFNγ.
E = C + neutralising antibodies to TNFα.
F = C + neutralising antibodies to IFNγ and TNFα.

Each panel represents the mean of at least 3 separate experiments. Statistical significance was observed between different co-culture conditions; † p=0.01, ** p=0.03; ‡ p=0.01, * p=0.04, ◊ p=0.04. Error bars represent mean ± s.e.m.
5.3.2. Upregulation of PDL1 on hepatocytes following direct and indirect cell co-culture with HBV-specific CD8+ T-cell clone

In order to establish whether cross talk between effector and target cells involved the PD-1/PDL1 pathway, PDL1 expression was assessed on HepG2.2.15 cells in the presence/absence of activated HBV-specific CD8+ T-cells. A significant increase in PDL1 expression was observed on HepG2.2.15 cells in the direct co-culture systems, compared to hepatocytes cultured alone (p=0.01) (Figure 31). This increase in PDL1 expression was dependent on the number of T-cells present with a higher concentration of T-cells (E:T ratio 1:6) resulting in increased PDL1 expression (Figure 31).
Figure 3: PDL1 expression on HepG2.2.15 cells in the presence of HBVc18-27 specific CD8+ T-cell clone in a direct co-culture model

Representative FACS dot plots demonstrate upregulation of PDL1 expression on the cell surface of HepG2.2.15 cells (target cells) in the presence of HBV-specific CD8+ T-cell clone in a direct co-culture model. This upregulation was confirmed at an mRNA level. Each panel is representative of at least 3 separate experiments. Error bars represent mean ± s.e.m.
Upregulation of PDL1 was also observed on hepatocytes despite physical separation from effector CD8+ T-cells in the indirect model (see Figure 32). This suggests the involvement of a soluble factor or factors produced by T-cells which mediate changes in PDL1 expression altering the “cross talk” that occurs between effector and target cells in this system. These results demonstrate that PDL1 upregulation on HepG2.2.15 cells is driven by CD8+ T-cells via a soluble factor, most probably a cytokine, produced by activated virus-specific CD8+ T-cells.
Figure 32: PDL1 expression on HepG2.2.15 cells in the presence of HBVc_{18-27} specific CD8+ T-cell clone in an indirect co-culture model

Representative FACS dot plots demonstrate upregulation of PDL1 expression on the cell surface of HepG2.2.15 cells (target cells) in the presence of HBV-specific CD8+ T-cell clone in an indirect co-culture model. This upregulation was confirmed at an mRNA level. Each panel is representative of at least 3 separate experiments. Error bars represent mean ± s.e.m.
5.4. Summary of Results

This study demonstrates that both cytolytic and non-cytolytic CD8+ T-cell effector functions are important in the effective control of HBV replication in this model of human HBV infection. In animal models of HBV infection IFNγ has been shown to carry out “purging” of infected hepatocytes, and these results suggest that this mechanism remains central in the control of viral replication in human HBV infection. This study reports that virus-specific CD8+ T-cells, engage in cross talk with infected hepatocytes via regulation of the expression of the PDL1 ligand on the surface of hepatocytes through the production of a soluble factor (probably a cytokine).

These results demonstrate that co-culturing activated virus-specific CD8+ T-cells with their target cells (infected hepatocytes) results in upregulation of PDL1 on the hepatocyte cell surface. This suggests that there is cross-talk between HBV-specific CD8+ effector T-cells and their target hepatocytes, and as this effect is dependent on the number of T-cells present and also seen in an indirect co-culture system, it appears to be driven by an as yet unidentified soluble factor that is produced by activated CD8+ T-cells.
The subsequent study (Chapter 6) of this thesis focused on the identification of this soluble factor(s), important in the elucidation of mechanisms involved in the cross-talk between hepatocytes and effector T-cells. In this study the functional consequences of manipulation of the PD-1/PDL1 pathway through the use of blocking antibodies on the balance between cytolytic and non-cytolytic CD8+ T-cell effector functions were also assessed.
Characterisation of a soluble factor that allows cross-talk between target cells (HepG2.2.15 cells) and effector cells (virus-specific CD8+ T-cells) and the role of the PD-1 pathway in determining the differential effector functions of virus-specific CD8+ T-cells in a model of Hepatitis B virus infection
6) Characterisation of a soluble factor that allows cross-talk between target cells (HepG2.2.15 cells) and effector cells (virus-specific CD8+ T-cells) and the role of the PD-1 pathway in determining the differential effector functions of virus-specific CD8+ T-cells in a model of Hepatitis B virus infection.

6.1. Background to study

Both cytolytic and non-cytolytic HBV-specific CD8+ T-cell effector functions play an important role in the effective control of viral replication in this purposely designed tissue culture model of human HBV infection. Although the importance of these effector mechanisms has been established in animal models of chronic infection, little is known of the factors which regulate the differential effector mechanisms of CD8+ T-cells.

We have also shown that activated HBV-specific CD8+ T-cells upregulate PDL1 expression on their target cells (HepG2.2.15 cells) through the production of a soluble factor(s).

This study reports a novel role for the PD-1 pathway as one of the key mechanisms by which CD8+ T-cells engage in cross-talk with the target cells (infected hepatocytes) to programme their effector functions. These results report a novel homeostatic and immunomodulatory role for IFNγ produced by HBV-specific CD8+ T-cells in regulating PDL1 expression on infected hepatocytes and therefore
impacting on the balance between cytolytic and non-cytolytic control of viral replication. This data challenges the existing dogma that infected hepatocytes are passive “targets” of the adaptive immune response to chronic hepatitis infection, and has profound implications for future immunotherapeutic approaches targeting the PD-1/PDL1 synapse.
6.2. Materials and Methods

Cell lines and T cell clone generation and co-cultures. HepG2.2.15 cells and HBV-specific CD8+ T-cells were propagated and co-cultured as described in Chapter 3 (See figure in Chapter 15).

Cytokine Bead Array (CBA) (BD Biosciences, Oxford) was performed to assess the level of a panel of 16 candidate cytokines in indirect and direct cell co-culture supernatants (TNFα/IFNγ/IL-10/IL-6/IL-8/IL-12/IL-5/IL-7/IL-12p70/IP-10/MIP-1B/RANTES/MCP-1/MIP-1A). CBA was also performed on supernatants of the cytokine neutralization co-cultures to confirm complete abolition of target cytokines by anti-IFNγ and anti-TNFα antibodies.

Real-Time PCR. HBV replication in 2.2.15 cells was assessed by quantitation of core-associated HBV-DNA (cytoplasmic DNA) and HBV-DNA from the viral particles secreted in the supernatants of these co-culture experiments (secreted DNA) as previously described (see Chapter 3).

Cytotoxicity. The proportion of apoptotic 2.2.15 cells was assessed using the TACS™ Annexin V-FITC Apoptosis detection kit (R&D Systems, Abingdon, UK). The degree of cytotoxicity was also evaluated through the measurement of ALT levels in the supernatants of cell co-cultures (as described in Chapter 3).
6.3. Results

6.3.1 Upregulation of PDL1 expression on hepatocytes correlates with IFNγ, and TNFα levels produced by HBV-specific CD8+ T-cell clone

In order to further elucidate the mechanisms by which PDL1 was upregulated and to determine which soluble factors/cytokines produced by activated HBV-specific CD8+ T-cells correlated with PDL1 expression on hepatocytes, exploratory analysis of 17 candidate cytokines/chemokines performed by cytometric bead array was performed on supernatants from co-cultures. Analysis was performed on supernatants of both direct and indirect co-cultures in the presence/absence of neutralising antibodies to IFNγ/TNFα/PD-1. Significant correlations were observed between PDL1 and IFNγ ($r=0.99$, $p=0.00006$) and PDL1 and TNFα ($r=0.80$, $p=0.02$) (Figure 33). These correlations suggested a significant role for anti-viral cytokines, produced by HBV specific effector T-cells, in mediating cross-talk between effector and target cells.

To confirm the role of IFNγ and/or TNFα, in mediating an increase in PDL1 expression, virus-specific CD8+ T-cells and target hepatocytes (HepG2.2.15) cells were co-cultured in the presence of neutralising antibodies to IFNγ and/or TNFα in the indirect co-culture system. The upregulation of PDL1 previously observed in the presence of HBV-specific CD8+ T-cells was significantly attenuated in the presence of anti-IFNγ Ab. ($p=0.01$) (Figure 34). TNFα neutralisation did not decrease PDL1 expression. As well as inhibiting the observed upregulation in PDL1 expression, these neutralising antibodies also abrogated the observed suppression of viral load (Figure 30 – Chapter 5). These results suggest a dual role for IFNγ whereby it
functions not only as an anti-viral cytokine “purging” infected hepatocytes, but also plays a key role in limiting the extent of immune-mediated target cell lysis through the upregulation of the co-inhibitory ligand PDL1.

Incubation of HepG2 and HepG2.2.15 cells with recombinant IFNγ revealed a dose dependent increase in PDL1 expression (Figure 35). The concentrations of cytokines used reflects those levels detected in the supernatants of the cell co-culture experiments by CBA. Recombinant TNFα did not result in an increase in PDL1 expression when cultured alone with HepG2.2.15 cells.
A direct correlation was observed between relative PDL1 expression as assessed by qT-PCR, and IFNγ levels ($r=0.99$, $p=0.00006$). A further direct correlation was observed between PDL1 expression and TNFα levels ($r=0.80$, $p=0.02$).
Figure 34: PDL1 expression on HepG2.2.15 cells in the presence of HBVc\textsubscript{18-27} specific CD8+ T-cell clone in an indirect co-culture model, in the presence / absence of neutralising antibodies to IFN\textgreek{y}/TNF\textalpha.

Representative FACS dot plots demonstrate that the increase in PDL1 expression observed on HepG2.2.15 cells in the presence of HBV-specific CD8+ T-cell clone is strongly attenuated in the presence of neutralising antibodies to IFN\textgreek{y}. This decrease was confirmed at an mRNA level. There is no change in PDL1 expression in the presence of anti-TNF\textalpha antibodies alone. Each panel is representative of at least 3 separate experiments. Error bars represent mean ± s.e.m.
A dose dependent increase in PDL1 expression was observed on HepG2.2.15 cells (Figure 6a) and HepG2 cells (Figure 6b) in the presence of rIFNγ. Hepatoma cell lines were cultured alone (A) or with 4 different concentrations of IFNγ (B)=100pg/ml, (C)=1000pg/ml, (D)= 5000pg/ml & (E)=10,000pg/ml. Each panel is representative of at least 3 separate experiments.
6.3.2. Blockade of the PD-1/PDL1 pathway resulted in an increase in the percentage of target cells (hepatocytes) undergoing apoptosis

To ascertain the impact of PD-1/PDL1 binding on the degree of cytolysis and anti-viral cytokine production by HBV-specific CD8+ effector T-cells these T-cells were co-cultured with their target hepatocytes (HepG2.2.15 cells) in the presence/absence of neutralising antibody to PD-1 in a direct co-culture model. Target cell apoptosis, anti-viral cytokine levels and HBV-DNA levels were assessed. A 54% increase in the percentage of hepatocytes, (gated on according to physical characteristics using forward and side scatter) undergoing apoptosis was observed in the presence of neutralising antibody to PD-1 (Figure 36a).

To further assess the degree of hepatocyte cytolysis in the presence of anti-PD-1 antibodies, ALT, a surrogate marker of hepatocyte apoptosis, was measured in the supernatants of these cell co-cultures. Blockade of PD-1 resulted in an elevation in ALT levels, suggestive of an increased rate of hepatocyte cytolysis (p=0.03). (Figure 36b).
There were no significant changes in the secretion of IFNγ or TNFα in the presence of neutralising antibody to PD-1 at either 24 or 48 hours (Figure 36c). Despite this shift in the balance between cytolytic and non-cytolytic CD8+ T-cell effector functions, significant changes in total levels of intracellular HBV-DNA in the presence of neutralising antibodies to PD-1 were not seen (Figure 36d). This suggests that activation of the PD-1 pathway, whilst decreasing the cytolytic arm of the immune response, does not initially impair the ability of the CD8+T-cells to clear infection through antiviral cytokine production (IFNγ & TNFα).
In the presence of anti-PD-1 antibodies there is an increase in the percentage of cells undergoing apoptosis following co-culture of HepG2.2.15 cells with virus-specific CD8+ T-cell clone for 24 hours as assessed by Annexin V and PI staining (Figure 36a). Hepatocytes were gated onto according to physical characteristics. Similarly, blockade of the PD-1 pathway resulted in an increase in ALT levels (a surrogate marker of hepatocyte apoptosis) (Figure 36b). Conversely there were no changes in IFNγ and TNFα production following blockade of the PD-1 pathway as assessed via cytokine bead array (Figure 36c). These changes in the balance between cytolytic and non-cytolytic CD8+ T-cell effector functions did not result in a change in control of HBV-DNA replication (Figure 36d). Each panel is representative of at least 3 separate experiments. Error bars represent mean ± s.e.m.
6.4. Summary of Results

This study demonstrates that in this tissue culture model of human HBV infection, upregulation of PDL1 expression on hepatocytes correlates with IFNγ, and TNFα levels produced by HBV-specific CD8+ T-cell clone.

This upregulation of PDL1 on target hepatocytes observed in direct and indirect effector/target cell co-cultures was significantly attenuated in the presence of neutralising antibodies to IFNγ.

Incubation of HepG2 and HepG2.2.15 hepatoma cells with recombinant IFNγ revealed a dose dependent increase in PDL1 expression. This data taken together demonstrates that virus-specific CD8+ T-cells, through IFNγ production, engage in cross talk with infected hepatocytes via regulation of the expression of the PDL1 ligand on the surface of hepatocytes.

These results provide evidence that the PDL1 pathway plays a central role in dictating the balance between cytolytic and non-cytolytic CD8+ T-cell effector functions, initially preserving non-cytolytic cytokine driven control of viral replication, whilst curtailing cytolytic functions, thereby limiting liver cell injury. Blockade of the PD-1 pathway in the direct co-culture model resulted in an increase in the number of cells undergoing apoptosis as assessed both by ALT measurements and by Annexin V/PI FACS analysis. There was no impact on anti-viral cytokine production observed
for 48hrs following blockade of the PD-1 pathway and no difference in viraemia levels was observed.

The results of this study show that hepatocytes are active participants in bidirectional, tissue-specific regulation of T-cell functions influencing the definition of the differential effector function commitment of HBV-specific CD8+ T-cells via the PD-1/PDL1 pathway, promoting effective viral control without extensive liver injury.
Hepatitis B virus upregulates hepatocyte expression of PDL1 to evade hepatotoxic adaptive immune responses
7) Hepatitis B virus upregulates hepatocyte expression of PDL1 to evade hepatotoxic adaptive immune responses

7.1. Background to study

There is a growing body of evidence suggesting the hepatitis B virus employs a variety of strategies aimed at overwhelming, evading or neutralising the host immune response to infection resulting in chronicity of infection (see Chapter 1).

Previous chapters of this thesis have demonstrated that infected hepatocytes can pre-programme differential effector functions of virus-specific CD8+T-cells through their cell surface expression of PDL1 (see Chapter 6). In this way target hepatocytes can themselves impact on the balance between CD8+T-cell cytolytic and non-cytolytic effector functions.

This study investigates whether HBV infection can itself impact on hepatocyte PDL1 expression. Through this potential manipulation of the PD-1 pathway, Hepatitis B virus may specifically target cells of the adaptive immune response, inhibiting components of CD8+ T-cell effector functions leading to virus-specific T-cell exhaustion and subsequent persistence of infection.

The impact of HBV infection on hepatocytic PDL1 expression was assessed through the transfection of a human hepatoma cell line (Huh-7) with Hepatitis B virus.
The varying levels of PDL1 expression on human hepatoma cell lines that constitutively express Hepatitis B virus compared to their parent cell lines was also assessed.
7.2. Results

7.2.1. Increased PDL1 expression following transfection of Huh7 cells with Hepatitis B virus

There was a significant increase in both intracellular and secreted HBV-DNA levels following transfection of Huh7 cells with HBV via a plasmid vector, confirming successful transfection of Hepatitis B virus (Figure 37). This increase in HBV-DNA levels was associated with a relative increase in PDL1 expression on transfected hepatocytes (Figure 37). There was no observed increase in PDL1 expression following transfection of hepatocytes with an empty vector, suggesting this observation was virus-specific. Following transfection there was no observed increase in PD-1 or PDL2 expression on target hepatocytes (remained undetectable).

7.2.2. Correlation between PDL1 expression and HBV-DNA levels following transfection of Huh7 cells with Hepatitis B virus

There was a significant correlation between PDL1 expression on hepatocytes and HBV-DNA levels quantified both intracellularly (cytoplasmic HBV-DNA) \( r=0.98, p=0.01 \) and HBV-DNA secreted in the supernatants \( r=0.908, p=0.048 \), following transfection of Huh7 cells with hepatitis B virus (Figure 38).
Figure 37: HBV-DNA levels and PDL1 expression on Huh7 cells following transfection with Hepatitis B virus infection

Extracellular (Figure 37a) and Intracellular (Figure 37b) HBV-DNA levels were elevated following transfection of Huh7 cells with Hepatitis B virus. Each panel is representative of at least 3 separate experiments.
Figure 38: Correlations between HBV-DNA levels and PDL1 expression following transfection of Huh7 cells with Hepatitis B virus

A close correlation was observed between both extracellular (secreted) (Figure 38a) & intracellular (cytoplasmic) (Figure 38b) HBV-DNA levels and relative expression of PDL1.
7.2.3. Increased PDL1 expression in hepatoma cell lines which constitutively express live virions (HepG2215 cell line) compared with HepG2 parent cell line

The relative expression of PDL1 on the cell surface of HepG2.2.15 cells (which constitutively express Hepatitis B virus) and HepG2 cells (the parent cell line for the HepG2.2.15 cells) was assessed. PDL1 expression was also assessed in a controlled tissue culture setting to ascertain whether it was constant over time. Neither of these cell lines were found to constitutively express detectable levels of PD-1 or PDL2 in culture.

Both cell lines expressed stable levels of PDL1, which did not significantly change over time. A higher level of basal PDL1 expression was observed on HepG2.2.15 cells compared with HepG2 cells (Figure 39).
Figure 39: HepG2215 have constitutively higher levels of expression of PDL1 than parent HepG2 cells

HepG2.2.15 cells constitutively have greater expression of PDL1 on the cell surface than their parent HepG2 cells.
7.2.4. Activation of HBV-DNA expression in an AD38 cell line via a tetracycline inhibited promoter region resulted in concurrent upregulation of PDL1 expression

Dynamic changes in PDL1 expression following activation of HBV-DNA expression in an AD38 cell line, through a tetracycline-dependent promoter region, were assessed. Activation of hepatitis B virus genome transcription in the AD38 cell line (by incubating in the absence of tetracycline) resulted in an increase in both HBV-DNA levels and PDL1 expression on the cell surface of these hepatoma cells (Figure 40). There was a close correlation between HBV-DNA levels and PDL1 expression in this “activated” AD38 cell line.
Figure 40: Activation of AD38 cell line resulted in an increase in HBV-DNA production, which correlated with an increased cell surface expression of PDL1.

HBV DNA and PDL1 expression in AD38. (a) Activation of AD38 cell line with Tet resulted in an increase in HBV-DNA production, which correlated with an increase in expression of PDL1. (b) Switching HBV DNA production off from day 1 resulted in a decrease in PDL1 expression. Switching HBV DNA production back on from day 8 resulted in a subsequent increase in PDL1. There was a strong correlation between PDL1 and HBV DNA levels (c).
7.3. Summary of Results

This study demonstrates that following transfection of Huh7 human hepatoma cells with Hepatitis B virus, there is a significant increase in the expression of PDL1 by these cells which was not observed following transfection with an empty vector.

A close correlation between PDL1 expression by Huh7 cells following transfection with hepatitis B virus and HBV-DNA levels (both excreted and intracellular) was observed over time.

An increased expression of PDL1 on hepatoma cell lines which constitutively express live virions compared with their parent hepatoma cell lines was observed, and activation of HBV-DNA expression in an AD38 cell line via a tetracycline switch resulted in concurrent upregulation of PDL1 expression.

These results suggest a novel mechanism that Hepatitis B virus may employ to evade immune responses, through the upregulation of PDL1 expression on host cells (infected hepatocytes).
Chapter 8

Discussion
8) Discussion

It is estimated that 2 billion people worldwide have been exposed to the hepatitis B virus and there are over 400 million people chronically infected with this virus. With the exception of interferon therapy, all currently available treatments directly target the HBV polymerase enzyme. Whilst these agents may control viral replication for a period of time there are significant short-comings in these therapies, principally relating to concerns over long-term usage with the resulting emergence of resistance, together with the cost of these therapeutics and the potential side effects of indefinite use of these agents. Furthermore, it is well established that a broad, strong immune response to infection is central to effective control of viral replication.

To this end there has been much interest in the immunopathogenesis of hepatitis B virus infection, specifically investigating which cells of the innate and adaptive immune response are central in the successful resolution of infection. Although these investigations have been hampered by a deficit of studies which have recruited patients with an acute HBV infection (due to the difficulty in recruiting this patient cohort), there is a growing body of evidence which points to the importance of a robust CD8+ virus specific T-cell response targetting multiple viral epitopes in dictating whether there is resolution or chronicity of infection following exposure to HBV.

It has also been demonstrated that the level of virus-specific CD8+ T-cell responses in terms of cytokine production, cytotoxicity and proliferation, depends on a balance
of stimulatory and inhibitory T-cell pathways. Barber et al. first described the potential importance of the Programmed Cell Death (PD-1/PDL1) pathway (one of the inhibitory T-cell costimulatory pathways) in a mouse model of Chronic Lymphocytic Choriomeningitis Virus (LCMV) infection. This group demonstrated, in a genome wide microarray, that PD-1 was upregulated on “exhausted” virus-specific T-cells compared with “functional” T-cells. Furthermore, it was shown that blockade of the PD-1 pathway resulted in restoration of T-cell functions with increased cytotoxicity, cytokine production and proliferation, and perhaps most importantly of all, reduction of viral load, with clearance of the virus from the spleen and blood of these mice. Further work in HIV (Day et al, Trautmann et al) demonstrated a similar role of reversible virus-specific T-cell dysfunction in HIV infection.

In this thesis the role of the PD-1 pathway in the immunopathogenesis of hepatitis B virus infection was investigated through several different approaches. Firstly, longitudinal changes in PD-1 expression in patients with chronic hepatitis B virus infection undergoing oral antiviral therapy was investigated. This provided the opportunity to analyse the relationship between viral load and PD-1 expression at both transcriptional and translational levels. Next, through the employment of a purposely-designed in vitro cell co-culture model of Hepatitis B virus infection the interactions between HBV-producing hepatocytes (target cells) and CD8+ T-cells (effector cells) was investigated. In addition, through the transfection of a human hepatoma cell line with hepatitis B virus and the analysis of various hepatoma cell lines that differentially express Hepatitis B virus, the impact of the Hepatitis B virus
on subsequent hepatocytic expression of PDL1 (the major ligand for PD-1) was investigated.

Although cell culture techniques are useful tools in investigating the immunopathogenesis of chronic hepatitis B infection, it should be remembered that there are limitations to these techniques. The most obvious short fall in the use of these co-culture models is the absence of other cells of the innate and adaptive immune responses that are so important in shaping immune responses and dictating host and immune interactions (e.g. dendritic cells etc). The other important limitation of these studies is the lack of the unique micro-environment and architecture of the liver. These co-culture models use cloned T-cell lines and hepatoma cell lines which leads to several concerns. Firstly, it is known that in-vivo a wide T-cell response targeting multiple epitopes is important in viral clearance and this model only has one T-cell clone in vitro. Furthermore, hepatoma cell lines are known to produce elevated levels of PDL1 compared to primary human hepatocytes, although examination of how this expression is modulated is of value and appears to transfer from hepatoma lines to primary human hepatocytes.

The interpretation of statistical correlations that arise from these co-cultures need to be performed with caution. The use of cell clones in tightly controlled conditions lead to highly reproducible results and subsequent statistical analysis can therefore suggest extremely close correlations between measured variables (e.g. stated correlations between IFNγ and PDL1 expression in cell culture models – see chapter
6). It should be remembered that these controlled cell culture techniques, whilst useful in the assessment of specific relationships, do not approximate what is happening in vivo and apparently strong causal relationships in these controlled experiments are almost certainly of less significance in vivo where multiple dynamic and variable factors may be involved.

*The programmed cell death pathway in patients with chronic hepatitis B undergoing anti-viral therapy:*

These studies have demonstrated that there is a direct correlation between viral load and PD-1 expression on effector virus-specific CD8+ T-cells, in a cohort of patients with CHB undergoing a course of oral anti-viral treatment. Furthermore, treatment-induced suppression of viraemia resulted in a significant decrease in PD-1 expression on virus-specific CD8+ T-cells both at a transcriptional level (mRNA expression by RT-PCR) and at a translational level (cell surface protein expression by flow cytometry).

Another important finding of this study was that the decrease in HBV-DNA with antiviral treatment and a concomitant decrease in PD-1 expression was associated with improvement in virus specific T-cell reactivity. Furthermore, HBeAg seroconversion was associated with a further decrease in PD-1 expression.
These findings extend recent observations in patients with acute hepatitis B showing decreased PD-1 expression on CD8+ T-cells of patients with spontaneous resolution of HBV infection, while in those with persistent HBV replication both PD-1 and HBV-DNA levels remained high (Boettler et al 2006, Boni et al 2007). Serial testing of seven patients with anti-HBe positive chronic hepatitis B infection, having spontaneous reactivation of the disease, demonstrated that viral load can directly influence HBV-specific T-cell repertoire (Boni et al 2007). The advantage of the present study design is that by monitoring longitudinally the impact of treatment-induced suppression of HBV replication with direct antivirals Telbivudine or Lamivudine, the cause-effect relationship between viral load, PD-1 expression and CD8+ T-cell function could be defined.

In this study cohort a close positive correlation between HBV-DNA levels and PD-1 expression on total & HBV-specific CD8+T-cells as well as on CD4+T-cells was demonstrated. A number of studies have demonstrated in a mouse model with LCMV infection and in humans infected with HIV, HBV or HCV, that blockade of PD-1 binding to its ligands results in functional restoration of virus-specific T-cells (Barber et al 2006, Trautmann et al 2006, Day et al 2006, Urbani et al 2006, Boni et al 2007, Penna et al 2007, Golden-Mason et al 2007). This has led to speculation that blockade of the PD-1/PD L-1 pathway may be a possible immunomodulatory approach for enhancing immune control of viral replication. However, the PD-1 pathway has potential harmful effects, such as precipitating autoimmunity or immunopathology and these should be considered before embarking on blockade of this pathway as a therapeutic approach. (Martinic et al 2008). Moreover, as
discussed later, manipulation of this pathway has a significant impact on the differential effector commitments of CD8+ T-cells potentially resulting in increased cytolysis, which in vivo could lead to fulminant liver damage. Indeed, it was demonstrated by Barber et al. 2006 that mice with knock out of the PD-1 gene (PD-1<sup>−/−</sup>) succumbed early to fatal immunopathogenesis. In order to avoid immune-mediated damage to the host, selective targeting of PD-1 on virus-specific T-cells may be a pre-requisite to achieving an acceptable risk/benefit balance with this approach to the control of viral replication.

It is recognised that high ALT levels are associated with an increased rate of HBeAg seroconversion, both during the natural history of the disease and with antiviral treatment (Perrillo et al 2002). If ALT is a surrogate marker of an ongoing “immune response” to HBV then a relationship between ALT at baseline and PD-1 expression might have been expected. However this study revealed no such relationship. This was in part due to the fact that PD-1 levels were significantly different at baseline between individual patients and although a relative decrease in PD-1 expression was observed over time, some patients had higher absolute PD-1 levels at timepoint 3 than other patients at baseline. It may also reflect that ALT is a marker of hepatocyte necrosis rather than the degree of inflammation per se. ALT levels in these patients are known to fluctuate and are affected by diverse factors. Analyses of HBV-specific CD8+T-cells in patients with both HBeAg positive and negative chronic hepatitis B showed lack of association between disease exacerbations and the frequency of circulating virus-specific T-cells (Webster et al 2004, Boni et al 2007). The present study extends the findings by Webster et al that whilst the repertoire of HBV-specific
CD8+T-cells is inversely proportional to the level of HBV replication, there is no direct correlation with the degree of liver damage (Webster et al 2004).

Treatment-induced suppression of HBV replication results in significant decrease in PD-1 expression in all patients – those with HBeAg seroconversion and patients remaining HBeAg positive. It has been demonstrated that HBeAg loss during treatment with adefovir dipivoxil is associated with both profound reduction of HBV-DNA levels, and an increase in CD4+T-cell reactivity, while patients with moderate reduction in serum HBV-DNA and no changes in CD4+T-cell responses remained HBeAg positive (Cooksley et al 2007). Thus, reduction of PD-1 expression, as a result of profound reduction of HBV viral load does not appear enough to fully restore CD4+ and CD8+T-cell response and to achieve HBeAg seroconversion in all treated patients.

The difference in PD-1 expression and the percentage of various memory subsets seen at baseline merits further comment. Analysis of the correlations between PD-1 expression and central memory, effector memory and effector phenotypes in core specific cells at baseline suggest that elevated PD-1 levels are associated with a decrease in effector cells and an increase in central memory and effector memory phenotypes. This adds support to the concept that PD-1 plays a central role in T-cell exhaustion and viral persistence. However, there was no significant relationship between frequency of different memory phenotypes of CD4+ and CD8+T-cells expressed at different time points, and HBV-DNA levels, e antigen status and PD-1
expression observed in this cohort of patients. It may be that PD-1 expression does not impact memory phenotypes as it is more a marker of CD8+ T-cell functionality and is dynamically responsive to the environmental factors (e.g. HBV-DNA levels – see later).

Nucleoside analogues are thought to interfere with viral replication, lowering HBV-DNA levels, but have not been proven to influence the development of effective memory T-cell differentiation and function, hence the need for long term therapy to control viral load (Marinos et al 1996). This study did not show any difference in the frequency of different memory phenotypes on CD4+ or CD8+ T-cells expressed at different time points. It did however show a significant increase in the frequency of IFNγ producing CD4+ and CD8+ T-cells over time, as well as a decrease in the frequency of IL-10 producing T-cells. This suggests that there may indeed be a shift in the cytokine profile of T-cells as HBV-DNA and PD-1 expression decrease with anti-viral therapy. Given the mechanism of action of Telbivudine or Lamivudine, which directly block viral replication, it seems likely that therapy results in a fall in HBV-DNA levels, and that this in turn leads to a decrease in PD-1 expression, which results in an improvement in cytokine production by HBV-specific T-cells. This is similar to what is reported in infection with HIV (Trautmann et al 2006, Day et al 2006).
Overall, these experiments highlighted the closely related mechanisms linking HBV replication and impaired T-cell functions in chronic hepatitis B. The data revealed strong correlation between HBV viremia and hyperexpression of PD-1 on all T-cells.

The physiological role of PD-1 pathway in dictating the balance of cytolytic and non-cytolytic effector T-cell function:

In order to further evaluate the dynamic interaction between virus-specific CD8+ T-cells and their target cells (infected hepatocytes) and the role of the PD-1 pathway in this interaction, a purpose designed in-vitro model with a HBV-specific CD8+ T-cell clone derived from a patient who had recovered from acute hepatitis B infection and a human hepatoma cell line that constitutively expresses fully infectious Dane particles (the HepG2215 cell line) was developed.

These studies revealed that both cytolytic and non-cytolytic CD8+ T-cell effector functions play an important role in the effective control of viral replication in this model of human hepatitis B virus infection and demonstrates the importance of non-cytolytic anti-viral cytokines in “purging” hepatitis B virus from infected hepatocytes. This data is further supported by an observed correlation between IFNγ production and reduction in serum and liver HBV-DNA in murine and chimpanzee models of acute HBV infection (Guidotti et al 1996 & 1999). Furthermore an inverse correlation between HBV-DNA levels and the frequency of IFNγ producing CD4+ T-cells was observed in patients with chronic hepatitis B undergoing treatment with recombinant interleukin-12 which is a potent inducer of IFNγ production (Rigopoulou et al 2005).
This data is supported by the observation by Jung et al (1999) that HBV-specific T-cells in patients with chronic hepatitis B had weak or absent antigen-specific IFNγ production, in contrast to a more robust IFNγ production from HBV-specific T-cells in acute resolving hepatitis B infection.

The importance of some degree of a cytolytic response should not, however, be underestimated. Perforin-deficient mice are unable to clear LCMV infection (Kagi et al 1994) and patients with an elevated ALT during acute hepatitis B are more likely to successfully resolve their infection.

These experiments also reveal that co-culturing activated virus-specific CD8+ T-cells with their target cells (infected hepatocytes) results in upregulation of PDL1 on the hepatocyte cell surface. This would suggest that there is cross-talk between HBV-specific CD8+ effector T-cells and their target hepatocytes, and as this effect is dependent on the number of T-cells present and also seen in an indirect co-culture system, it appears to be driven by a soluble factor that is produced by activated CD8+ T-cells. Subsequent studies reported in Chapter 6 demonstrated that the soluble factor responsible for this observed upregulation of PDL1 was IFNγ.

Following an acute hepatitis B virus infection, Type 1 interferons are produced by several cell types, including infected hepatocytes, which both directly inhibit HBV replication (Caselmann et al 1992) and stimulate CCL3 production from Kupffer cells, resulting in the recruitment of NK and NKT cells (Crispe 2003). Once activated these
NK cells produce IFNγ in significant quantities, resulting in a substantial increase in intrahepatic IFNγ levels during acute HBV infection (Hodgson et al 2001). Indeed, the number of NK cells simultaneously peaks with HBV replication, before the appearance of a virus-specific T-cell infiltrate in the liver 2-4 weeks later (Webster et al 2000).

These results suggest that in the context of a hepatitis B virus infection, IFNγ production by cells of the innate immune response may result in upregulation of PDL1 on hepatocytes. Subsequently, when CD8+ T-cells are exposed to direct contact with their target cells (infected hepatocytes), this upregulation of PDL1 results in attenuation of the destructive adaptive immune response, limiting CD8+T-cell driven cytolytic liver damage with initial preservation of non-cytolytic cytokine-mediated control of viral replication. This “priming” of hepatocytes, with upregulation of PDL1 may explain why virally infected hepatocytes show resistance to perforin/granzyme-mediated killing (Kafrouni et al 2001) whilst remaining sensitive to antiviral cytokine mediated control of viral replication.

In parallel to this suppression of cytolysis through upregulation of PDL1 on target cells, IFNγ carries out a vital anti-viral “purging” of infected hepatocytes as demonstrated by our data with neutralizing antibodies to IFNγ (see chapter 5).

Whilst it has been demonstrated previously that IFNγ applied directly to primary human hepatocytes results in upregulation of PDL1 (Muhlbauer et al 2006) for the
first time this study demonstrates that this upregulation is dose dependent, can be
driven at concentrations of IFNγ produced by virus-specific T-cells, and crucially that
this upregulation has functional consequences on the balance between cytolytic and
non-cytolytic CD8+ T-cell effector functions.

Adoptive transfer of an HBV-specific CD8+ T-cell clone into a transgenic mouse
model of HBV infection resulted in severe necroinflammatory liver disease that
interest, repeated infusions of these HBV-specific T-cells did not result in further
episodes of hepatitis, if they were readministered within 3-4 weeks of first infusion
(Wirth et al 1995). Our model suggests that the intrahepatic IFNγ production from the
initial infusion upregulates PDL1 on hepatocytes, priming them, and leaving them
resistant to further CD8+ T-cell cytolysis.

There is already some data suggesting that manipulation of the PD-1/PDL1 pathway
has consequences for the delicate balance between cytolytic and non-cytolytic CD8+
effector T-cell functions. Blockade of this synapse with a monoclonal antibody in
transgenic murine models of herpes stromal keratitis significantly exacerbated the
keratitis (Jun et al 2005) and blockade in a further model which expresses OVA as a
self-antigen throughout the small bowel, resulted in a highly specific CD8+ T-cell
mediated fatal auto-immune enteritis (Reynoso et al 2009).
Previous reports examining the role of the programmed death pathway during established chronic viral infections, have implicated hyperexpression of PD-1 in the observed exhaustion of virus-specific CD8+ T-cells. Moreover, PD-1 blockade has resulted in restoration of both cytolytic and non-cytolytic CD8+ T cell effector functions (Barber et al 2006, Trautmann et al 2006, Golden-Mason et al 2007).

Taken together, these observations suggest a possible temporal difference whereby PD-1 engagement during acute viral infection initially limits immunopathogenesis, whilst anti-viral cytokine production is at first preserved and indeed drives the further predominance of this pathway through the upregulation of PDL1 on target cells. Preservation of cytokine-mediated control of viral replication is also seen in a transgenic mouse model of chronic hepatitis B infection where antibody mediated blockade of the PD-1/PDL1 pathway does not result in a significant difference in IFNγ production by intrahepatic lymphocytes 5 days after adoptive transfer of virus-specific CD8+ T-cells (Maier et al 2007). In contrast, once chronic infection with hepatitis B has been established, virus-specific CD8+ T-cells are phenotypically exhausted, with impairment of both curative and destructive effector functions as a consequence of ongoing viral replication.

These results suggest a physiological homeostatic role of IFNγ in maintaining the balance between antiviral suppression of viral replication through the activation of intracellular antiviral pathways, and suppression of CD8+ T cell cytolytic effector functions through a negative feedback loop via the upregulation of PDL1 on target
hepatocytes and may in turn explain the apparent ineffectiveness of IFNγ as a therapeutic approach to the treatment of chronic viral hepatitis (Kakumu et al 1991, Lau et al 1991).

Overall these findings provide evidence of an important mechanism by which hepatocytes, in response to IFNγ produced by cells of the immune system, play an active role in the tight regulation of CD8+ T cell effector function delivery through upregulation of PDL1 expression. This role is pivotal in maintaining the fine equilibrium between effective control of viral replication, and avoiding excessive tissue injury.

**HBV hijacks PD-1 pathway**

The Hepatitis B virus has evolved effective strategies for resisting immune responses through overwhelming, neutralising or evading innate and adaptive immune responses to infection (see Chapter 1 Section 1.3.3.1).

These studies have demonstrated that hepatoma cell lines which constitutively express HBV, have higher levels of PDL1 expression than their parent cell lines. Furthermore, activation of HBV-DNA transcription in an AD38 cell line through a tetracycline on/off switch results in an increase in PDL1 expression. Finally, transfection of a human hepatoma cell line (Huh7 cell line) with HBV-DNA via a plasmid, results in upregulation of PDL1 expression which increased from 6 hours
post transfection through to 72 hours. This increase in PDL1 expression directly correlated with both intracellular and excreted HBV-DNA levels, suggesting that viral load predicts the degree of upregulation of PDL1. Cell surface PDL1 expression was assessed with flow cytometry in this transfection model, but no upregulation of PDL1 expression was observed, despite those changes observed at an mRNA level. This may reflect differences in the effective translation and translocation of PDL1 by Huh7 cells. This phenomenon has also been observed in iNOS expression following IFNγ treatment of Huh7 cells (Proto et al 2008).

These results suggest that Hepatitis B virus replication can itself result in upregulation of PDL1 on infected hepatocytes and this phenomena may in turn result in skewing of the balance between cytolytic and non-cytolytic immune responses, contributing to viral persistence.

In conclusion, these studies demonstrate that the PD-1 pathway plays an important role in the immunopathogenesis of chronic hepatitis B virus infection. The main findings of this thesis are summarised in Figure 41 and represent a possible model by which the PD-1 pathway is involved in the establishment of chronic HBV infection.

In patients with chronic hepatitis B on anti-viral therapy, viral load directly correlates with PD-1 expression on CD8+ and CD4+ T-cells in vivo. Furthermore the Hepatitis B virus itself upregulates PDL1 expression on infected hepatocytes in vitro. Both cytolytic and non-cytolytic CD8+ T-cell effector functions are important in effective
control of viral replication, and blockade of the PD-1 pathway distorts the balance between these differential effector functions in vitro. The PD-1 pathway has evolved along with other co-inhibitory pathways as an evolutionary break on the adaptive immune response, and the Hepatitis B virus has probably evolved to take advantage of this inhibitory T-cell pathway allowing it to persist with chronicity.

Future areas of study will undoubtedly include clinical trials of fully humanised anti-PD-1 antibody and may also involve neutralising antibodies to other co-inhibitory T-cell pathways (e.g. CTLA-4 etc). Indeed there is currently an ongoing clinical trial with a fully humanised anti-PD-1 antibody in 34 patients with chronic hepatitis C virus infection, who have failed standard treatment strategies. These trials must be conducted with caution as the risk of excessive immune-mediated host damage is well recognised. These studies will need to encompass other chronic viral infections (e.g. HIV/HCV).

It is also crucial to establish the intracellular mechanisms by which Hepatitis B virus infection upregulates PDL1 expression on hepatocytes as this may lead to the development of further immunotherapeutic targets.
This figure summarises the main findings of this thesis and represents a possible model by which the PD1 pathway is involved in the establishment of chronic HBV infection.

Finding 1. PD-1 hyperexpression is associated with dysfunctional CD8+ T-cells. These studies in patients with CHB undergoing a course of oral antiviral therapy confirmed that the observed paralysis of T-cell function was reversible and associated with HBV-DNA levels and hyperexpression of PD-1.

Finding 2. PD-1 engagement impacts on differential effector functions of HBV-specific CD8+ T-cells. These studies in a purposely-designed model of human hepatitis B virus infection demonstrated that engagement of the PD-1/PDL1 pathway resulted in a decrease in cytolytic CD8+ T-cell function, with initial preservation of non-cytolytic cytokine production.

Finding 3. Hepatitis B virus exploits the PD-1 pathway which may favour viral persistence. These studies on human hepatoma cell lines which express components of HBV and transfection of hepatoma cell lines with HBV demonstrate that HBV infection results in upregulation of PDL1 expression on the cell surface of infected hepatocytes, with subsequent functional impairment of T-cell reactivity, possibly favouring chronicity of infection.
Chapter 9

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