# Immune Control of Hepatitis B Virus infection by innate lymphocytes

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**Chapter 6: Reciprocal interactions between Vδ2 T cells and epithelial cells**

**Declaration**

I, Mellissa Conroy, declare that this thesis is my own work and has not been submitted in any form for another qualification at any university or other institute of education, with the exception of the cytotoxicity assays which were performed by Ross McNicholas as part of his MD.

Information derived from the work of others has been acknowledged and cited in the text.

Signed: _____          Date: _____

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## Abbreviations

| | |
|---|---|
| $^{51}$Cr | Chromium |
| ALT | Alanine aminotransferase |
| APC | Antigen presenting cell |
| APC | Allophycocyanin |
| BFA | Brefeldin A |
| BSA | Bovine serum albumen |
| CBA | Cytometric Bead Array |
| cccDNA | Covalently closed circular DNA |
| CCN | Corrected copy number |
| CD | Cluster of Differentiation |
| CMV | Cytomegalovirus |
| CN | Copy number |
| CRC | Colorectal carcinoma |
| $C_t$ | Threshold cycle |
| CTL | Cytotoxic T lymphocyte |
| DC | Dendritic cell |
| dH$_2$O | Distilled water |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxyribonucleotide triphosphates |
| DTT | Dithiothreitol |
| EBV | Epstein Barr virus |
| EtBr | Ethidium bromide |
| FCS | Foetal calf serum |
| FITC | Fluorescein isothiocyanate |
| FSC | Forward Scatter |
| GM-CSF | Granulocyte-macrophage-colony stimulating factor |
| HAV | Hepatitis A virus |
| HBcAg | Hepatitis core antigen |

| | |
|---|---|
| **HBeAg** | Hepatitis B 'e' antigen |
| **HBsAg** | Hepatitis B surface antigen |
| **HBSS** | Hanks Balanced Salt Solution |
| **HBV** | Hepatitis B virus |
| **HCC** | Hepatocellular carcinoma |
| **HCV** | Hepatitis C virus |
| **HDV** | Hepatitis D virus |
| **HEV** | Hepatitis E virus |
| **HIV** | Human Immunodeficiency virus |
| **HLA-DR** | Human leukocyte antigen DR |
| **HLA-E** | Human leukocyte antigen E |
| **HMBPP** | (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate |
| **HSV** | Herpes simplex virus |
| **ICAM-1** | Intercellular adhesion molecule-1 |
| **IFN** | Interferon |
| **Ig** | Immunoglobulin |
| **IL** | Interleukin |
| **iNKT cell** | Invariant natural killer T cell |
| **IPP** | Isopentenyl pyrophosphate |
| **IU** | International units |
| **LCMV** | Lymphocytic choriomeningitis |
| **mAb** | Monoclonal antibody |
| **MFI** | Mean Fluorescence Intensity |
| **mg** | Milligram |
| **MHC** | Major histocompatibility complex |
| **MICA / B** | Major histocompability complex class I-related chain A/B |
| **ml** | Millilitre |
| **MMLV reverse transcriptase** | Moloney murine leukemia virus reverse transcriptase |
| **mRNA** | Messenger RNA |
| **NEAA** | Non-essential amino acids |

| | |
|---|---|
| **NK cell** | Natural Killer cell |
| **NKG2A** | Natural killer group 2A |
| **NKG2C** | Natural killer group 2C |
| **NKG2D** | Natural killer group 2D |
| **NKR** | Natural killer receptor |
| **NKT cell** | Natural Killer T cell |
| **nm** | Nanometre |
| **NT cell** | Natural T cell |
| **OD** | Optical density |
| **PBMC** | Peripheral blood mononuclear cells |
| **PBS** | Phosphate buffered saline |
| **PE** | Phycoerythrin |
| **PE-Cy5** | Phycoerythrin-Cyanine 5 |
| **PEG-IFN** | Pegylated interferon |
| **PerCP** | Peridinin-chlorophyll protein |
| **PFA** | Paraformaldehyde |
| **pg** | Picogram |
| **PHA** | Phytohaemagglutinin |
| **PMA/I** | Phorbol 12-myristate 13-acetate and Ionomycin |
| **qRT-PCR** | Quantitative real-time polymerase chain reaction |
| **rcDNA** | Relaxed circular DNA |
| **RNA** | Ribonucleic acid |
| **RPM** | Revolutions per minute |
| **RPMI** | Roswell Park Memorial Institute medium |
| **SEM** | Standard Error |
| **SSC** | Side scatter |
| **TCR** | T cell receptor |
| **TGF-β** | Transforming growth factor-Beta |
| **Th cell** | T helper cell |
| **TNF-α** | Tumor necrosis factor-alpha |
| **Treg cell** | Regulatory T cell |

**ULBP**                    UL16-binding protein

**VSV**                    Vesicular stomatitis virus

**Summary**

Hepatitis B virus (HBV) infects over 300 million people worldwide and despite the availability of a vaccine, it remains the second biggest carcinogen in the world and a serious global health problem. The majority of adult infections are resolved while 90% of neonates fail to clear infection. Persistently-infected individuals harbour a risk of developing liver disease and hepatocellular carcinoma (HCC). Treatment of HBV is met with several problems including poor tolerance and antiviral resistance and therefore, novel well-tolerated and effective therapies are required.

Innate lymphocytes include NK, NT, NKT and γδ T cells and comprise a group of MHC-unrestricted lymphocytes that can elicit potent cytokine production and cytotoxicity at a very early stage of virus infection. We proposed that a better understanding of the role of innate lymphocytes in the control of HBV infection might eventually facilitate the development of new cell-based immunotherapies.

We have quantified the frequencies and cytokine profiles of natural killer (NK), natural T (NT), natural killer T (NKT) and γδ T cells in a group of HBV patients with relatively low viral load and little evidence of liver disease in order to ascertain the role of these cells in controlled HBV infection. We found expansions in the frequencies of circulating NK and NT cells and the Vδ2 and Vδ1 subsets of γδ T cells in these patients, compared to uninfected control subjects. Furthermore, expansions of interferon-γ (IFN-γ)-producing NT, Vδ2 T and conventional T cells were significantly higher in this group as were the frequencies of total IFN-γ-producing lymphocytes. Higher levels of IFN-γ expression in HBV were confirmed using qRT-PCR and we, therefore, propose that such expansions are indicative of an active antiviral immune response and that IFN-γ plays an important role in the control of HBV replication. Higher frequencies of IL-10-producing NK cells observed in the HBV patients may indicate a possible regulatory role of these cells in asymptomatic HBV infection. IL-17-producing NT and T cells exhibit enhanced responses to *in vitro* stimulation in HBV suggesting that IL-17 plays a supporting role in the control of HBV infection and that such Th17-biased cells can expand rapidly following sufficient stimulation. Since IL-10 and IL-17 can elicit inhibitory effects on IFN-γ production, we propose that the increased potential of IL-10 and IL-17 production in these patients might represent a regulatory mechanism that limits the antiviral immune response to prevent liver injury. We have also found reduced expression of the cytotoxicity-associated receptor NKG2D by Vδ2 T cells

which may be another mechanism by which immune-mediated damage is limited in these HBV patients. Furthermore, we have found that HBV proteins have negative effects on IFN-γ-production by Vδ2 T cells suggesting that HBV can inhibit the effector mechanisms of these cells.

HCC is a serious endpoint of chronic HBV infection for which there are few curative options. We performed a preliminary study to survey the potential of HMBPP/IL-2 expanded γδ T cells as an immunotherapy against HCC by investigating the interactions between Vδ2 T cells and the HCC-derived Hep3B cells. We found that the expression of NKG2D by Vδ2 T cells and its ligand MICA/B on Hep3B cells was downregulated following co-culture. We propose that the shedding of MICA/B by the carcinoma-derived cells might be the mechanism by which NKG2D is downregulated and the effector functions of Vδ2 T cells are subverted. Quantification of cytokine expression in Hep3B cell / Vδ2 T cell co-culture supernatants revealed higher IL-10 expression and reduced IFN-γ and TGF-β1 expression. From our analysis of these findings, we suggest that Hep3B cell-derived IL-10 might inhibit IFN-γ production by Vδ2 T cells as an immune evasion strategy while the Vδ2 T cells may suppress TGF-β1 expression by the epithelial cells to avoid the immunosuppressive effects of the growth factor.

From this study, we propose that we have identified a new model of the non-cytolytical control of HBV replication in which NT and Vδ2 T cells minimise viral load without causing significant liver pathology via mechanisms that involve enhanced but regulated IFN-γ production. These results place HBV infection as a candidate disease that might benefit from cellular therapies involving innate T cells.

# Chapter 1

# Introduction

## 1.1 An Introduction to Hepatitis B Virus

### 1.1.1 The discovery of Hepatitis B virus

The discovery of hepatitis B virus (HBV) is owed to the work of Dr. Baruch Blumberg; a physician, clinical research scientist and ex-navy officer. In 1967, Blumberg and his colleagues were researching the genetics of disease susceptibility when an unusual finding arose. An antibody in a New York haemophiliac was specific to an antigen in an Australian aborigine (Blumberg *et al.* 1967). This antigen was called the 'Australia antigen' and was later shown to be the Hepatitis B surface antigen (HBsAg) (Millmann *et al.* 1970). After discovering the virus, Blumberg and his colleagues at the Fox Chase Cancer Centre went on to develop sensitive tests to screen for hepatitis B in donated blood. They also, developed a vaccine based on inoculation with a viral subunit (Millman & Blumberg 1978). This was a novel form of vaccination at the time and HBsAg is now the viral subunit used to vaccinate against hepatitis B virus with over 90% efficacy. Blumberg went on to win the Nobel Prize in 1976 for his contribution to human health.

### 1.1.2 The prevalence and prognosis of HBV

HBV along with Hepatitis C virus (HCV) is one of the major causes of liver disease worldwide (Lok & McMahon 2001; Lauer & Walker 2001). It is estimated that 2 billion people have been exposed to HBV and over 300 million of these are persistent carriers of HBsAg (Takano *et al.* 1995; Evans & Landon 1998; Christina & Page 2001; Kao & Chen 2002). Furthermore, over 1 million deaths per year are attributed to HBV infection (Pisani *et al.* 1990; Evans & Landon 1998; Christina & Page 2001). The majority of adult HBV infections are acquired horizontally (intravenous drug use, sexual transmission, blood transfusions, accidental needle stick injuries) and are resolved while, most neonates acquire infection vertically (perinatally) and an alarming 95% develop persistent infection (Chisari & Ferrari 1975; Stevens *et al.* 1975; Rehermann & Nascimbeni 2006).

Infection with HBV is followed by a 6-24 week incubation phase during which patients may experience a range of symptoms including nausea, vomiting, diarrhea, anorexia, fever, skin rash, headaches and jaundice (World Health Organisation (WHO) 2008). This acute illness can be followed by clearance of infection or life-long infection. Persistent infection may be asymptomatic or can lead to the development of chronic hepatitis. Progression to liver cirrhosis occurs in 2-5% of HBV patients each year and

the rate of progression is augmented by co-infection with hepatitis C virus (HCV), hepatitis delta virus (HDV), human immunodeficiency virus (HIV) or a high hepatitis B viral titre (Poynard *et al.* 1997; Mathurin et al 1998; De Franchis *et al.* 2003; Iloeje *et al.* 2006; Rehermann & Nascimbeni 2006). Cirrhosis is a serious disease which can lead to the development of hepatocellular carcinoma (HCC) and often leads to liver failure. HCC is a common endpoint of chronic HBV infection with a 100-fold increased risk in chronically infected persons compared to age-matched uninfected controls. Approximately 5% of chronically infected patients who develop liver cirrhosis are diagnosed with HCC while only 0.2% of asymptomatic carriers develop this malignancy (Beasley *et al.* 1981; Beasley 1988; Rehermann & Nascimbeni 2006). While the majority of infected adults recover from HBV infection and others develop lifelong infection, 0.5% of subjects succumb to fulminant hepatitis − an acute illness marked by liver inflammation which can lead to death (Stevens *et al.* 1975). Individuals aged below 11 or above 40 years have poor prognosis in fulminant hepatic failure and would often be considered for liver transplant (Grady *et al.* 1991).

The highest prevalence of HBsAg carriers exists in developing countries with limited healthcare. Many infected individuals in Africa and Asia contract HBV in childhood and in some parts, HBsAg carrier rates can be 10-15%. Countries with the highest living standards have the lowest prevalence, for example, UK, USA and Scandinavia (World Health Organisation (WHO) 2008).

Although a vaccine is available for HBV, new accessible and effective therapeutics are needed to treat the vast number of people already infected worldwide. Such therapies could limit the spread of infection, reduce the overall incidence of HBV and associated illnesses and contribute to a possible eradication of the virus. The prevalence of the disease among children in developing countries is of major concern and with limited curative options, such individuals are burdened with a life-long infection and a high risk of liver disease. A better understanding of the immune responses in HBV infection may facilitate the development of an effective immunotherapy for HBV.

### 1.1.3  The biology of HBV

**1.1.3.1 The structure of HBV**

HBV is a 42 nm enveloped non-cytopathic, hepatotropic DNA virus which belongs to the hepadnaviridae family of viruses (Tiollais *et al.* 1985; Seeger & Mason 2000; Rehermann & Nascimbeni 2006). The structure of the virion is shown in Figure 1.1. It consists of an outer envelope made up of 3 proteins; S, M and L. Each of these proteins contain the S domain called HBV surface antigen i.e. HBsAg which, by itself or together with other envelope proteins forms a filamentous and spherical antigen that is secreted from infected cells in 100-fold excess to complete virions (Kim & Tilles 1973; Stibbe & Gerlich 1983a; Stibbe & Gerlich 1983b; Heermann *et al.* 1984). The nucleocapsid (core protein HBcAg) lies within the viral envelope and contains the partially double stranded DNA viral genome. The genome consists of ~3200 nucleotides with a full-length negative strand and a shorter positive strand. The viral reverse transcriptase is covalently linked to the 5' end of the negative strand where as an oligoribonucleotide is linked to the 5' end of the positive strand. The nucleocapsid open reading frame contains two start codons therefore, encoding 2 proteins – the nucleocapsid (HBcAg) and a longer protein called pre-core. Pre-core is translocated to the endoplasmic reticulum where removal of its amino-terminal 29 amino acids and trimming of the carboxyl terminus gives a polypeptide that is secreted as HBeAg (Ou *et al.* 1986; Roossinck *et al.* 1986; Rehermann & Nascimbeni 2006).

**1.1.3.2 The life cycle of HBV**

HBV primarily infects hepatocytes but it has also been shown to infect peripheral blood mononuclear cells (PBMC), spleen, kidney, pancreas, skin and bone marrow cells (Chemin *et al.* 1994; Seeger & Mason 2000; Zoulim 2004; Ganem & Prince 2004).

HBV interacts with a cellular receptor on the hepatocyte, fuses with the cell membrane and then releases its nucleocapsid into the cytoplasm. The nucleocapsid translocates to the nucleus where the viral DNA is transformed from a relaxed circular DNA molecule (rcDNA) into a covalently closed circular proviral DNA molecule (cccDNA) (Zoulim 2004; Rehermann & Nascimbeni 2006). The cccDNA is supercoiled in the host chromatin and has a long half-life. It is the template from which 4 viral RNAs are transcribed using the host polymerase. These RNAs are exported to the cytoplasm and translated to form the nuclecapsid, envelope, reverse transcriptase and polymerase proteins. The reverse transcriptase binds its own mRNA (the pregenome

RNA) and is subsequently packaged into the nucleocapsid where new partially double-stranded viral genomes are reverse-transcribed from the pregenome RNA (Zoulim 2004; Rehermann & Nascimbeni 2006).

The nucleocapsid matures via a dephosphorylation process that increases its affinity to bind DNA. It then becomes enveloped within the envelope proteins as it passes through the endoplasmic reticulum and/or Golgi apparatus to form a complete virion that can be released (Zoulim 2004; Rehermann & Nascimbeni 2006).

Sometimes the mature nucleocapsid is not released. This occurs when the large L envelope protein accumulates in excess of the other two envelope proteins in the endoplasmic reticulum and disrupts trafficking through the Golgi and normal shedding of the viral particles. This can sometimes lead to oxidative stress and cytopathy so to overcome this, the DNA-containing nucleocapsid can be recycled back to the nucleus where its DNA is transformed into cccDNA. This is believed to maintain the cccDNA pool and as many as 30-50 copies of cccDNA can be found in the nucleus of an infected hepatocyte. It is believed to serve as a reservoir for viral replication and spread of infection. These reservoirs of cccDNA are important in the maintenance of chronic HBV infection and are believed to be a major hindrance in the durability of antiviral treatment (Zoulim 2004; Rehermann & Nascimbeni 2006). The formation of cccDNA is not fully understood but it is believed that the removal of the RNA transcriptase from the relaxed circular DNA (rcDNA) is crucial (Gao & Hu 2007). Further investigation into the biology of HBV may have to be performed before the complete elimination of cccDNA and successful eradication of HBV can be achieved.

Viral DNA can be integrated into the host genome but not as a means of viral replication. This together with the oxidative stress can lead to the development of HCC via genetic alterations that may result in uncontrolled hepatocyte proliferation (Zoulim 2004; Farazi & DePinho 2006). The life cycle of HBV is illustrated in Figure 1.2.

FIGURE 1.1

FIGURE 1.2

### 1.1.3.3 Genetic variation of HBV

It is estimated that $10^{10}$ incorrect nucleotides are incorporated into viral DNA everyday in a HBV-infected individual due to the absent proofreading capability of reverse transcriptase (Summers *et al.*1982). This leads to genetic variation and some variants may be selected based on their replication fitness, immune escape potential and susceptibility to antiviral medication. Such variants are known as quasispecies (Zoulim 2004).

HBV is divided into 8 groups based on its genetic heterogeneity i.e. genotypes A – H (Stuyver *et al.* 2000; Arauz-Ruiz *et al.* 2002; Fung & Lok 2004). Genotypes A and D are most prevalent in Europe and North America while genotypes B and C occur mostly in Asia. Compared with genotype C, B is associated with spontaneous, less active liver disease, a slower rate of progression to cirrhosis and less frequent development of HCC (Kao *et al.* 2000; Chu *et al.* 2002; Sumi *et al.* 2003). Differences in responses to treatment have also been observed between different HBV genotypes, however, the findings are conflicting among different studies. Most reports show no differences in responses to antiviral therapy (Pichoud *et al.* 1999; Nafa *et al.* 2000; Seigneres *et al.* 2000; Westland *et al.* 2003). However, Kao *et al.* (2002) found that lamivudine treatment is more effective in patients with genotype B, compared to those with genotype C. In addition, patients with genotype A and B have higher responses to pegylated interferon treatment than those with genotype C and D (Wai *et al.* 2002; Erhardt *et al.* 2005). The reduced efficacy of treatment in patients with genotype C is not surprising, since this genotype is associated with higher HBV titers and alanine aminotransferase (ALT) levels (Gu *et al.* 2009). However the emergence of genetic variants during treatment is the most troublesome problem and this problem is discussed in section 1.1.5 below.

### 1.1.4 The course of HBV infection

The different stages of HBV infection can be distinguished by the presence or absence of particular HBV antigens and/or antibodies specific for them. A combination of biochemical, serological and virological tests, and histological features can be used to diagnose and classify HBV infection (de Franchis *et al.* 2002). Assays for serum aminotransferases, HBV antigens (HBsAg and HBeAg) and antibodies (anti-HBs, anti-HBc [total and IgM] and anti-HBe), are widely available. It should be noted that

individuals that have received the HBV vaccine will also be positive for anti-HBs. Serum HBV DNA may be detected by DNA hybridisation, with or without signal amplification, while PCR-based assays for HBV RNA in serum are highly sensitive tools for the diagnosis of HBV infection. The assessment of a liver biopsy is an integral part of the diagnosis and management of patients with HBV infection, and in grading the severity of inflammation and the stage of fibrosis.

HBV infection is defined by the presence of HBsAg or HBV DNA in serum. Persistently undetectable or low serum HBV-DNA levels are associated with inactive disease, but high serum HBV-DNA levels may or may not be associated with active disease. Because of the fluctuating course of chronic HBV infection, serial determinations are necessary to ascertain HBV replication status of individual patients. Occult HBV infection is characterised by undetectable serum HBsAg but detectable HBV-DNA in serum or liver.

Diagnosis of acute hepatitis B is based on the history, raised serum aminotransferase levels and the presence of serum HBsAg and anti-HBc IgM. In chronic hepatitis B there is persistent hepatic inflammatory injury. In mild chronic hepatitis B aminotransferase levels are normal or minimally elevated and biopsy reveals minimal inflammation and absent fibrosis. In moderate to severe chronic hepatitis B aminotransferase levels are raised and there is moderate to severe inflammation and fibrosis. The presence of HBeAg in serum indicates that viral replication is taking place and anti-HBe is undetectable. In HBeAg negative chronic hepatitis B infection, anti-HBe is present and HBeAg is absent in serum. In the inactive HBsAg carrier state, HBsAg and anti-HBe are present in serum, but serum aminotransferase levels are persistently normal and there is little or no necro-inflammatory activity on liver biopsy. Such patients have either low or undetectable levels of HBV-DNA in serum (de Franchis *et al.* 2002). Table 1.1 lists these serological markers and the clinical relevance of their presence or absence in HBV infection and therefore, provides a good overview of their importance in the understanding of the course of HBV infection.

A quiescent 4-7 week phase follows HBV infection. Then, in acute HBV infection, the virus begins to replicate aggressively causing viral load to increase rapidly, reaching levels of $10^9$ copies /ml within 1-2 weeks and infecting most hepatocytes in the process (Jilbert *et al.* 1992; Kajino *et al.* 1994; Guidotti *et al.* 1999; Webster *et al.* 2000;

Thimme *et al.* 2003; Chang & Lewin 2007). This is followed by the generation of virus-specific T cells and antibodies causing the viral load to decrease. The decrease in viral load marks the immunoactive phase which is usually characterized by an increase in the level of ALT. ALT is an enzyme which is released into the blood stream upon hepatocyte damage or death and it is widely used as an indicator of hepatocyte injury in hepatitis. However, in some cases no elevation of ALT levels is detected. Seroconversion to HBsAg and HBeAg both occur soon after the decline in HBV viral load and HBV-specific T-cell responses are also detected conferring protective immunity against HBV (Rehermann & Nascimbeni 2006; Chang & Lewin 2007, Figure 1.3a).

Spontaneous HBeAg seroconversion occurs in 8-15% of patients in western countries (de Franchis *et al.* 2002). HBeAg seroconversion is usually associated with improved long term outcome because the expression of the 'e' antigen is associated with active viral replication (De Jongh *et al.* 1992; Bonino & Brunetto 2003). However, mutations (commonly G-A transition at position 1896 of the pre-core region) can give rise to hepatitis B variants with defective HBeAg production and this can lead to HBeAg, negative chronic hepatitis. HBeAg-negative HBV can manifest as a recurrent form with exacerbations and periods of remission (45%), an unremitting form (36%) or an unremitting form with acute exacerbations (20%) (Brunetto *et al.* 2002; Bonino & Brunetto 2003). Fulminant hepatitis due to HBV has been linked to mutations in the genes encoding HBeAg and often occurs in HBeAg-negative patients (Omata *et al.* 1991).

In chronic HBV infection, the virus can persist for many years during what is known as an immune tolerant phase during which HBsAg and HBeAg are present and HBV DNA levels are usually greater than $10^5$ copies/ml. At this time hepatic inflammation is mild and ALT levels are normal or mildly elevated. After several years of chronic HBV infection, a spontaneous or therapy-induced switch from an immune tolerant to an immune active phase can occur. HBeAg seroconversion may occur during this phase and the HBV-infected individual enters a low replicative phase where there is both reduced HBV viral load as well as normal ALT. If infection is not cleared or a genetic variant of HBV emerges, HBV DNA and ALT can rise and fall again over the course of a life-long infection (Chang & Lewin 2007, Figure 1.3b).

**Table 1.1**

**The serological markers of HBV infection:**

The markers described below are routinely used to distinguish between the different stages of HBV infection.

| HBV antigen | Antibody |
|---|---|
| **HBsAg (Hepatitis B surface antigen)** | **Anti-HBs** |
| The earliest indicator of acute infection. Indicates chronic infection if its presence persists for more than 6 months | The specific antibody to hepatitis B surface antigen. Its appearance 1-4 months after onset of symptoms indicates clinical recovery and subsequent immunity to HBV |
| **HBcAg (Hepatitis B core antigen)** | **Anti-HBc** |
| Not detectable in the bloodstream but HBcAg peptides can be expressed on the surface of hepatocytes. A marker of the infectious viral material and the most accurate index of viral replication. | The specific antibody to hepatitis B core antigen. Class IgM and IgG antibodies which do not neutralize the virus. IgM identifies an early acute infection. In the absence of HBsAg and anti-HBs, it shows recent infection. IgG with no IgM is present in chronic and resolved infections. Used to identify all previously infected persons, including HBV carriers, but does not differentiate carriers and non-carriers. |
| **HBeAg (Hepatitis B e antigen)** | **Anti-HBe** |
| Appears during weeks 3 - 6 and indicates an acute active infection at its most infectious period. Its presence means that the patient is infectious. Its persistence beyond 10 weeks shows progression to chronic infection and infectiousness. Secreted into the serum. Mutant strains of HBV exist that replicate without producing HBeAg i.e. HBeAg-negative HBV.* | The specific antibody to hepatitis B e antigen. During the acute stage of infection the seroconversion from HBeAg to anti-HBe indicates resolution of infection. Its presence in the patients blood along with anti-HBc and in the absence of HBsAg, anti-HBs and core HBV mutants indicates low contagiousness. |

* HBV DNA is routinely used to test for HBV infection when mutant strains escape detection by other methods. The loss of HBV DNA usually indicates the loss of HBV's replication capabilities. Data obtained from WHO (2008).

**FIGURE 1.3**

### 1.1.5 Treatment of HBV infection and antiviral resistance

The easiest way to control HBV and viral transmission is through vaccination with HBsAg (Purcell & Gerin 1975; Mahony 1999). Vaccination with HBV envelope antigens can also prevent liver disease when administered post-exposure to the virus. (Iwarson *et al.* 1988). Efficacy of the HBsAg vaccine is over 90% with 5-10% of infected vaccinees failing to mount an adequate immune response after immunization. This can be marked by the absence of detectable specific antibodies and such individuals remain at risk to HBV infection (Coates *et al.* 2001; Dienstag *et al.* 1984). HBsAg-specific B and T cells, NK and NT cells are involved in the immune responses to HBV vaccine and T cells from individuals who fail to respond to the vaccine (non-responders) exhibit inadequate proliferation in response to HBsAg (Chang *et al.* 1984; Chedid *et al.* 1997; Albarran *et al.* 2005). However, immunomodulatory and antiviral therapies are also available.

Interferon-α (IFN-α) is a naturally occurring cytokine with immunomodulatory antiviral properties and was licensed for treatment of HBV infection in the early 1990s (Haria & Benfield 1995). The addition of a polyethylene glycol (PEG) moiety to IFN gave rise to the pegylated form of interferon which has an extended half-life and therefore, a more sustained antiviral response (Craxi & Cooksley 2003). Pegylated IFN (PEG-IFN) leads to loss of HBeAg in 35% of treated patients and seroconversion to anti-HBe in 29-32% of patients (Cooksley *et al.* 2003; Chan *et al.* 2005; Lau *et al.* 2005). In HBeAg negative patients, trials have shown that 36% of patients show a reduction of HBV DNA to levels below $10^4$ copies / ml and exhibit normal ALT at the end of the follow-up, while 10-20% of HBeAg negative virological responders lose HBsAg and seroconvert to anti-HBsAg (Janssen *et al.* 1993; Marcellin *et al.* 2004; Lau *et al.* 2005). While effective in many subjects, the side effects of PEG-IFN can be quite harsh and include; headache, fatigue, nausea, anorexia, weight loss, influenza-like symptoms, alopecia, neuropsychiatric symptoms such as irritability, insomnia and depression, neurological symptoms and thyroid dysfunction (Janssen *et al.* 1990; van Zonneveld *et al.* 2005). Therefore, PEG-IFN is not always a preferential choice of treatment.

Lamivudine triphosphate was the first nucleotide analogue licensed for the treatment of chronic HBV. It is a cytosine analogue and it competes with this nucleoside for incorporation into growing DNA chains thus causing termination of DNA chain elongation and therefore, inhibiting reverse transcriptase culminating in inhibition of

viral replication. Lamivudine has been shown to reduce progression to liver disease and HCC in patients with advanced fibrosis or cirrhosis (Liaw *et al.* 2004). After 1 year of therapy HBeAg seroconversion and a reduction of HBV DNA to levels below $10^5$ copies / ml occur in 16-22% of treated patients compared to 4-13% in untreated patients. After 2 years, HBeAg seroconversion increases to 29% and further increases of 40% and 47% are seen after 3 and 4 years of therapy, respectively. Decreases in HBV DNA occur in 98% of patients. Once HBV DNA is below a detectable level and HBeAg seroconversion has occurred, lamivudine treatment can be discontinued (Lai *et al.* 1998; Dienstag *et al.* 1999; Liaw *et al.* 2000; Schalm *et al.* 2000; Leung *et al.* 2001; Schiff *et al.* 2003; Chang *et al.* 2004). However, HBeAg seroconversion is less durable than that observed following PEG-IFN treatment and relapse rates are considerably high. Furthermore, response rates in HBeAg negative patients decline over a treatment period of 4 years. (Hadziyannis *et al.* 2000; Buti *et al.* 2001; Perrillo *et al.* 2002; van Nunen *et al.* 2003; Gaia *et al.* 2004). Therefore, other antivirals have become available over the years and are often administered to HBV patients who have not been treated previously for HBV or to those who have received lamivudine but have developed resistance. Adefovir dipivoxil, entecavir, emtricitabine**,** telbivudine and tenofovir disoproxil are all nucleot(s)ide analogues that are used with varying rates of responsiveness and resistance. The highest incidence of antiviral resistance is still associated with lamivudine treatment while resistance to adefovir and entecavir is less common (Buster & Janssen 2006; Zhang & Wang 2009).

Long term off-treatment control has not been achieved in many patients. PEG-IFN-based therapies have the highest chance of sustained off treatment response. On the other hand, prolonged treatment with nucleoside analogues is feasible and even considered indefinitely. Therefore, in many cases PEG-IFN is the drug of choice due to the durable response associated with it and the removed risk of antiviral resistance. However, the harsh side effects of the therapy and a response rate of less than 50% means that a new immunomodulatory therapy with fewer side-effects and improved efficacy would be desirable for the treatment of HBV infection. For now, strategies are being employed to maximize the durability of antiviral treatment, for example, using more than one antiviral at a given time to reduce the risk of complete resistance to treatment. In addition, cloning of HBV genomes isolated from patients into vectors to design assays for detecting HBV drug sensitivity *in vitro* is taking place. This would facilitate prescription of existing antivirals or development of new antivirals to be used

against circulating strains (Durantel *et al.* 2004; Yang *et al.* 2004).

### 1.1.6 Other Hepatitis viruses

The development of hepatitis is the dominant common trait of the hepatitis viruses A-E. The viruses belong to different virus families, have different modes of transmission and the associated symptoms vary along with their abilty to cause hepatitis.

Hepatitis A virus (HAV) is a non-enveloped RNA picornavirus that infects hepatocytes and usually causes an acute asymptomatic infection. However, in some cases a fulminant course of infection can occur and may result in morbidity; age over 40 and a pre-existing liver disease (often HBV or HCV infection) can lead to this (Lemon 2000). HAV is transmitted via the fecal-oral route and can be spread through contaminated food, with overcrowding and poor personal hygiene contributing to its transmission (Hutin *et al.* 1999). A vaccine against HAV exists and has contributed to the worldwide decline in the incidence of infection.

Hepatitis C virus (HCV) is a non-cytopathic RNA flavivirus that infects hepatocytes and has infected approximately 170 million people worldwide. Like HBV, it is a blood born pathogen acquired through intravenous drug use, sexual transmission, blood transfusions, accidental needle stick injuries and perinatally. The clinical endpoints of HCV are similar to HBV but the prognosis is less favourable with 70% of infected individuals developing persistent infection (Chisari 2005). Of these chronically infected HCV patients, 10-20% will develop cirrhosis and subsequent liver failure while, 1-5% will develop HCC (WHO 2008). There is no vaccine for HCV and treatment is effective in only 55% of patients. Therefore, HCV is a major cause of death worldwide and the most common cause of liver failure in the United States (Chisari 2005). Impaired innate and adaptive immune responses have been associated with the persistence of HCV infection (Cooper *et al.* 1999; Lechner *et al.* 2000; Deignan *et al.* 2002; Golden-Mason *et al.* 2008). Furthermore, its genetic heterogeneity and multiple immune evasion strategies pose serious problems for the clearance and treatment of HCV infection (Chisari 2005). The findings of the vast amount of HCV research led to many of our hypotheses in HBV infection and inspired numerous experiments in the current study.

Hepatitis D virus or hepatitis delta agent can only form an infectious particle when the cell it has infected is co-infected with HBV. Its RNA genome encodes a single protein known as the delta antigen and HDV must acquire the HBsAg of HBV in order

to enter / infect another cell. HDV infection of a chronically infected HBV carrier usually causes an acute and self-limiting infection but in 5% of co-infection cases, chronic HDV infection occurs (Hadziyannis 1997). Progression to liver cirrhosis occurs in 60-80% of individuals with chronic HDV while the incidence of HCC is the same as that in HBV infection. The mortality rate for HDV infections are 2-20%, which are significantly higher than for hepatitis B (Purcell & Gerin 1996; Hadziyannis 1997). At the present time, treatment of HDV is only effective via interferon-α treatment of HBV and prophylactic measures via HBV vaccination are probably the best option (WHO 2008).

Hepatitis E virus (HEV) is a non-enveloped RNA virus which was previously classified as a calcivirus but is unclassified at present. Like, HAV, HEV is transmitted via the fecal-oral and is prevalent in areas with poor sanitation. It usually manifests as an acute illness which is characterised by jaundice, nausea, fever, vomiting, anorexia and abdominal pains. No cases of chronic infection with HEV or a chronic carrier state have yet been reported. Fulminant hepatitis is perhaps, the most serious outcome with a mortality rate of 0.5-4% and highest risk of death in the third trimester of pregnancy. Furthermore, co-infection of young children with HEV and HAV can cause acute liver failure (Purcell & Ticehurst 1988; Purcell 1996; Mast *et al.* 1998; WHO 2008).

## 1.1.7 Hepatocellular carcinoma

### 1.1.7.1 Problem, disease and treatment

As the third most frequent oncological cause of death in the world, HCC is a major global health problem and its incidence is increasing (Bruix & Llovet 2003; Stefaniuk *et al.* 2010). Viral hepatitis is a major cause of HCC and 80 – 90% of HCC patients have underlying cirrhosis (Fattovich *et al.* 2004; Paraskevi *et al.* 2006). The annual incidence of HCC is approximately 2% in European patients with cirrhosis, with a 5 year cumulative incidence of ~10%. This is increased to ~3% in Asian patients with cirrhosis, with a 5 year cumulative incidence of ~15% (Fattovich *et al.* 2004). Older age, male sex, active liver disease, high HBV DNA level, HBeAg positivity, co-infection with HCV or HIV, or alcohol abuse are all factors which increase the chances of HCC (Yang *et al.* 2002; Fattovich *et al.* 2004; Iloeje *et al.* 2005; Chen *et al.* 2006).

HCC often recurs after successful surgical or non-surgical treatment and progresses to an advanced stage, and its survival rate of only 5 years is mostly due to tumour invasion and metastasis (Sun *et al.* 1999; Jinushi *et al.* 2005; Toutirais *et al.*

2009). There are limited curative options for HCC and surgical resection is really only possible for small HCC malignancies (Jinushi *et al.* 2005; Stefaniuk *et al.* 2010). Liver transplantation is often the best option because it removes the tumour and in many cases, the underlying cirrhosis. Living donor liver transplantations account for over 96% of liver transplants in Asia where HCC is the leading cause of cancer death (De Villa & Lo 2007). Due to the limitations of treatment for HCC, careful monitoring of high risk groups is performed so that the malignancy can be treated in time. Alpha-fetoprotein levels and radiographic examinations are used to monitor for HCC in high risk HBV patients but new biomarkers are warranted due to the low sensitivity of alpha-fetoprotein (Lok & McMahon 2001; Stefaniuk *et al.* 2010). TGF-β1 has been implicated as a more sensitive indicator of small HCCs (Elliott & Blobe 2005). These data show that new immunotherapies are also required for the successful treatment of liver cancer.

### 1.1.7.2 HBV association with HCC

There is a strong association between the development of HCC and viral hepatitis with a reported 100-fold increased risk of HCC development in HBV-infected persons (Beasley *et al.* 1981). There are several mechanisms whereby HBV or HCV could contribute to HCC development and they are as follows;

1) HBV or HCV infection may lead to an inflammatory response that in turn leads to hepatocyte necrosis. The resulting cycle of hepatocyte necrosis and regeneration increases the possibility of genetic alterations which may lead to the development of tumour cells and HCC.

2) HBV- or HCV-infected hepatocytes may also be subjected to oxidative stress under which mutagens may activate cancer-relevant signalling pathways leading to genetic alterations and the development of HCC.

3) HBV- or HCV-associated cirrhosis can cause micro-environmental changes that can favour tumour development

4) HBV or HCV viral factors and/or host factors may inactivate the tumour suppressor gene p53 resulting in uncontrolled hepatocyte proliferation, genetic alterations and HCC (Farazi & DePinho 2006).

**1.2 Immunology of HBV infection**

**1.2.1    The antiviral immune response**

The antiviral immune response requires the recognition of the virus and activation of effector cells which can kill the virus-infected cells and inhibit viral replication via non-cytolytic mechanisms. A brief overview of this response is described here and the process is also illustrated in Figure 1.4.

In viral infection, a virus enters a cell, is uncoated and starts to synthesise viral RNA and proteins. The double-stranded RNA can trigger the production and the secretion of type 1 interferons i.e IFN-α/β, and the production of various IFN-stimulated gene-encoded proteins that can inhibit viral replication. While viral replication is controlled within the infected cell, the secreted IFN-α/β can signal to other cells to amplify the antiviral immune response (Sen 2001). For example, macrophages can be activated to produce cytokines that recruit natural killer (NK) cells to the site of infection. The IFN-α/β produced by the infected cells also activates the NK cells and once they home to the site of infection, they can directly recognise virus-infected cells and kill them through the induction of apoptosis. NK cells may also produce cytokines such as TNF-α and IFN-γ which can have direct antiviral effects, or IL-2 which enhances T cell proliferation (Guidotti & Chisari 2001).

IFN-α/β also induces upregulation of MHC Class 1 molecules and increases antigen presentation capabilities thus making virus infected cells more visible to T cells and antigen presentation cells. Dendritic cells can recognise double stranded RNA and viral antigens presented on the surface of the infected cells and once activated, they can produce cytokines (IL-12, TNF-α, IFN-γ) and present antigen to T cells thus stimulating T cell differentiation and proliferation. The recruitment of cytotoxic T lymphocytes (CTLs) and helper T cells takes significantly longer than the activation and appearance of NK cells. The CTLs can then induce apoptosis of the target cells while the helper T cells produce cytokines (IL-2, IFN-γ) that serve to sustain and amplify the immune response as well as exerting direct antiviral effects (Guidotti & Chisari 2001; Barry & Bleackley 2002).

**FIGURE 1.4**

**1.2.2 CD8^{+} T cells in HBV infection**

CD8^{+} T cells or cytotoxic T lymphocytes (CTL) are crucial to immune responses against intracellular pathogens, most notably viruses. CTLs are primed by professional antigen presentation cells (APC) in the lymphoid organs and require processed antigens that have been endogenously produced within or phagocytosed by the APC. When viruses, for instance, do not infect APCs, tissue-derived dendritic cells (DC) can internalise apoptotic virally-infected cells and debris and migrate to the regional lymph nodes to prime CTLs (Steinman *et al.* 1999; Sallusto & Lanzavecchia 1999). Upon recognition of their specific antigen, MHC-restricted T cells can induce apoptosis of virus-infected cells and can also elicit non-cytolytic effector functions via cytokine production. CD8^{+} T cells predominantly produce IFN-γ which can block viral replication and 'purge' viruses from cells without inducing cell death (Guidotti & Chisari 1999). TNF-α and IL-2 are also produced by the CD8^{+} T cells which facilitates amplification of the antiviral response. Once a CD8^{+} T cell has encountered its specific antigen, a pool of memory CD8^{+} T cells specific to that antigen are generated and these long-lived cells confer immunity against subsequent challenge with that antigen (Weninger *et al.* 2002; Santana & Rosenstein 2003). The cytolytic effector functions of CD8^{+} T cells can be mediated through perforin and granzyme, Fas ligand or TNF-α. Perforin and granzyme are contained in specialised lysosomes known as cytotoxic granules which are released by CD8^{+} T cells following antigen priming. The perforin serves to deliver the granzyme into the cytoplasm of the infected cell where the granzyme can activate apoptosis via its protease capabilities (Smyth *et al.* 2001). Fas ligand and TNF-α bind to the receptors Fas (CD95) and TNFR-I, respectively, which are expressed on the surface of the target cells. Both receptors contain cytoplasmic death domains which initiate a signalling cascade upon ligand binding and ultimately recruit caspases that promote cell death (Aggarwal 2003).

Resolution of HBV infection is associated with strong, polyclonal and multi-specific CTL responses directed against epitopes within the polymerase, envelope and core and pre-core proteins of HBV (Bertoletti *et al.* 1991; Penna *et al.* 1991; Missale *et al.* 1992; Nayersina *et al.* 1993; Rehermann *et al.* 1995; Maini *et al.* 1999; Thimme *et al.* 2003). CTLs usually appear in infected tissues 5-7 days after viral exposure where they elicit their cytolytic and non-cytolytic activity. CTLs often employ non-cytolytic mechanisms of viral clearance in large vital organs such as the liver (Harty *et al.* 2000).

This was shown in the HBV transgenic mice where HBV-specific CTLs secreted IFN-γ and tumour necrosis factor-α (TNF-α) which helped to evict the virus from cells non-cytopathically, as well as carrying out some killing activity (Guidotti *et al.* 1994; *et al.* 1996; Guidotti & Chisari 1999). This non-cytolytic mechanism of viral eradication was not surprising as inhibition of HBV gene expression in the liver of transgenic mice by TNF-α and IFN-α/β had already been observed (Gilles *et al.* 1992; Guidotti *et al.* 1994). Furthermore, HBV replication could be inhibited following transfer of HBV-specific CTLs from perforin-deficient & Fas ligand-deficient mice thus suggesting that both cytolytic pathways are needed for control of HBV (Guidotti *et al.* 1996; Nakamoto *et al.* 1997). However, recent work by Yang *et al.* (2009) has revealed that Fas and TNFR1, but not perforin, are required for clearance of HBV DNA from liver in transgenic mice.

The antiviral T cell responses are maintained for decades after spontaneous resolution of HBV infection. Trace amounts of HBV DNA and virus specific CTLs often persist in the blood suggesting that viral replication is minimized but the virus is never completely eradicated (Michalak *et al.* 1994; Penna *et al.* 1996). Further work has revealed that HBV-specific CD8^{+} T cells have memory T cell phenotypes suggesting that HBV-specific memory CD8^{+} T cells are vital in the clearance of HBV from patients with acute HBV infection (Sobao *et al.* 2002).

HBV-specific CD8^{+} T cell numbers are significantly lower in those individuals who develop chronic HBV infection (Maini *et al.* 2000; Sobao *et al.* 2002, Yang *et al.* 2009). Chronic HBV patients have lower numbers of virus specific CD8^{+} T cells as well as impaired IL-2 production and proliferation of CD8^{+} T cells (Chisari 1997, Maini *et al.* 2000; Reignat *et al.* 2002; Das *et al.* 2008). However, one study found that the CD8^{+} T cells with impaired proliferative capacity and IL-2 production still produced IFN-γ and TNF-α in chronic HBV and the authors proposed that these cells contribute to liver inflammation but do not facilitate viral clearance (Das *et al.* 2008). This theory is supported by earlier work which found large numbers of non-antigen specific CD8^{+} T cells in the livers of patients with uncontrolled HBV infections (Bertoletti & Maini 2000; Reignat *et al.* 2002).

CTLs are also involved in the immune responses that result from vaccination and therapy. CTLs specific for HBsAg are present post-vaccination (Hohn *et al.* 2002) while the strength of the CTL response differs between responders and non-responders to IFN-α treatment (Rehermann *et al.* 1996).

Viral parameters such as viral load and the genotype of the virus are believed to influence CTL responses. It was found that adequate stimulation of HBV-specific $CD8^+$ T cells can be achieved in chronic HBV carriers with low levels of serum HBV-DNA but not in those with high levels of serum HBV-DNA (Sobao *et al.* 2002). Also, lower HBV-specific CTL responses were observed in patients infected with genotypes C, compared to those infected with HBV genotype B (Gu *et al.* 2009). Most research, however, has focused on the influence of host parameters on CTL responses. Defective T cell priming by DC has been reported but this impairment could be restored using a cytokine cocktail of IL-1β, IL-6, TNF-α and prostaglandin $E_2$ (PGE2) that induced DC maturation, thus suggesting that the cause of impaired T cell responses in chronic HBV may be due to defects in DC maturation. These data are conflicting with other reports which indicate that DC maturation and functions are in intact in HBV-infected individuals (Duan *et al.* 2006). Regulatory T (Treg) cells have also been implicated in the inadequate $CD8^+$ T cell responses in HBV. An accumulation of Treg cells in HCC patients was concurrent with a significantly decreased infiltration of $CD8^+$ T cells in tumour regions compared with non-tumour regions. Other studies have shown that Treg cells from HCC patients could inhibit anti-CD3/CD28 mAb-induced proliferation, activation, degranulation, and production of granzyme-B and perforin by $CD8^+$ T cells. Increased frequencies of Treg cells were also associated with high mortality and reduced survival time of HCC patients. This suggests that Tregs may promote disease progression in HCC patients by inhibiting $CD8^+$ T cell functions (Fu *et al.* 2007). HBV variants carrying mutations in cytotoxic T cell epitopes have also been detected in chronic HBV thus showing that the evolution of the virus faciliates immune evasion and contributes to its persistence (Bertoletti *et al.* 1994; Rehermann *et al.* 1995).

There is significant evidence implicating the importance of CTLs in the resolution of HBV infection, however, there is no correlation between ALT, viral load and the frequencies of HBV-specific $CD8^+$ T cells in chronic HBV infection thus suggesting that they are not the main determinant in immunity against HBV or the main cause of immunopathogenesis (Yang *et al.* 2009).

### 1.2.3 $CD4^+$ T cells in HBV infection

Whereas $CD8^+$ T lymphocytes are committed to becoming CTLs upon recognition of their specific antigen, $CD4^+$ T cells can differentiate into a number of different effector T cells. Their differentiation is mostly determined by the signals provided by the APC at

the time of antigen presentation. These signals are transduced via co-stimulatory molecules expressed on the surface of the APC and via the secretion of cytokines and while, they promote the differentiation of one type of effector CD4$^+$ T cell they can also inhibit the differentiation of another. Effector CD4$^+$ T cells are classified into 4 main groups based on their cytokine profiles; T-helper 1, 2 and 17 (Th1, Th2, Th17) cells and the regulatory T (Treg) cells (Abbas *et al.* 1996; Glimcher & Murphy 2000; Ansel *et al.* 2003).

Th1 cells produce IL-2, TNF-α, IL-12 and IFN-γ and are involved in immune responses against intracellular pathogens such as *Mycobacterium tuberculosis* and viruses. Through the expression of cytokines and co-stimulatory signals, Th1 cells can promote activation of CD8$^+$ T cells as part of the antiviral response and activation of macrophages as part of the anti-mycobacterial immune responses. They can also stimulate antibody production by B cells in immune responses against extracellular pathogens. Through cytokine production, Th1 cells can also promote further Th1 differentiation while suppressing Th2 responses (Mosmann & Coffman 1989; Abbas *et al.* 1996).

Th2 cells produce IL-4, IL-5, IL-10 and IL-13. They stimulate antibody production facilitating the elimination of extracellular pathogens and can suppress Th1 immune responses and promote further generation of Th2 cells via cytokine production (Mosmann & Coffman 1989).

Th17 cells have been identified more recently than the other T-helper cells. They are distinguished based on their production of the pro-inflammatory cytokine IL-17. These cells are involved in immune responses against bacterial, fungal, parasitic and viral infections and have also been implicated in the pathogenesis of several autoimmune diseases (Mills 2008; McGeachy & Cua 2008; McKinstry *et al.* 2009; Gutkowski & Hartleb 2009; Zhang *et al.* 2010).

Treg cells produce the anti-inflammatory cytokine IL-10 and/or the immunomodulatory growth factor TGF-β and are said to be the modulators of cellular immune responses. These CD4$^+$ T cells are often further characterized by the intracellular expression of the transcription factor FOXP3 and the surface marker CD25. Their abundance can determine the course of immune responses to infections and they are crucial in the prevention of immune-mediated damage (Sakaguchi 2005; Bi 2009).

CD4$^+$ T cell-deficient mice have impaired HBV-specific CD8$^+$ T cell numbers and elicit severely weakened antiviral responses thus suggesting that CD4$^+$ T cells are a

crucial to the induction of the CD8$^+$ T cell response in HBV infection (Yang *et al.* 2009). A direct, cytokine-dependent antiviral role of Th1 cells has also been shown in HBV transgenic mice (Franco *et al.* 1997). Furthermore, HBV appears to subvert Th1 responses via the over-production of noninfectious subviral particles such as the nucleocapsid hepatitis B e antigen. This has been shown to induce a preferential Th2 cytokine profile by deleting Th1 cells (Milich *et al.* 1997; 1998). Th17 cells are expanded in the blood and livers of chronic hepatitis B patients and furthermore, their frequencies correlate with viral load levels and the severity of liver damage thus, implicating them in the immune responses against HBV and the pathogenesis of disease (Zhang *et al.* 2010). The frequencies of CD4$^+$CD25$^+$ Tregs are similar in controls and asymptomatic HBsAg carriers but they are significantly higher in chronic hepatitis B patients. They are thought to modulate antiviral responses in HBV and therefore, aid viral persistence (Peng *et al.* 2007). Fu et al (2007) showed that Treg cells can suppress CTL responses and that their expansions in HCC patients correlate with the reduced infiltration of CTLs in tumour regions and high mortality and reduced survival time of HCC patients.

### 1.2.4 Antigen presentation cells in HBV infection

"Professional" antigen presentation cells comprise the MHC-class II-expressing B cells, macrophages and dendritic cells (DC). It can be hypothesised that the deficient T cell responses associated with HBV infection may be linked with deficiencies in the APC and impaired T cell priming.

DC are the most important APC and these cells take up and process antigen and then, migrate to the lymphoid organs where they present the antigen to the T cell (Steinman & Cohn 1973; Steinman 1991). DC are generated from precursor cells e.g. monocytes or macrophages, via signals involving cytokines such as IL-4 and granulocyte-macrophage-colony stimulating factor (GM-CSF). Upon their generation, DC are said to be immature and serve a phagocytic function until they receive the correct signal which stimulates their differentiation into a mature phenotype. For instance, in viral infection, the recognition of viral double stranded RNA can trigger the differentiation of DC. Once mature, DC upregulate numerous adhesion and antigen presentation molecules and then migrate to the lymph node where their primary function is antigen presentation. They also produce numerous cytokines to recruit other arms of the immune response (Banchereau *et al.* 2000). The findings concerning

dendritic cells (DC) in HBV are conflicting. Defective function and immature phenotype of DC has been observed in HBV infection (Wang *et al.* 2001). However, Tavkioli *et al.* (2004) studied monocyte derived DC (MDDC) and found that while there were a few minor phenotypical alterations and slightly reduced IL-12 production by MDDC in HBV, their T cell stimulatory capacity was unaffected. Another study found that the expression of co-stimulatory molecules on DC and their capacity to stimulate T cells was impaired in chronic HBV infection (CHB) but could be restored using a cytokine cocktail (Duan *et al.* 2006). In 2007, Tavakioli *et al.* published their research on myeloid DC and plasmacytoid DC in which they found no quantitative, phenotypic or functional defects in chronic HBV carriers, compared to uninfected control subjects. The role of DC has not yet been elucidated in the clearance or persistence of HBV infection, however, IFN-α treatment has been shown to increase frequencies of circulating DC and increase the expression of  HLA-DR, CD80 and CD54 (ICAM-1) by such cells (Yu *et al.* 2006). One interesting report found that plasmacytoid DC from patients chronically infected with HBV induced the generation of a higher proportion of $CD4^+CD25^+$ Treg cells compared to those from uninfected controls or HBV resolvers (Hong *et al.* 2009).

Macrophages are a second type of "professional" APC. They are long-lived, phagocytic cells that circulate in blood as monocytes & reside in organs & tissues. These phagocytic cells engulf microorganisms, red cells, immune complexes and endotoxins and present the processed antigens to T cells (Laskin & Pendino 1995). Once activated by infected viruses or by NK, NKT or T cell derived cytokines, macrophages can produce cytokines that have direct (IFN α/β, TNF-α, nitric oxide) or indirect (IL-1, 6, 8, 10, 12, 08, GM-CSF ) antiviral effects (Laskin & Pendino 1995; Dinarello 1999). Macrophage-derived IFN α/β, TNF-α, IL-12 and nitric oxide can inhibit HBV gene expression and replication in HBV transgenic mice (Cavanaugh *et al.* 1997; Guidotti *et al.* 2000; Pasquetto *et al.* 2000).

B cells are the third type of "professional" APC. They internalize specific antigen by receptor-mediated endocytosis and subsequently present the antigen to helper T cells which can then stimulate antibody production by the B cells. The central role of B cells is in the antibody response which is discussed below. B-cell mediated immune responses are crucial to the elimination of HBV (Klenerman *et al.* 2008).

### 1.2.5 Humoral response in HBV infection

By coating viruses with antibodies (neutralizing and non-neutralizing) the physical interactions of many viruses with their receptors can be blocked and complement can be activated to destroy viruses (Cooper & Nemerow 1983; 1984). The Fc portion of virus-bound antibodies can interact with the Fc receptors on the surface of macrophages and NK cells and accelerate the removal of virions from circulation (Cooper & Nemerow 1983; 1984; Backmann & Zinkernagel 1997; Burton *et al.* 2000).

In general, following primary infection, the IgM response occurs (usually within the first few days). Neutralizing IgM responses significantly reduce the blood titer of several viruses such as vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV) and Friend virus (Brundler *et al.* 1996; Seiler *et al.* 1998; Super *et al.* 1998). As IgM levels drop, a T-cell dependent isotype switch to IgG occurs (including neutralizing IgG). This can occur as soon as 1 week after infection in infections such as VSV but peaks at a later time in HBV infection (Chisari & Ferrari 1995; Bachmann & Zinkernagel 1997). The neutralizing IgG helps remove extracellular virus and is considered to be very important in controlling long term infections that are not completely cleared such as HBV infections (Michalak *et al.* 1994). HBV may never be completely cleared and small traces may persist indefinitely but the antibodies are believed to serve to inhibit re-emergence of the virus by blocking extracellular spread (Rehermann *et al.* 1996).

Clearance of HBV is associated with production of anti-envelope antibodies and sera containing high levels of such antibodies can prevent chronic HBV infections (Alberti *et al.* 1978; Grady *et al.* 1978). Activation of the virus-specific cellular immune response is followed by the humoral response at least 10-12 weeks after HBV infection (Fong *et al.* 1994). In HBV infection, anti-HBc is the first antibody to appear. This is an antibody against the core antigen and its presence is used as a marker for HBV infection (past or present). IgM anti-HBc is present in high titer during acute infection and usually disappears within 6 months whereas IgG anti-HBc generally remains detectable for a lifetime. Anti-HBe antibody appears after anti-HBc antibody and its presence indicates a decrease in viral replication and therefore, in infectivity. Anti-HBe completely replaces HBeAg in the resolution of disease. Anti-HBs replaces HBsAg as the acute HBV infection is resolving and anti-HBs persists for a lifetime in over 80% of patients and indicates immunity (WHO 2008). However in $1 - 5\%$ of HBeAg negative patients, biochemical and histological activity persists with high serum HBV DNA

levels. In such patients, HBeAg is undetectable because of the predominance of HBV strains that cannot express HBeAg. These patients constitute a group with HBeAg negative chronic hepatitis in which HBsAg and anti-HBe are present in serum (de Franchis *et al.* 2002).

### 1.2.6 Innate lymphocytes in HBV infection

Innate lymphocytes are early responding lymphocytes that are not MHC-restricted and unlike B and T lymphocytes, they are activated in the absence of prior priming with an antigen. This is due to the expression of invariant receptors that recognize conserved antigens or 'danger'-associated molecules on the surface of infected or tumour cells. Once activated, innate lymphocytes can carry out cytotoxic activity or can produce cytokines which can contribute to the elimination of the pathogen / tumour and modulate adaptive immune responses.

### 1.2.6.1 Natural Killer cells in HBV infection

Natural Killer (NK) cells display powerful cytotoxic activities and are potent producers of pro-inflammatory cytokines such as IFN-γ. They constitute 10-15% of peripheral blood lymphocytes and are characterized by the presence of the immunoglobulin superfamily molecule CD56 and the absence of CD3 (Robertson & Ritz 1990). The two main subsets of NK cells are distinguished based on the density of their CD56 expression. $CD56^{BRIGHT}$ NK cells make up ~10% of the NK cell population. They express high levels of CD56 and are the main producers of IFN-γ while the majority of NK cells are $CD56^{DIM}$ and they are primarily responsible for natural cytotoxicity (Robertson & Ritz 1990; Cooper *et al.* 2001). NK cells also express a set of invariant stimulatory, co-stimulatory and inhibitory receptors (NKRs) and it is the combination of signals transmitted through these receptors that determines whether an NK cell is activated or not. Such receptors bind components of pathogens, host cells or cytokines. For example, virus-infected cells and activated resident macrophages produce chemoattractant factors which recruit NK cells to infected tissues (Biron *et al.* 1999). A variety of signals can then activate the NK cell to induce apoptosis of the virally-infected cell via perforin release. Activated NK cells also produce antiviral cytokines such as IFN-γ and TNF-α which can have direct antiviral effects and influence subsequent adaptive immune responses. However, in the absence of infection or

malignancy, the inhibitory receptors will have more bound ligand than the activating receptors and therefore, the NK cell is not inappropriately activated (Kos & Engleman 1995; 1996; Biron *et al.* 1999; Cooper *et al.* 2001; Guidotti & Chisari 2001; Picciolo *et al.* 2002; McQueen & Parham 2002; Lanier 2005).

NK cells are involved in immune responses against a wide variety of viruses including; cytomegalovirus, herpes simplex virus, adenovirus, influenza virus, vaccinia virus and coxsackie virus (Quinnan *et al.* 1982; Bukowski *et al.* 1983; 1984; Sheil *et al.* 1984; Stein-Streilein & Guffee 1986; Godeny & Gauntt 1987; Biron *et al.* 1989; Orange *et al.* 1995; Orange & Biron 1996; Durantel *et al.* 2004; Yang *et al.* 2004). Several studies have investigated NK cell responses in HBV infection but their role is still not fully understood.

It is thought that NK cells may play an important role in the early defence against HBV (Guidotti & Chisari 2001). They have been implicated in the inhibition of HBV replication and resolution of acute infection (Echevarria *et al.* 1991; Pasquetto *et al.* 2000; Kakimi *et al.* 2000; Kimura *et al.* 2002). However, their involvement in the immunopathogensis of the disease is also well documented with evidence suggesting that they play a central role in liver injury in both chronic HBV infection and in fulminant hepatic failure (Kakimi *et al.* 2001; Sitia *et al.* 2002; Dunn *et al.* 2007; Zou *et al.* 2010). The role of NK cells in HBV immunity and immunopathogenesis is believed to be mediated by the activating receptor NKG2D and the cytokines IL-2, IFN-γ and TNF-α (Echevarria *et al.* 1991; Dunn *et al.* 2007; Zou *et al.* 2010). IFN-α levels in CHB patients with liver inflammation have been shown to be sufficiently high to induce cytotoxic activity of NK cells and such IFN-α is presumably secreted by the infected hepatocytes (Dunn *et al.* 2007). The importance of NK cells in immunity against HBV is further evident in their enhanced activation status and IFN-γ production in responders to HBsAg vaccination, when compared to non-responders (Albarran *et al.* 2005).

From the evidence above, one may hypothesise that NK cells are important in early defense against HBV and that deficiencies in such cells may lead to the subsequent impairment of adaptive responses that are characteristic of chronic HBV infection. The findings of Zeng *et al.* (2009) support this hypothesis. They found that cytotoxicity and NKG2D expression by NK cells was reduced in HCC patients. It was also lower in HBV patients compared to controls. Our recent work in HCV infection also supports this hypothesis; it was found that frequencies of circulating NK cells are

reduced in individuals chronically infected with HCV, compared to those who resolve infection. Depletions and expansions of specific subsets of NK cells observed in the chronic HCV patients suggested that antibody-dependent cytotoxicity was impaired and inhibitory signals to the NK cells were amplified. From this, it was concluded that the alteration in NK cell frequencies may lead to further impairment in the overall cellular immune responses (Golden-Mason *et al.* 2008). Other studies have elucidated a mechanism by which HCV inhibits NK cell functions (Tseng *et al.* 2002; Crotta *et al.* 2002). In this study, the importance of NK cells in the control of HBV infection was assessed. This was achieved by investigating the frequencies and cytokine profiles of NK cells in a group of asymptomatic HBsAg carriers who, as a model of immune control, would help to inform us of the role of NK cells in HBV immunity.

### 1.2.6.2 Natural T cells in HBV infection

A proportion of human T cells, known as CD56^{+} T cells or natural T (NT) cells, express the NK cell surface molecule CD56, and can be classified as innate T cells because their cytolytic activity is not MHC-restricted, meaning that they are much earlier responders than their CD56^{-} counterparts (Schmidt *et al.* 1986; Kelly-Rogers *et al.* 2006). Such cells usually constitute 5% of PBMC but can account for up to 50% of T cells in the liver, thus making them of particular interest in the study of liver disease (Norris *et al.* 1999; Ishihara *et al.* 1999). Although NT cells are said to be innate lymphocytes and are classified as such in this study, at least some NT cells differentiate from classical CD8^{+} T cells (Kelly-Rogers *et al.* 2006). Furthermore, their activation sometimes requires the ligation of both the stimulatory NKRs and the TCR. Therefore, it is important to distinguish between the true innate nature of NK cells and the dual capacity of NT cells to respond in both an innate and adaptive manner. NT cells can respond to a diverse array of cytokines including IFN-γ, IFN-α, IL-1, IL-2, IL-12, IL-15 and IL-18 and are potent producers of IFN-γ, TNF-α and IL-4 (Lu & Negrin 1994; Satoh *et al.* 1996; Jin *et al.* 1998; Zoll *et al.* 1998; Dunne *et al.* 2001; Loza *et al.* 2002; Kelly *et al.* 2004). The rapid production of cytokines and cytolytic activity by NT cells in response to infection and malignancy poises them as ideal therapeutic candidates. As well as their own antiviral activities, the cytokine profiles of NT cells enables their modulation of subsequent adaptive responses – a concept that is of particular interest in chronic HBV infection where adaptive responses are weakened.

NT cells have been implicated in the immune response to HBV infection and the liver pathology associated with it, while their depletion has been associated with the persistence of HCV infection and hepatic malignancies (Barnaba *et al.*1994, Deignan *et al.*2002; Albarran *et al.* 2005; Norris *et al.* 2003). It was hypothesized that NT cells play a role in immunity to HBV and therefore, studied a group of HBsAg carriers without or with mild liver disease and low viral load, to determine if such cells were involved in HBV immune control.

**1.2.6.3 Invariant Natural Killer T cells and CD1 in HBV infection**

Another subset of innate T lymphocytes that respond rapidly and elicit potent IFN-$\gamma$ production and cytotoxic activity are the classical or invariant NKT (iNKT) cells. They express a restricted TCR repertoire consisting of a V$\alpha$14J$\alpha$18 $\alpha$-chain in mice and a V$\alpha$24J$\alpha$18 $\alpha$-chain in humans, paired with a limited number of $\beta$-chains (Bendelac *et al.* 1997). Such cells also express a number of cell-surface markers typically expressed on NK cells, hence the name NKT cell, and recognize lipid antigen presented by the MHC-like glycoprotein, CD1d (Bendelac *et al.* 1997; Exley *et al.* 1997; Brigl & Brenner 2004; Gumperz 2006). These cells are therefore, referred to as CD1d-restricted T cells.

CD1d is one of 5 isoforms of CD1; CD1a-e. All CD1 proteins possess a sequence of hydrophobic amino acids in their antigen-binding groove which facilitate the binding and presentation of lipid antigens. They are expressed on a variety of specialized APC including monocytes, B cells and dendritic cells (Porcelli *et al.* 1998).

Although several endogenous and bacterial lipid antigens have found to be presented by CD1, the most potent activator of iNKT cells isolated to date is the marine sponge-derived glycolipid $\alpha$-galactosylceramide ($\alpha$-galcer), which is presented by the CD1d molecule. Therapeutic activation of iNKT cells with $\alpha$-galcer in mice results in potent anti-tumour cytotoxic activity and the rapid release of cytokines leading to the inhibition of tumour growth and the elimination of a wide variety of viruses (Kawano *et al.* 1997; Lynch *et al.* 2009). However, the glycolipid was much less effective in humans due to the difference in the numbers of iNKT cells between humans and the transgenic mouse models used in these studies. While, iNKT cells account for 5% of circulating and 30% of hepatic T cells in mice, they only constitute 0.02-0.8% of circulating T lymphocytes and less than 1% of liver T lymphocytes in humans (Kenna *et al.* 2004). The human omentum is the only organ reported to have a high proportion

of such cells with numbers in healthy subjects averaging 10% (Lynch *et al.* 2009). Therefore, although the studies in mice appear promising, the elucidation of new antigenic lipids and novel subsets of NKT cells may be the key to finding a lymphocyte subset with equivocal potency in humans.

There is more evidence showing the role of iNKT cells in intracellular bacteria, parasitic infections and tumours than in viral infection but their potent cytotoxic activity, production of IFN-γ, rapid activation of NK cells and modulation of subsequent adaptive responses, suggests that they have an important role to play in antiviral immune responses (Sieling *et al.* 1995; Denkers *et al.* 1996; Cui *et al.* 1997; Bendelac *et al.* 1997; Schofield *et al.* 1999; Apostolou *et al.* 1999; Carnaud *et al.* 1999).

Studies in mouse and chimpanzee models have shown that invariant NKT cells are important in the inhibition of HBV replication and viral clearance (Guidotti *et al.* 1999; Kakimi *et al.* 2000). Baron *et al.* (2002), however, showed that non-invariant NKT cells which are still CD1d-restricted responded to hepatocytes expressing HBV antigens in a mouse model and led to liver injury in such animals. They hypothesized that such non-classical NKT cells are important early responders in HBV infection and may be responsible for the significant decline in HBV DNA that preceded the CTL influx in HBV infection (Guidotti *et al.* 1999; Baron *et al.* 2002). *Baron et al.* (2002) also concluded that NKT cells may modulate subsequent adaptive responses and/or play a role in the development of liver disease in HBV patients. However, the differences in cell frequencies cited above mean that the antiviral mechanism of such cells in humans may not be as effective as those in mice and further work is needed to evaluate the need and potential of such cells in HBV infection in humans.

From the findings documented above, one may hypothesise that NKT cells are important in the control of HBV infection and that deficiencies in such cells may lead to inadequate adaptive immune responses and unfavourable outcomes of HBV infection. Depleted numbers of iNKTs have already been reported in the livers of chronic HCV patients and in HIV type 1 infection and such deficiencies could pre-dispose HBV-infected individuals to persistent infection (Deignan *et al.* 2002; van der Vliet 2002). Alternatively, HBV may interfere with iNKT functions to permit development of persistent infection. Such interference could be mediated through the CD1d molecule as in herpes simplex virus (HSV) and HIV infections (Yuan *et al.* 2006; Chen *et al.* 2006). Therefore, it was decided to investigate the frequencies of circulating iNKT cells and

CD1a, b, c and d$^+$ cells in a group of chronic HBV patients with mild liver disease and low viral load.

**1.2.6.4 γδ T cells in HBV infection**

Another group of unconventional early responding T cells are the γδ T cells which express a TCR consisting of a γ- and δ-chain, unlike the α- and β-chain of the classical T cell receptor. There are a multitude of differences between γδ and αβ T cells; αβ T cells take 4-96 hours to respond to antigen while γδ T cells can respond in under an hour, αβ T cells recognise peptide antigens complexed with MHC molecules while γδ T cells recognise non-peptide antigens in a MHC-unrestricted manner. Furthermore, while αβ T cells possess receptors for chemokines that promote their homing to the spleen and lymph nodes, γδ T cells are rarely found at these locations (Groh *et al.* 1998; Hayday 2000). While γδ T cells are potent producers of pro-inflammatory cytokines and elicit a strong cytotoxic effector function, their functions appear to exceed the normal constraints of T cells with reports of antigen presentation and cellular repair functions (Brandes 2005; Jameson and Havran 2007; Brandes 2009). It must be noted that many studies performed on γδ T cells are done in murine models but one must note the differences that exist between γδ T cell repertoires in humans and mice. In humans, γδ T cells are divided into 3 main groups based on their δ chain usage i.e. Vδ1, Vδ2 and Vδ3 T cells. Vδ2 T cells being the most prevalent in circulation while Vδ1 subsets are more abundant in the tissues and mucosal surfaces (e.g. intestine) and Vδ3 subsets appearing to be confined to the liver (Kenna *et al.* 2004).

One striking difference is the absence of Vγ9Vδ2 T cells in mice, which as the main circulating subset in humans and the centre of a number of therapeutic studies, is of great importance. Circulating γδ T cells in mice are predominantly Vγ1Vδ5/6 or Vγ2Vδ5 T cells. Therefore, it is advisable to take these differences on board when considering studies performed in murine models.

The exact mechanisms of γδ T cell antigen recognition is not fully understood but their rapid reaction time has always indicated that the classical antigen presentation process whereby αβ T cells are activated is not true for γδ T cells. Furthermore, studies

have revealed the activation of γδ T cells in the absence of MHC Class I and II and CD1 (Morita *et al.* 2001; Wei *et al.* 2008). This is due to the expression of several activating and inhibitory receptors called Natural Killer Receptors (NKRs) that facilitate the recognition of conserved antigens and danger-associated molecules (Constant *et al.* 1994; Tanaka *et al.* 1995; Morita *et al.* 1995; Battistini *et al.* 1997; Jomaa *et al.* 1999; von Lilienfeld-Toal *et al.* 2006; Toutirais *et al.* 2009). The NKR NKG2D, for instance, facilitates the recognition of MICA and MICB and mediates activation of γδ T cell cytotoxicity while NKG2A binds to HLA-E and induces inhibitory signals that prevent unnecessary killing.

Recognition of phosphoantigens such as the the pyrophosphate HMBPP ((E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate) is believed to be mediated through the Vγ9Vδ2-TCR and it induces potent activation of the cells, so much so that several phosphoantigens are being evaluated for cell-based therapies against malignancy (Eberl *et al.* 2002; Allison *et al.* 2001; Bennouna *et al.* 2008). Aminobisphosphonates and alkylamines have also been shown to be effective stimulators of Vγ9Vδ2 T cells and are also in clinical trials for the treatment of cancer. They stimulate Vγ9Vδ2 T cells indirectly by causing the accumulation of pyrophosphates in antigen presentation cells (Kunzmann *et al.* 1999; Scotet *et al.* 2005; Thompson *et al.* 2006; Dieli *et al.* 2007; Abe *et al.* 2009).

The importance of γδ T cells in immune responses against a wide range of bacteria, fungi, parasites and viruses is well documented (Bukowski *et al.* 1998; Morita *et al.* 2007). Their role in antiviral immunity has been evident in various studies, with elevated frequencies observed in EBV, HIV and HSV infections (De Paolo *et al.* 1990; Maccario *et al.*1995; Poccia *et al.* 1999). However, of particular relevance to this study, is the role of γδ T cells in hepatitis. Studies have revealed that γδ T cells may actually play a role in the liver injury associated with HCV infection (Nikolopoulou *et al.* 1995; Tseng *et al.* 2001). There is also evidence that the frequencies of circulating Vδ2 T cells are depleted in chronic HBV infection, along with lower IFN-γ secretion and γδ T cell cytotoxicity (Chen *et al.* 2008). Moreover, increases in the frequencies of CD4^{+} and CD8^{+} cells expressing the γδ TCR have been observed in HBV patients who seroconverted (Sing *et al.* 1998). Another study found no differences between controls and HBV patients when frequencies of Vδ1^{+} T cells were investigated where as the

frequencies of such cells were said to be higher in the liver of HCV patients (Rossol *et al.* 1998; Agrati *et al.* 2001).

Since γδ T cells can modulate adaptive immune responses via cytokine production and have even been implicated in an antigen presentation role, it was hypothesised that a depletion of such innate lymphocytes could lead to an impairment in subsequent adaptive immune responses in chronic HBV. Therefore, the frequencies of γδ T cells were studied in a group of asymptomatic HBsAg carriers and a group of un-infected control subjects to examine the role of such cells in a model of immune control. Furthermore, as the Vγ9Vδ2 T cell subset are already the centre of several clinical trials, their potential against HBV-associated HCC was investigated by assessing whether a human HCC cell line could modulate Vγ9Vδ2 T cell responses *in vitro*.

### 1.2.6.5 Activation of innate lymphocytes - NKRs

Innate lymphocytes express a set of invariant stimulatory, co-stimulatory and inhibitory receptors known as NKRs and it is the combination of signals transmitted through these receptors than can determine whether an innate lymphocyte cell elicits an effector function or not (Biron *et al.* 1999).

NKG2D is an activating receptor found on the surface of NK, NKT, CD8^{+} and γδ T cells whose loss often has detrimental effects in cancer (Clayton *et al.* 2008). The receptor is a type II transmembrane glycoprotein that binds stress-inducible molecules such as MICA and MICB and ULBP1-5 and triggers cytotoxic effector functions by NK cells but acts as a costimulatory molecule on CD8+ T cells. The NKG2D ligands are over-expressed in tumor cells and virally-infected cells and their expression is induced by numerous cellular stresses (Gonzalez *et al.* 2008). Many studies have highlighted the importance of NKG2D in tumor recognition and its tumor-mediated suppression on NK cells is well documented. Furthermore, the frequencies of circulating NKG2D-expressing NK cells are depleted in HCC, HBV and cervical cancer (Arreygue-Garcia *et al.* 2008; Zeng *et al.* 2009).

NKG2A (CD94) is an inhibitory NKR often expressed by NK cells and γδ T cells and upon recognition and binding to its ligand, the non-classical MHC class I molecule human leucocyte antigen (HLA)-E, inhibits lysis of an un-infected, non-tumor cell (Mistry & O'Callaghan 2007). The loss of HLA-E would abolish the inhibitory signal to the effector cells and according to the 'missing self' hypothesis would allow

the dominance of the activating signal and thus induce lysis of the target cell (Mistry & O'Callaghan 2007). NKG2A has even been implicated in γδ$^+$ T cell-mediated regulation of αβ$^+$ T cells in the intestine (Bhagat *et al.* 2008).

In this study, NKG2D expression by γδ T cells was investigated in HBV-infected patients as part of the elucidation of the role of γδ T cells in the immune control of the virus. NKG2D and NKG2A surface expression by HMBPP-expanded Vγ9Vδ2 T cells after co-culture with HCC-derived cells was also investigated to determine if there were any changes that would indicate tumor cell-mediated immune suppression in HBV-associated HCC.

**1.2.7 Cytokines in HBV infection**

**1.2.7.1 Type 1 Interferons in HBV infection**

Recognition of dsRNA by most cell types (a product of the replication process of RNA and DNA viruses) is a common trigger of type 1 IFNs (IFN-α/β) (Vilcek & Sen 1996). IFN α/β is usually produced within a few hours of viral infection and can inhibit viral entry or inhibit transcription, translation, assembly and secretion of several DNA and RNA viruses (Vilcek & Sen 1996; Stark *et al.* 1998; Thomson 1998). IFN α/β can also inhibit cell division, stimulate effector functions of NK cells, CTLs and macrophages upregulate MHC class I and II expression and induce antibody synthesis in B cells (Thomson, 1998).

It is suspected that HBV clearance is due to the direct antiviral mechanism of IFN α/β (Guidotti *et al.* 1995; Shimize *et al.* 1998). IFN-α/β has been shown to inhibit the formation of RNA-containing capsids in HBV infection thus abrogating HBV replication without affecting transcription, translation, capsid maturation or secretion (Wieland *et al.* 2000). Studies in HBV-transgenic mice have shown that IFN α/β is needed to control HBV infection. (Kamijo *et al.* 1994; McClary *et al.* 2000; Deonarain *et al.* 2000). IFN α/β can stimulate the proliferation of memory-phenotype T cells and those HBV-specific CTLs found in acute HBV infection are of an effector memory phenotype (Tough *et al.* 1999, Sobao *et al.* 2002).

**1.2.7.2 IFN-γ and TNF-α in HBV infection**

In animal models where infection is self-limited, an increase in viral replication is accompanied by IFN-γ and TNF-α production which leads to an adaptive immune

response sufficient to resolve infection (Guidotti *et al.* 1999; Thimme *et al.* 2003). IFN-γ and TNF-α can recruit and activate macrophages, NK cells and T cells, polarise T cell responses to ensure that the antiviral response occurs and upregulate antigen processing, transport and MHC expression in infected cells (Guidotti & Chisari 2001). These cytokines can also exert direct antiviral activity by disrupting viral replication or by purging the virus from the infected cell, for example, 2'5' oligoadenylate synthethase-induced RNase L degrades viral RNA and double standard RNA activated protein RNase (PKR) inhibits viral protein synthesis (Guidotti & Chisari 2001).

The relevance of these mechanisms in HBV were revealed when the intrahepatic induction of IFN-γ and TNF-α was shown to trigger the degradation of pre-formed HBV RNA in the nucleus of hepatocytes (Tsui *et al.* 1995). The importance of IFN-γ in immunity against HBV has been shown in the findings of several studies (Guidotti *et al.* 1999, Guidotti & Chisari 1999; Albarran *et al.* 2005).

### 1.2.7.3 Other cytokines in HBV infection

IL-2 has been implicated in the clearance of acute HBV infection (Echevarria S 1991, Biron 1991, Das *et al.* 2008). More recently IL-10 and IL-12 have been implicated in spontaneous HBeAg seroconversion in HBeAg-positive patients and furthermore, polymorphisms in the genes encoding IL-10, IL-12, IL-2 and IFN-γ have been associated with spontaneous resolution or persistence of HBV infection (Wu *et al.* 2009; Gao *et al.* 2009). The early stage of acute HBV infection is marked by a transient inhibition of NK and T cell responses that coincide with an increase in IL-10 accompanying HBV viremia. This suggests that HBV may induce IL-10 to evade antiviral immune responses (Dunn *et al.* 2009). Furthermore, CD4^{+} T cells from chronically infected patients were shown to produce higher levels of IL-10 in response to HBcAg compared to resolvers of HBV infection thus indicating that these cells may play a role in viral persistence (Barboza *et al.* 2009). This is not surprising as IL-10 has been manipulated by several viruses in order to evade antiviral immune responses (Taoufik *et al.* 1997; Stockl *et al.* 1999; Brady *et al.* 2003). This is in agreement with the findings above where Treg cells inhibit CD8^{+} T cell functions. TGF-β1 has been implicated in HBV-related liver fibrogenesis while IL-13 is thought to be involved in fibrosis in HCV infection (Weng *et al.* 2009). IL-17 is a pro-inflammatory cytokine whose role in antiviral immunity is still not fully understood. The induction of antigen-

specific Th17 cells have been observed in influenza and HCV infection. Furthermore, it was found that HCV NS4 protein could induce IL-10 and TGF-β to subvert antiviral responses via inhibition of IL-17 (Rowan *et al.* 2008). More recently, it has been reported that IL-17 is involved in the liver damage associated with chronic HBV infection with increases in circulating and intrahepatic Th17 cells positively correlating with disease progression and negatively correlating with circulating Th1 cell frequencies (Zhang *et al.* 2010, Ge *et al.* 2010). IL-17 has also been implicated in HBV-related liver fibrosis (Xu *et al.* 2009).

Due to the lack of cytokine profiling of innate lymphocytes in HBV infection, the cytokine profiles of innate lymphocytes in HBV was investigated by examining their production of a Th1 (IFN-γ), Th2 (IL-13), Treg (IL-10) and a Th17 cytokine (IL-17). It was hypothesized that any deficiencies / expansions in the specific cytokine producing populations of these early-responding cells could dictate the overall outcome of HBV infection. For example, a depletion of IFN-$γ^+$ NK cells may lead to uncontrollable infection whereas an expansion in the frequencies of such cells may lead to viral clearance, immune control or liver inflammation. Since a group of HBsAg patients with mild liver disease and low viral load were investigated in this study, it was expected that any differences observed between this group and uninfected controls would be an indication of the necessary measures for immune control without the occurrence of immune-mediated damage.

### 1.3 Rationale of this study

Since CD8$^+$ T cell responses are deficient in chronic HBV infection and innate lymphocytes can modulate adaptive responses, it was hypothesised that deficiencies in innate lymphocytes can lead to an impaired adaptive response and the development of persistent infection. We, and others have previously shown such deficiencies existed in another persistent infection characterised by weakened CTL responses i.e. HCV infection (Deignan *et al.* 2002; Golden-Mason *et al.* 2008).

The aims of our study were to examine circulating innate lymphocyte numbers, phenotypes and functions in patients with HBV infection and to compare them with those in healthy subjects. This involved the study of NK, NT, iNKT and γδ T cells in a cohort of individuals persistently infected with HBV. All patients in our study group had low viral load, mild liver disease, no comorbidities and were not receiving any

treatment for HBV infection. This group can therefore be considered as a model of persistent viral infection where the immune system nevertheless exerts effective control of viral replication while inflicting minimal pathology. The differences in the frequencies of innate lymphocytes, compared to those observed in uninfected control subjects, can inform us about the cells that control virus spread, that mediate pathology and liver disease and that are targeted by HBV. By comparing the cytokine profiles of innate lymphocytes in HBV-infected subjects to those in uninfected controls, it was aimed to determine what cytokines were involved in early responses against HBV. Our patient cohort was racially-diverse and since the demographics of our uninfected control subjects were mostly unknown, a group of ethnically-matched uninfected controls were also studied to confirm that any differences observed were due to HBV infection and not the demographics of our study group.

There were several limitations to our study, most notably, the use of circulating innate lymphocytes instead of hepatic innate lymphocytes. In the study of liver disease, it would be ideal to study intrahepatic cells but access to such liver samples is always a constraint. Peripheral blood samples were readily available which facilitated the study of significantly higher numbers of patients than a liver study would permit, particularly a study in which the patients have relatively little liver disease. Furthermore, obtaining healthy liver samples for our control group would be another significant challenge.

The findings of this study should serve to inform us of the early immune responses in HBV and of the responses required to achieve immune control. Alterations in the functions and frequencies of innate lymphocytes in HBV infection may indicate why the subsequent adaptive responses are impaired. This study should also act as a platform for subsequent similar research projects in which other groups of HBV-infected subjects can be investigated, most notably those who clear the virus and those with both chronic HBV infection and severe liver disease. The investigation of innate lymphocytes in the livers of all patient groups would also be desirable. It is only through the study of these groups that the immune responses against HBV can be fully understood and an effective and durable immunotherapy with few side effects can be developed. Innate lymphocytes would be ideal candidates for immunotherapy since they are easily cultured and do not require any specific antigen stimulation to become activated and most importantly, they are already the focus of several clinical trials in cancer. The potential of innate T cells in the treatment of HBV-associated HCC was

also assessed here, in a preliminary study that investigated reciprocal interactions between Vγ9Vδ2 T cells and HCC cells *in vitro.*

The overall aim of this study was to gain information that would aid the development and/or improvement of cell-based therapies for HBV and associated disease.

## 1.4 Aims of this study

- To examine the frequencies and phenotypes of circulating NK cells, NK cell subsets, NT cells, γδ T cells and their subsets, iNKT cells and CD1-expressing cells in patients with HBV infection and to compare them with those in healthy subjects.

- To investigate the cytokine profiles of circulating innate lymphocytes in patients with HBV infection and to compare them with those in healthy subjects by examining their production of a Th1 (IFN-γ), Th2 (IL-13), Treg (IL-10) and a Th17 cytokine (IL-17).

- To investigate the differences in the frequencies, phenotypes and cytokine profiles of the above mentioned cell subsets based on gender, age, viral load and ALT levels.

- To examine the natural and cytokine-induced cytotoxic capabilities of whole PBMC in patients with HBV infection and to compare them with those in healthy subjects.

- To investigate reciprocal interactions between Vγ9Vδ2 T cells and HCC cells *in vitro* by examining phenotypic changes and cytokine secretion following co-culture.

# Chapter 2


# Materials & Methods

## 2.1 Materials and equipment

General reagents used in this present study are shown in Table 2.1. Monoclonal antibodies (mAbs) used for flow cytometry and receptor cross-linking are shown in Tables 2.2 and 2.3, respectively. Mitogens and antigens used in cell culture are listed in Table 2.4. Table 2.5 comprises a list of reagents used in polymerase chain reaction. The reagents used in the cytometric bead array are listed in Table 2.6. Plastic-ware and equipment used during this study are listed in Table 2.7 and 2.8, respectively. Software applications used in the present study are listed in Table 2.9. Cell lines used are listed in Table 2.10. The cell lines GRM and HT29 were a gift from Dr. Stephen Todryk (Universifty of Northumbia, Newcastle). The cell line Hep3B was a gift from Dr. Steven Gray (IMM, Trinity College Dublin).

### Table 2.1

**General reagents:** The General reagents used in this study and their commercial sources.

| Product | Company |
|---|---|
| **Bovine Serum Albumin (BSA)** | Sigma-Aldrich, UK |
| **Brefeldin A (BFA)** | Sigma-Aldrich, UK |
| **Cell dissociation solution** | Sigma-Aldrich, UK |
| **Dimethyl sulfoxide (DMSO)** | Sigma-Aldrich, UK |
| **Dulbecco's Modified Eagle Medium** | Gibco, NZ |
| **Ethidium Bromide (EtBr)** | Sigma-Aldrich, UK |
| **Foetal Calf Serum (FCS)** | Sigma-Aldrich, UK |
| **Foetal Calf Serum (FCS)** | Hyclone |
| **Fungizone** | Gibco, NZ |
| **Hanks Balanced Salt Solution (HBSS)** | Gibco, NZ |
| **Hepes** | Gibco, NZ |

| | |
|---|---|
| **Ionomycin** | Sigma-Aldrich, UK |
| **Lymphoprep** | Nycomed, Norway |
| **Non-essential amino acids** | Gibco, NZ |
| **Paraformaldehyde (PFA)** | Sigma-Aldrich, UK |
| **Penstreptmycin** | Sigma-Aldrich, UK |
| **Phosphate Buffered Saline (PBS)** | Sigma-Aldrich, UK |
| **Polymyxin B** | Sigma-Aldrich, UK |
| **Roswell Park Memorial Institute medium (RPMI)** | Gibco, NZ |
| **Saponin** | Sigma-Aldrich, UK |
| **Sodium azide** | Sigma-Aldrich, UK |
| **Sodium Pyruvate 100mM** | Gibco, NZ |

**Table 2.2**

**Fluorescence-labelled monoclonal antibodies:** The monoclonal antibodies used for flow cytometry, their commercial sources and clone names.

| Antibody | Company | Clone |
|---|---|---|
| **Allophycocyanin (APC) labelled CD27** | ImmunoTools GmbH | LT27 |
| **APC labelled CD19** | BD Biosciences UK | SJ25C1 |
| **APC labelled CD3** | BD Biosciences UK | UCHT1 |
| **APC labelled CD54** | BD Biosciences UK | HA58 |
| **APC labelled IgG1** | BD Biosciences UK | MOPC-21 |
| **APC labelled IgG1** | ImmunoTools GmbH | PPV-06 |
| **APC labelled MICA/B** | Biolegend | 6D4 |
| **APC labelled NKG2A** | R&D Systems Europe | 131411 |
| **APC labelled NKG2D** | eBioscience | 1D11 |
| **Fluorescein isothiocyanate (FITC) labelled IgG1** | BD Biosciences UK | X40 |
| **FITC labelled CD1b** | BD Biosciences UK | HI149 |
| **FITC labelled CD1c** | Miltenyi Biotec GmbH | AD5-8E7 |
| **FITC labelled CD4** | BD Biosciences UK | SK3/RPA-T4 |
| **FITC labelled CD45RA** | ImmunoTools GmbH | MEM-56 |
| **FITC labelled CD56** | BD Biosciences UK | NCAM16.2 |
| **FITC labelled HLA-DR** | BD Biosciences UK | G46-6(L243) |
| **FITC labelled IFN-γ** | BD Biosciences UK | 25723.11 |
| **FITC labelled IgG1κ** | BD Biosciences UK | MOPC-21 |

| | | |
|---|---|---|
| **FITC labelled IgG2a** | BD Biosciences UK | X39 |
| **FITC labelled V$\alpha$24** | Immunotech France | C15 |
| **FITC labelled V$\gamma$9** | BD Biosciences UK | B3 |
| **FITC labelled $\gamma\delta$ TCR** | BD Biosciences UK | 11F2 |
| **FITC labelled Ber-EP4** | DakoCytomation Denmark | Ber-EP4 |
| **Phycoerythrin (PE) labelled IgG1** | BD Biosciences UK | X56 |
| **PE labelled V$\beta$11** | Immunotech France | C21 |
| **PE labelled 6B11** | BD Biosciences UK | 6B11 |
| **PE labelled CD1a** | BD Biosciences UK | HI149 |
| **PE labelled CD1d** | BD Biosciences UK | CD1d42 |
| **PE labelled CD8** | BD Biosciences UK | HIT8a |
| **PE labelled HLA-E** | BD Biosciences UK | 3D12HLA-E |
| **PE labelled IgG1$\kappa$** | BD Biosciences UK | MOPC-21 |
| **PE labelled IL-10** | BD Biosciences UK | JES 3-9D7 |
| **PE labelled IL-13** | BD Biosciences UK | JES 10-5A2 |
| **PE labelled IL-17** | R&D Systems Europe | 41802 |
| **PE-labelled anti-mouse IgG (secondary reagent)** | R&D Systems Europe | LXP08 |
| **PE labelled V$\delta$2** | BD Biosciences UK | B6 |
| **Phycoerythrin-Cyanine 5 (PE-Cy5) labelled IgG1** | BD Biosciences UK | MOPC-21 |
| **PE-Cy5 labelled CD14** | BD Biosciences UK | TUK4 |
| **PE-Cy5 labelled CD3** | BD Biosciences UK | UCHT1 |

| | | |
|---|---|---|
| **PE-Cy5 labelled CD40** | BD Biosciences UK | 5C3 |
| **PE-Cy5 labelled IgG1κ** | BD Biosciences UK | MOPC-21 |
| **PE-Cy5 labelled IgG2a** | BD Biosciences UK | G155-178 |
| **Peridinin-chlorophyll protein (PerCP) labelled CD3** | Biolegend | S K7 |
| **PerCP labelled NKG2C** | R&D Systems Europe | 134591 |
| **Unconjugated Vδ1** | Becton Dickinson | 11F2 |

**Table 2.3**

**Stimulating monoclonal antibodies:** Monoclonal antibodies used for receptor cross-linking and their commercial sources.

| Antibody | Company |
|---|---|
| **Anti-human-CD3** | BD Biosciences, UK |
| **Anti-CD28 mAb Anti-human** | BD Biosciences, UK |

**Table 2.4**

**Stimulants used in cell culture:** Mitogens and antigens used in cell culture and their commercial source.

| Product | Company |
|---|---|
| **(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP)** | Dr. Hassan Jomaa, Universitatsklinikum Gieben und Marburg GmbH, Germany |
| **Isopentenyl pyrophosphate (IPP)** | Sigma-Aldrich, UK |
| **Phytohaemagglutinin (PHA)** | Sigma-Aldrich, UK |
| **Phorbol 12-myristate 13-acetate (PMA)** | Sigma-Aldrich, UK |

**Table 2.5**

**RT-PCR:** All buffers, primers, probes and reagents used in RT-PCR and their commercial sources.

| Product | Company |
|---|---|
| **Lysis buffer** | Sigma |
| **β-Mercapthoethanol** | Sigma |
| **RNase Zap** | Sigma |
| **RNeasy Mini-kit (RNA extraction kit)** | Qiagen |
| **RNase-free DNase set** | Qiagen |
| **First strand buffer** | Invitrogen |
| **DTT** | Invitrogen |
| **Dimethyl sulfoxide (DMSO)** | Sigma |
| **Deoxyribonucleotide triphosphates (dNTPs)** | Invitrogen |
| **RNasin** | Promega |
| **MMLV Reverse Transcriptase** | Invitrogen |
| **Random Hexamers** | Invitrogen |
| **dH$_2$O** | Sigma |
| **β-actin primers** | A gift from Genomic Research Laboratory, IMM |
| **IFN-γ primers** | A gift from Genomic Research Laboratory, IMM |
| **β-actin probe** | A gift from Genomic Research Laboratory, IMM |

| | |
|---|---|
| **IFN-γ probe** | A gift from Genomic Research Laboratory, IMM |
| **IL-10 pre-customised primers and probes** | ABI |
| **IL-17 pre-customised primers and probes** | ABI |
| **Taqman® Universal PCR mastermix** | ABI |

**Table 2.6**

**Cytometric Bead Array**: The reagents used in the cytometric bead array and their commercial sources.

| Reagent | Company |
|---|---|
| **Cytometric Bead Array Human Soluble Protein Master Buffer Kit** | BD Biosciences, UK |
| **Human TGF-β1 Single Plex Flex kit** | BD Biosciences, UK |
| **Human IFN-γ Single Plex Flex kit** | BD Biosciences, UK |
| **Human IL-4 Single Plex Flex kit** | BD Biosciences, UK |
| **Human IL-6 Single Plex Flex kit** | BD Biosciences, UK |
| **Human IL-10 Single Plex Flex kit** | BD Biosciences, UK |
| **Human IL-12 Single Plex Flex kit** | BD Biosciences, UK |
| **Human IL-13 Single Plex Flex kit** | BD Biosciences, UK |

**Table 2.7**

**Plastic-ware:** Items of plastic-ware used in this study and their commercial sources.

| Product | Company |
|---|---|
| **24-well tissue culture plate** | Nunc, Denmark |
| **48-well tissue culture plate** | Nunc, Denmark |
| **40μm Nylon mesh needle filter** | Falcon 2340, USA |
| **6-well tissue culture plate** | Nunc, Denmark |
| **96-well u-bottomed tissue culture plate** | Nunc, Denmark |
| **96-well flat bottomed microplate** | Nunc, Denmark |
| **5 ml polystyrene round bottom Falcon tube** | BD Biosciences, UK |
| **50 ml Falcon Tubes** | BD Biosciences, UK |
| **RNase-free eppendorfs** | Qiagen |
| **RNase-free collection tubes** | Qiagen |
| **PCR plate** | ABI |

**Table 2.8**

**Equipment:** Pieces of equipment used in this study and their commercial sources.

| Equipment | Company |
|---|---|
| **Centrifuge 5810** | Eppendorf |
| **Centrifuge 5415D** | Eppendorf |
| **FACSCalibur system for flow cytometry** | BD Biosciences, UK |
| **Haemocytometer** | Neubauer |
| **MACS LS Column** | Miltenyi Biotec, GmbH |
| **MACS Separator** | Miltenyi Biotec, GmbH |

| | |
|---|---|
| **Thermo Forma Incubator** | Thermo Scientific |
| **Waterbath** | Clifton |
| **Olympus CK40-SLP Light microscope** | Olympus, Japan |
| **Nikon Eclipse E200 UV Light microscope** | Nikon, Japan |
| **8 sample spectrophotometer ND-800** | Nanodrop |
| **PTC-100 Programmable Thermo Controller** | MJ Research, Inc. |
| **7000 sequence detection system** | ABI Prism |
| **P100 Pipette** | Eppendorf |
| **P1000 Pipette** | Eppendorf |
| **P200 Pipette** | Eppendorf |
| **P20 Pipette** | Eppendorf |
| **Pipette-aid** | Drummond |
| **Tecan Sunrise Microplate Reader** | Tecan |
| **Thermo IEC Micromax centrifuge** | Thermo Scientific |

**Table 2.9**

**Software**: Software applications used in this study and their commercial sources.

| **Software** | **Company** |
|---|---|
| **CellQuest Software** | BD Biosciences, UK |
| **Summit Version 4.3** | Dako Colorado, Inc., USA |
| **GraphPad Prism 5.0.0.288** | GraphPad Software, Inc., USA |
| **FCAP Array Software** | BD Biosciences, UK |

**Table 2.10**

**Epithelial cell lines:** Epithelial cell lines used in this study, the organ from which they originated and the disease they cause.

| Cell line | Source | Disease |
|-----------|--------|---------|
| HT29 | Colon | Colorectal adenocarcinoma |
| Hep3B | Liver | Hepatocellular carcinoma |
| GRM | Skin | Melonoma |

**2.2 Subjects**

**2.2.1 HBV patients**

One hundred and two consecutive patients infected with Hepatitis B virus (HBV), attending the Hepatology Outpatient clinic at St. James Hospital, Dublin were studied. The numbers of patients studied in each analysis (n) are shown in each results chapter. Therefore, the frequencies or cytokine profiles of any cells of interest were not investigated for all 102 patients. The patient cohort was diverse in race and comprised 42 Africans, 40 Caucasians and 20 Asians. ALT levels ranged from 8 to 143 with 75 patients having ALT below 40 and 27 patients having abnormal ALT level above 40. Very few patients had very high ALT levels and thus most patients were regarded as having no or mild liver disease. No patient investigated was receiving treatment. The viral load was measured at or close to the time at which the sample was taken − this measurement varied from a minimum of less than 7 to $4.5 \times 10^8$ but was less than 20,000 copies/ml in the majority of patients. Viral load data in exact international units were not available but there are approximately $5 - 6$ HBV copies in each international unit. All 102 patients were sAg positive while none were eAg positive. The patient group included 18 females aged between the $18 - 30$ years and 33 females were aged between $30 - 60$ years. Eighteen males were aged between 18 and 30 years and 33 were aged between 30 and 60 years. All patients were free from HIV and HCV infection. The only co-morbidity recorded was Down's syndrome in two patients. Data on the infecting HBV genotypes were not available. Ethical approval for this study was obtained from the Research Ethics Committees of St James Hospital, Dublin and NUI Maynooth.

**2.2.2 Control subjects**

**2.2.2.1 General control subjects**

Our control population consisted of 66 peripheral blood samples of which the majority were obtained from the Irish Blood Transfusion Board. The samples were in the form of buffy coat packs however, several fresh blood samples were used also.

**2.2.2.2 Demographically matched control subjects**

As discussed in section 2.2.1, our patient population was diverse in race. The demographics of our population of general control subjects were mostly unknown.

Therefore, to confirm that any differences observed between patients and controls were due to HBV infection and not demographics, some phenotypical analysis on a cohort of demographically-matched healthy donors was also carried out. Blood samples from 15 Africans, 15 Irish and 10 Asian healthy control subjects were obtained from the GUIDE Clinic, St. James Hospital, Dublin. Ethical approval for this study was obtained from the Research Ethics Committees of St James Hospital and Adelaide and Meath Hospital incorporating the National Children's Hospital, Dublin.

### 2.2.3 Collection of subject material

Venous blood from patient and control subjects was taken into heparinised tubes and processed immediately. Peripheral blood mononuclear cells (PBMC) were prepared from these blood samples, and these cells formed the basis of analysis for this study. PBMC from 50 of the 60 control subjects were also prepared from buffy coat packs, obtained from the Irish Blood Transfusion Board, St. James Hospital, Dublin. Serum samples for both patient and control subjects were also obtained by taking blood into tubes containing no anti-coagulant.

### 2.3 General tissue culture procedure

The handling of human tissue which is a potential biohazard and the handling of cell cultures which require sterility was confined to class 2 biosafety cabinets, in an area of the laboratory called the tissue culture facility. Human tissues include all bloods, tissue specimens and primary cell cultures derived from these tissues. All human tissues are potentially virus-positive and should be handled as such.

White coats and gloves were always worn while working in the tissue culture facility. The work surface of the laminar air flow hood was wiped with 70% alcohol before use. Gloved hands were sprayed with 70% alcohol before working in hoods or handling anything from the $CO_2$ incubators. All bags of sterile plasticware were resealed with masking tape after use.

Blood spillages were soaked with HBSS and then, absorbed and inactivated with Virkon disinfectant. The area was then swabbed with alcohol. Small sharp objects and glass were disposed in sharp boxes which were then closed, sealed and replaced with a new sharps box once full. Large pipettes and other implements that might puncture plastic disposal bags were disposed of in solid yellow disposal containers. All liquids, including blood, were discarded in a plastic jug containing Virkon at a concentration of

10g / litre water. The jug was kept inside the flow hood for at least 20 minutes after pouring the waste, before its contents could be discarded down the sink. All biohazard material was disposed of in autoclavable bags. All sterilins and containers were rinsed with Virkon and sealed before disposal in autoclavable bags. Non-hazardous waste was disposed of in a black bag, as general refuse.

Two users were designated to clean and maintain the tissue culture facility fortnightly. This comprised a thorough cleaning of the incubator, laminar flow-hoods, water bath, centrifuge, bench areas and floor. A stock check of general reagents and plastic ware was also carried out and such items were replaced and/or reordered if necessary.

### 2.3.1 Peripheral blood mononuclear cells (PBMC) isolation

PBMC were isolated from heparinised blood by density gradient centrifugation. This method is based on exploiting the difference in density between lymphocytes / monocytes and erythrocytes / granulocytes.

Heparinised blood was diluted 1:1 with HBSS containing 1% FBS which was pre-warmed and carefully layered onto 20 millilitres (ml) Lymphoprep in a 50 ml Falcon tube. Tubes were centrifuged at 400 x g for 25 minutes with the brake off at room temperature. Under these conditions, the denser cells (erythrocytes / granulocytes) aggregate at the bottom while lymphocytes / monocytes remain above the Lymphoprep.

After the centrifugation, the buffy coat above the Lymphoprep layer was collected using a sterile Pasteur pipette and transferred to a clean, labelled 50 ml Falcon tube. The tube was topped up with HBSS to 50 ml and centrifuged at 80$0$ x g for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended in 50 ml HBSS and centrifuged at 40$0$ x g for 10 minutes. The cell pellet was finally re-suspended in complete RPMI medium (cRPMI), i.e. Roswell Park Memorial Institute medium (RPMI) supplemented with 2% Hepes, 0.8% Fungizone, 0.8% Penstreptimycin and 10% foetal calf serum (FCS). When buffy coat packs were used, anticoagulant was added before dilution with HBSS. Five millilitres of 5% ethylene-diaminetetraacetate (EDTA) was added per 50 ml of blood to prevent coagulation. The blood was then diluted 1:7 with HBSS. After these two steps, the protocol was followed as above

### 2.3.2 Enumeration of cells and viability testing

To evaluate cell viability and numbers, ethidium bromide (EB) and acridine orange (AO) was added to the cells. A working EB/AO solution was made up by mixing EB (0.8 ml of 4 mg/ml) and AO (2 ml of 1 mg/ml solution) stock solutions and adding 200 ml 0.85% (w/v) sodium chloride. One hundred and ninety microlitres (µl) of EB/AO was added to 10 µl of cells in cRPMI, giving a 1/20 dilution. This solution was vortexed and 10 µl was transferred to a haemocytometer for counting using a microscope with a ultraviolet (UV) light. Dead cells stain with EB and appear orange under UV light. Viable cells exclude EB but take up acridine orange and appear bright green under UV light. The numbers of cells in an area corresponding to $2 \times 0.1 mm^3$ were counted and the numbers of cells per ml were calculated taking into account the 1/20 dilution. Only samples with a cell viability of > 90% were used in the experiments.

### 2.3.3 Cryopreservation and recovery of cells

PBMC were centrifuged at 15$0 \; x \; g$ for 5 minutes. The supernatant was discarded and the pellet was re-suspended in a freezing medium which consisted of 90% FCS and 10% DMSO (dimethyl sulfoxide). Cells were cryopreserved at a concentration of $5 \times 10^6$ cells per ml. 1 ml of cells in freezing medium was aliquoted to each cryovial. The aliquots were stored at -80°C for 24 hours and then transferred to liquid nitrogen.

Before recovering cells from the liquid nitrogen, sterilins of cRPMI were pre-heated for 10 minutes in a water bath at 37°C. The cryovials of cells were removed from the liquid nitrogen and once they were semi-thawed, a sterile pastette was used to drop some warmed cRPMI onto the semi-thawed cells. Then the cells were taken up slowly and released back into the cryovial using the pastette several times. Once fully thawed, all contents of the cryovial were transferred into a sterilin containing 10 ml of warmed cRPMI. The cells were centrifuged at 30$0 \; x \; g$ for 10 minutes and all of the liquid was discarded, removing all of the DMSO. The pellet was suspended in cRPMI. Cell counts were performed as detailed in section 2.6 to determine cell viability.

### 2.3.4 Cell lines

The GRM and HT29 cells were cultured in T75 tissue culture flasks in cRPMI. Hep3B cells were cultured in T75 tissue culture flasks in complete DMEM medium (cDMEM) i.e. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.1 mM non-essential amino acids (NEAA), and 1.0 mM sodium pyruvate and 10% FCS. An Olympus CK40-SLP light microscope was used to examine the cells' confluency each

day. Before the cells became confluent, 90% of the cells were discarded while 10% were re-cultured in a new T75 tissue culture flask. This was achieved by firstly, removing all medium from the flask and performing a single wash with fresh medium to remove all dead cell debris. Two millilitres of non-enzymatic cell dissociation solution was then added to the flask and the flask was gently rocked from side to side to ensure that the fluid reached every cell. After 3-4 minutes, the layer of cells had broken away from the flask and, was removed in the cell dissociation fluid. This was topped up to ~ 20 ml with medium. The cells were centrifuged at $300 \times g$ for 8 min. The supernatant was discarded and the pellet was re-suspended in 10 ml of medium. 1 ml of this was added to a fresh flask and this was topped up with warm medium. The medium was replenished every 5 days for all cell lines.

## 2.4 Flow cytometry

## 2.4.1 Principles of flow cytometry

A flow cytometer facilitates simultaneous analysis of several properties of particles ranging in size from 0.2 to 150 μm (Figure 2.1). In cellular immunology, this technique is mainly use to analyse cells. Particles are acquired in a single suspension, hydrodynamically focussed to allow cells to pass, one by one, in front of a laser beam. Properties analysed include size, granularity and fluorescence (protein expression). As the cells pass by the laser beam, light scatter is used to determine the size and granularity of each cell. Protein expression is determined using specific antibodies conjugated to fluorescent markers called fluorochromes. Fluorochromes become excited to a higher energy state when they encounter certain wavelengths of light. When this happens, the fluorochromes emit photons of light which are then measured by dedicated detector photodiodes. Different fluorochromes emit light at different wavelengths. For example, FITC, PE, PE-Cy5 and PerCP become excited at wavelengths of 518, 575, 695 and 675 nm, respectively and can therefore be used on machines with a 488nm excitation laser. Fluorochromes such as APC and APC-Cy7 require a higher excitation wavelength i.e. 660 and 760nm, respectively and therefore they require a different excitation laser i.e. a 633nm excitation laser. The FACS Calibur used in this study (Figure 2.1) has both a blue 488nm and a red 633 nm excitation laser. Fluorochrome-conjugated antibodies can be used to detect a wide range of proteins, from cell surface markers to intracellular components. As fluorochromes vary with respect to the signal intensity they generate, it is recommended that the fluorochromes with the highest

signal intensity are used for the least abundant target proteins.  PE and APC have two of the highest signal intensities. Therefore, PE-Cy5- or PerCP-labelled CD3 could be used to detect T cells while PE-labelled or APC-labelled Vδ2 could be used to detect Vδ2 cells because they are the less abundant population. Various steps involving surface and/or intracellular surface staining with fluorochrome-conjugated antibodies is required prior to use of the flow cytometer. These steps shall be discussed in detail in the subsequent sections.
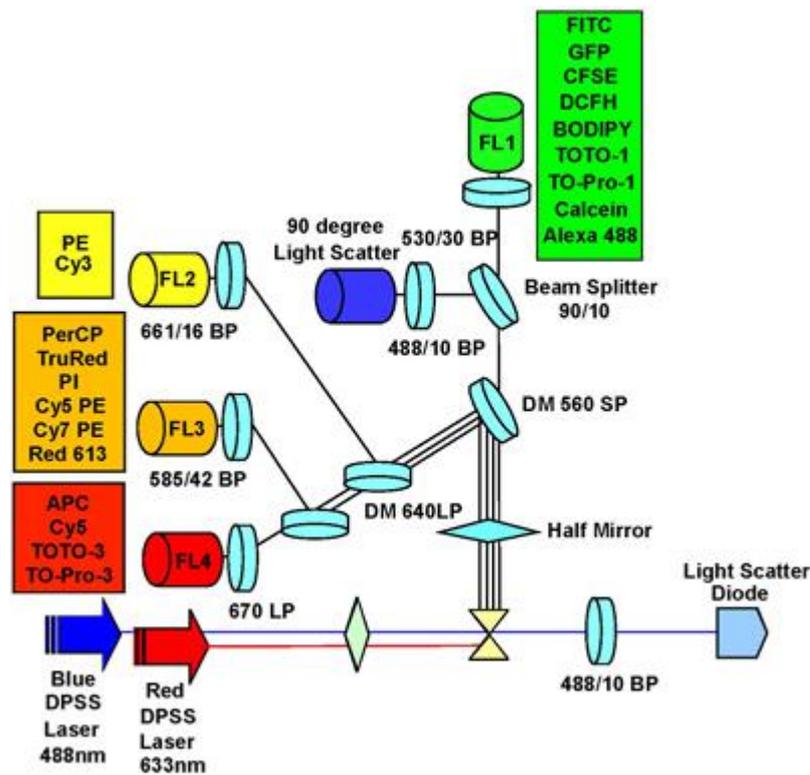


**Figure 2.1 Schematic diagram of FACs Calibur (BD):** This schematic diagram shows internal components of the FACs Calibur Flow Cytometer. The 'Blue DPSS Laser 488nm' is a low-power (15mW) argon laser that emits blue light at 488nm. The 'Red DPSS Laser 633nm'is a low-power (30mW) helium-neon diode that emits red light at 633 nm. The two '488/10' detectors for laser-light scatter are used to determine size (bottom) and granularity (top). The four detectors for fluorescence in the green, orange

and red/dark red regions of the colour spectrum are used to determine protein expression levels. Figure adopted from www3.niaid.nih.gov

## 2.4.2 Use of flow cytometry for the purpose of this study

### 2.4.2.1 Direct cell surface staining of cells with fluorochrome-conjugated mAbs

The required numbers of cells were centrifuged at $450 \, x \, g$ for 8 minutes. The cells were suspended in phosphate buffered saline (PBS) containing 0.33% bovine serum albumin (BSA) and 0.02% sodium azide (PBA buffer), at a density of $1x10^5$ cells per 50 μl PBA for each flow cytometry tube. The appropriate mAbs were added to each 50 μl aliquot of cells, therefore, labelling the cells for the surface markers listed in Table 2.12. Samples were incubated in the dark at 4°C for 20 minutes and then washed with 2 ml PBA. Labelled cells were fixed in 0.5 ml 1% paraformaldehyde (PFA).

### 2.4.2.2 Indirect cell surface staining of cells with unconjugated anti-Vδ1 mAb and determination of the optimal concentration to be used

The unconjugated anti-Vδ1 mAb was added to the appropriate tube containing 50 μl aliquot of cells in PBA buffer, therefore, labelling the cells. Samples were incubated in the dark at 4°C for 15 minutes, washed with 2 ml PBA and then 50 μl of PBA and 5 μl PE-labelled anti-mouse IgG was added to each tube. Samples were incubated for 15 minutes in the dark and a subsequent wash with 2 ml PBA was performed. Fifty microlitres of mouse serum was then added to each tube at a concentration of 200 μg/ml. Another 15 minute incubation and wash with PBA was performed and finally, the cells could be fixed in 0.5 ml 1% PFA.

Optimal concentration of the unconjugated anti-Vδ1 mAb was first determined by adding 0.5, 0.25, 0.1 and 0.05 μg to $1x10^5$ PBMC in 50 μl of PBA. The optimisation experiment was performed on PBMC from two healthy donors and, a concentration of 0.25 μg/ml was found to be the optimum concentration (Figure 2.2).

Figure 2.2

**2.4.2.3 Phenotypic analysis of surface stained cells**

Cell surface staining was used to investigate PBMC phenotypes in HBV groups and controls. Appropriate fluorescence-labelled isotype-matched control antibodies were used to correct for any background staining. Single staining of cells with fluorescence labelled anti-CD4, anti-CD8, anti-CD3 and anti-CD19 were used as additional controls to compensate for the interference of individual fluorochromes with one another during flow cytometry. For the identification of T cells, PE-Cy5 or PerCP-labelled anti-CD3 was used. This antibody was used in conjunction with other antibodies to identify specific types of T cells. For example, PE-Cy5- or PerCP-labelled anti-CD3 with both FITC-labelled anti-V$\alpha$24 and PE-labelled anti-V$\beta$11, or with PE-labelled anti-6B11 alone, was used for the identification of invariant NKT (iNKT) cells. PE-Cy5- or PerCP-labelled anti-CD3 and PE-labelled anti-CD56 was used to identify Natural T (NT) cells. Gamma delta T cells were identified using PE-Cy5- or PerCP-labelled anti-CD3 with FITC-labelled anti-$\gamma\delta$-TCR. The antibodies used to identify $\gamma\delta$ T cell subpopulations were FITC-labelled anti-V$\gamma$9, PE-labelled anti-V$\delta$2 and PE-labelled anti-V$\delta$1. The memory and effector T cell populations among these cells were identified using APC-labelled anti-CD27 and FITC-labelled anti-CD45RA. For the identification of B cells, APC-labelled anti-CD19 was used. Then, FITC-labelled anti-CD1b and PE-labelled anti-CD1a, or FITC-labelled anti-CD1c and PE-labelled CD1d were added to identify B cells expressing these CD1 isoforms. Likewise, for the identification of monocytes, PE-Cy5-labelled anti-CD14 was used. Then, FITC-labelled anti-CD1b and PE-labelled anti-CD1a or FITC-labelled anti-CD1c and PE-labelled CD1d were added to identify monocytes expressing these CD1 isoforms.

**Table 2.12**

**Phenotyping lymphocyte and monocyte subpopulations by flow cytometry:** The fluorescence-labelled mAbs used for cell surface staining of PBMC for phenotypic analysis by flow cytometry.

| Tube Number | FITC | PE | PE-Cy5* | APC |
|---|---|---|---|---|
| 1 | IgG1 | IgG1 | IgG1 | IgG1 |
| 2 | CD4 | CD8 | CD3 | CD19 |
| 3 | | CD56 | CD3 | |
| 4 | Vα24 | Vβ11 | CD3 | |
| 5 | CD56 | 6B11 | CD3 | |
| 6 | γδ | | CD3 | |
| 7 | Vγ9 | Vδ2 | CD3 | NKG2D |
| 8 | | Vδ1 | CD3 | |
| 9 | CD56 | Vδ2 | CD3 | |
| 10 | CD56 | Vδ1 | CD3 | |
| 11 | CD45RA | Vδ1 | CD3 | CD27 |
| 12 | CD45RA | Vδ2 | CD3 | CD27 |
| 14 | CD1b | CD1a | CD14 | CD19 |
| 15 | CD1c | CD1d | CD14 | CD19 |

* PerCP was used instead of PE-Cy5 in some experiments.

**2.4.3 Flow cytometry acquisition and analysis**

Acquisition and analysis of cell surface stained cells was carried out using the FACS Calibur flow cytometer and Cell Quest Software. Lymphocytes were gated using FSC (forward scatter, size) and SSC (side scatter, granularity) parameters (R1 in Figure 2.3). Monocytes were gated using FSC and SSC parameters, also (R2 in Figure 2.3), as where epithelial cell lines. Cells in tubes containing mAbs against both lymphocyte and monocyte surface markers were acquired twice, first with compensations and fluorescent detectors optimised for R1 and a second time with the acquisition parameters optimised for R2.

For the identification of lymphocyte subpopulations, 30,000 cells were acquired for each sample, with the exception of samples containing cells stained for invariant natural killer T (iNKT) cell receptors. Up to 200,000 cells were acquired for such samples because these iNKT cells are present in very low numbers in peripheral blood (~ 0.01% of T lymphocytes) (Kenna *et al.* 2004). The cells were first gated on the lymphocytes (R1 in Figure 2.3) and then, the T cells were identified as those cells which stained positively with PE-Cy5-labelled anti-CD3 mAb within R1. Such cells were gated in the region called 'T cells' (Figure 2.4). NT cells were identified as those cells within the region called 'T cells' that were positive for FITC-labelled anti-CD56 (Figure 2.4). NK cells were identified as all lymphocytes outside the 'T cells' region which were positive for FITC-labelled anti-CD56 (Figure 2.4). Invariant NKT (iNKT) cells were defined as those T cells which stained positive for FITC-labelled anti-V$\alpha$24 and PE-labelled anti-V$\beta$11, or PE-labelled anti-6B11. Similarly, gamma delta cells were identified as those T cells that stained positive for FITC-labelled anti-$\gamma\delta$-TCR, FITC-labelled anti-V$\gamma$9, PE-labelled anti-V$\delta$2 or PE-labelled anti-V$\delta$1. FITC-labelled anti-CD45RA and APC-labelled anti-CD27 were used to identify naïve, central memory, terminally differentiated and effector memory subsets of V$\delta$2$^+$, V$\delta$1$^+$ and CD8$^+$ T cells populations. This will be explained in more detail in the results section.

B cells were identified as APC-labelled anti-CD19 cells within R1 and were gated in a region called 'B cells'. CD1a$^+$, CD1b$^+$, CD1c$^+$ or CD1d$^+$ B cells were then

identified as those B cells that were positive for PE-labelled anti-CD1a, FITC-labelled anti-CD1b, FITC-labelled anti-CD1c or PE-labelled anti-CD1d, respectively.

Using FSC and SSC properties, the cells were first gated on the larger and more granular PBMC population (R2 in Figure 2.3) and then, the monocytes were identified as those cells which stained positively with PE-Cy5-labelled anti-CD14 mAb within R2. $CD1a^+$, $CD1b^+$, $CD1c^+$ or $CD1d^+$ monocytes were then identified as those $CD14^+$ cells in R2 that were positive for PE-labelled anti-CD1a, FITC-labelled anti-CD1b, FITC-labelled anti-CD1c or PE-labelled anti-CD1d, respectively.
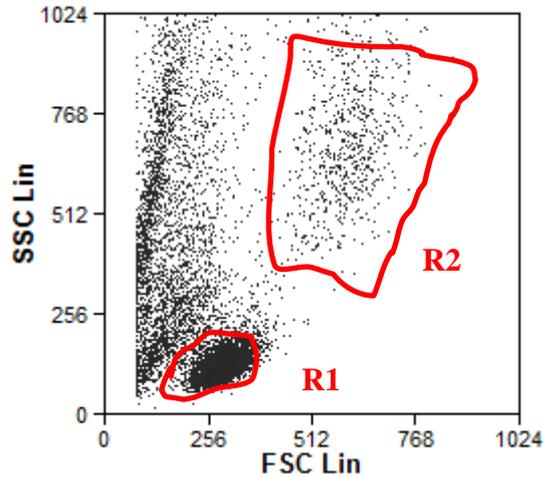
**Figure 2.3 Light scattering properties of human PBMC:** Measurements of cell size (FSC) are positioned on the X-axis, while measurements of cell granularity (SSC) are positioned on the Y-axis. Based on their size and granularity, all lymphocytes are gated within R1 and all monocytes are gated within R2.
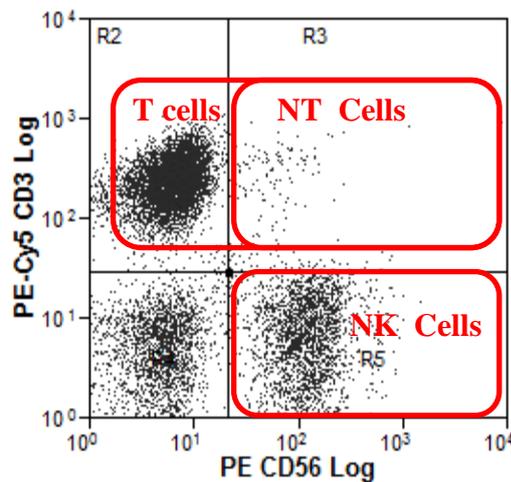


**Figure 2.4 CD3 and CD56 expression by human lymphocytes**: All cells represented here have been gated in R1 and are lymphocytes. Frequencies of $CD3^+$ lymphocytes are quantified in the right-hand and left-hand upper quadrants, while frequencies of $CD56^+$ lymphocytes are quantified by counting all cells in the right-hand-upper and right-hand lower quadrants. All $CD3^+$ lymphocytes are gated in a region called 'T cells'. All $CD56^+CD3^+$ lymphocytes are gated in a region called 'NT Cells'. All $CD56^+CD3^-$ lymphocytes are then gated in a region called 'NK Cells'.

**2.5 Analysis of cytokine production**

**2.5.1 Stimulation of PBMC *in vitro***

PBMC were suspended in cRPMI at densities of $0.5 \times 10^6$ cells per ml and 1 ml was placed in each well of a 24-well plate. Three different stimulations were performed. Some PBMC were incubated in medium alone. To induce cytokine production, 10 ng/ml phorbol 12-myristate 13-acetate and 1 µg/ml ionomycin (PMA/I) were added to PBMC in certain wells. In other wells, 9 µg anti-CD3 mAb and 9 µg anti-CD28 mAb were added to 3 ml 0.1 M $Na_2HPO_4$ (disodium hydrogen phosphate) binding buffer, and 1 ml was added to each well to give a final concentration of 3 µg anti-CD3 and anti-CD28 per well. The mAbs were left to bind overnight in a Thermo Forma $CO_2$ Incubator at 37ºC. The following day each well was washed 3 times with 1 ml PBS. Then, PBMC were added to the plate bound mAbs in each well. Ten micrograms per millilitre of Brefeldin A (BFA) was added to PBMC in all wells to prevent cytokine release. It achieves this by blocking protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus. The prevention of cytokine secretion in this assay allows them to become concentrated to detectable levels within cells. Cells were incubated in $CO_2$ incubator at 37ºC for 4 hours before intracellular cytokine staining was carried out.

**2.5.2 Cytokine analysis by intracellular cytokine staining and flow cytometry**

Following stimulation of PBMC *in vitro* and a subsequent 4 hour incubation, $1.5 \times 10^6$ cells for each type of stimulation were transferred to four separate 5 ml polystyrene round-bottom Falcon tubes and centrifuged at $450 \times g$ for 8 minutes. Cells were re-suspended in PBA at a cell density of $1 \times 10^5$ cells per 50 µl PBA and 50 µl was transferred to each flow cytometry tube. The appropriate mAbs were added to each 50 µl aliquot of cells to label them for the FITC-, PE- and PE-Cy5- or PerCP-conjugated cell surface markers as listed in Table 2.13. Samples were incubated in the dark at 4°C for 20 minutes and subsequently, washed with 2 ml PBA buffer. Cells were fixed in 0.5 ml 4% PFA and incubated in the dark at room temperature for 10 minutes. Following incubation, cells were washed with 2 ml PBA. Cells were then permeabilised by adding 1 ml 0.2% saponin and incubating in the dark at room temperature for 10 minutes. Cells were centrifuged at $450 \times g$ for 8 minutes, the supernatants were removed and the cells were stained for the presence of intracellular cytokines by adding 0.2 µg PE-conjugated IL-10, IL-13 or IL-17 in 50 µl saponin or 0.2 µg FITC-conjugated IFN-γ in 50 µl

saponin to each tube. Corresponding control mAbs were added at this time, also. Cells were incubated in the dark at 4°C for 20 minutes, washed with 2 ml PBA and centrifuged at 45*0 x g* for 8 minutes. Labelled cells were then fixed in 0.5 ml 4% PFA and samples were refrigerated before acquisition on the flow cytometer.

The lymphocyte subpopulations of interest were first identified as detailed in section 2.4.3. Cytokine producing cells within such populations were identified as those NK, NT or T cells which were also positive for FITC-labelled IFN-γ or PE-labelled anti-IL-10, IL-13 or IL-17. Figure 2.5 is a representative plot of IFN-γ production by T lymphocytes and non-T lymphocytes. Every cell gated in R5 is identified as an IFN-γ producing lymphocyte.

**Table 2.13**

**Flow cytometric analysis of cytokine production by innate lymphocytes:**
The fluorescence-labelled mAbs used for intracellular cytokine staining of PBMC for subsequent analysis by flow cytometry.

| Tube | FITC | PE | PE-Cy5* |
|------|------|------|------|
| 1 | IgG1 | IgG1 | IgG1 |
| 2 | CD4 | CD8 | CD3 |
| 3 | IFN-γ | CD56 | CD3 |
| 4 | CD56 | IL-10 | CD3 |
| 5 | CD56 | IL-13 | CD3 |
| 6 | CD56 | IL-17 | CD3 |
| 7 | IFN-γ | Vδ2 | CD3 |

* PerCP was used instead of PE-Cy5 in some experiments.
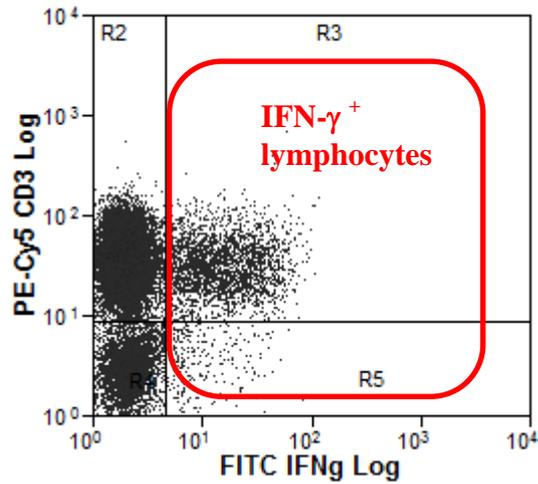
**Figure 2.5 IFN-γ production by PMA/I stimulated human lymphocytes** All cells represented here have been gated in R1 (Figure 2.1). Frequencies of CD3^{+} lymphocytes are quantified in the right-hand and left-hand upper quadrants, while frequencies of IFN-γ producing lymphocytes are quantified in the right-hand upper and right-hand lower quadrants. All IFN-γ producing lymphocytes are gated in a region called 'IFN-γ[+] lymphocytes'.

## 2.5.3 Cytokine analysis by quantitative real-time polymerase chain reaction (RT-PCR)

Quantitative RT-PCR is widely used to measure cytokine mRNAs in many cells including PBMC (Blaschke *et al.* 2000, Hartel *et al.* 2001, Kruse *et al.* 2001, Stordeur *et al.* 2002). In this study, the technique was used to compare the cytokine profiles of whole PBMC in HBV patients and healthy control subjects.

### 2.5.3.1 Cell lysis

PBMC were isolated from the blood of HBV patients and controls as described in section 2.3.1. The PBMC were re-suspended in 200 µl of HBSS and then placed in an RNase-free 1.5 ml eppendorf tube. The cell suspension was micro-centrifuged in a Thermo IEC Micromax centrifuge for 10 min at 13,400 x g. The HBSS was removed from the pellet and 600 µl of lysis buffer (Sigma) was added to the tube. Full reconstitution in the lysis buffer was ensured by gently pipetting the solution until all particulate had been dissolved. Ten microlilitres of B-mercapthoethanol was added to the cell lysates and the mixture was vortexed for 10 seconds, before being placed in a -80°C freezer.

### 2.5.3.2 RNA extraction

It was first ensured that all work surfaces, gloved hands and pipettes were RNase free by swabbing with IMS and RNase Zap (Sigma). The cell lysates were thawed on ice and then, homogenised until all precipitate was dissolved. The RNeasy mini-kit (Qiagen) was used to extract the RNA. The contents of the eppendorf tubes were first transferred to QIA shredder tubes and micro-centrifuged at 13,400 x g for 2 min. The spin column containing the cell debris was discarded and the collection tube was retained. An equal amount of 70% ethanol was added to the tube to match the volume of the contents and this was mixed by pipetting gently. This mixture was transferred to an RNeasy mini column and then, micro-centrifuged at 13,400 x g for 15 seconds. The flow through was discarded and 350 µl RWI buffer was added to the RNeasy mini column and then, micro-centrifuged at 13,400 x g for 15 seconds. The flow through was dispensed. DNase was diluted 1:7 in RDD buffer and 80 µl of this working concentration was placed very carefully onto the RNase silica gel membrane of the RNeasy mini column. After 15 min at room temperature, 350 µl RWI buffer was added to the mini column

and this was followed by a 15 second centrifugation at 13,400 x g. The flow through was discarded and the mini column was transferred to a new RNase-free collection tube. Five hundred micro litres of RPE buffer was added to the column and another 15 second centrifugation at 13,400 x g followed. The flow through was discarded and another 500 µl RPE buffer was added to the column followed by a 2 minute centrifugation at 13,400 x g, to dry the RNeasy Silica gel membrane. The mini column was transferred to a new RNase-free collection tube and centrifuged for 1 min at 13,400 x g. Again, the mini column was transferred to a new RNase-free eppendorf and 40 µl of RNase free water was added onto the membrane. This was followed by another centrifugation for 1 min at 13,400 x g. The same 40 µl of RNase free water was added onto the membrane and again, this was followed by centrifugation for 1 min at 13,400 x g. The flow through contained the RNA so the column was discarded and the eppendorf was retained and placed on ice.

A sample of RNA was taken to measure the yield and purity of the RNA using an 8 sample spectrophotometer ND-800 (Nanodrop). Spectrophotometry was carried out in duplicate to achieve accurate results. Following RNA extraction from $30 \times 10^6$ PBMC, spectrophotometry revealed that the RNA yields from both control subjects and HBV patients were sufficient. RNA purity was also tested. Pure preparations of RNA have $OD_{260}/OD_{280}$ values of greater or equal to 2. If there was contamination with proteins such as nucleases, for instance, the ratio would be less than 2. RNase contamination was the primary concern but contamination with any proteins would prevent accurate quantification of RNA. Similarly, pure preparations of RNA have $OD_{260}/OD_{230}$ values of 2. If there was contamination with phenolate ions or other organic compounds then, this ratio would be less than 2, and as with protein contamination, accurate quantification of RNA would not be possible. The $OD_{260}/OD_{280}$ and $OD_{260}/OD_{230}$ values were greater than but close to 2 for all the RNA samples of interest. Once the yield had been determined, the appropriate amount of RNA could be reverse transcribed to give 1000 ng of cDNA. This was the amount of cDNA needed to perform qRT-PCR for IFN-γ, IL-10, IL-17A and the house-keeping gene β-actin.

### 2.5.3.3 Reverse Transcription

Before PCR could be performed, the RNA samples required reverse transcription into DNA. The work area, pipettes and gloved hands were swabbed with RNAzap to ensure

the area was RNA-free. The RNA samples and reagents were then thawed on ice. A mastermix comprising 32% first strand buffer, 15.9% DTT (0.1M), 23.8% DMSO, 7.9% deoxyribonucleotide triphosphates (dNTPs), 3.2% RNAsin (1/4 dilution), 6.6% MMLV reverse transcriptase and 10.6% random hexamers was made and 18.85 µl of this solution was aliquoted to each of the appropriate number of eppendorfs required. Each RNA sample was reconstituted with distilled water (dH$_2$O) so that only 500 ng was added to 18.85 µl of mastermix. The eppendorfs were placed in the PTC-100 thermocycler which was programmed at 42°C for 1 hour. The cDNA was then removed from the PTC-100 and stored in a -20°C freezer.

### 2.5.3.4 PCR

Once the cDNA was obtained, PCR could take place. Firstly, PCR reaction mixtures were made. PCR reaction mixtures comprise a forward and reverse primer, a probe, a Taqman® Universal PCR mastermix, dH$_2$O and cDNA. β-actin and IFN-γ forward and reverse primers and probes were designed by Stordeur *et al.* (2002) and with permission, were made in-house by the Genomic Research Laboratory (IMM). The sequences are shown below in Figure 2.6. IL-10 and IL-17 forward and reverse primers and probes were purchased from ABI in a pre-customised form.

The IL-10 and IL-17 cDNA standards were obtained from ABI while the β-actin and IFN-γ cDNA standards were made in-house by the Genomic Research Laboratory (IMM). Serial dilutions of cDNA standards ranging from $10^3$ to $10^8$ were performed to create a standard curve for each cytokine and the hosue-keeping gene.

A volume of 7.5 µl of PCR reaction was added to the assigned wells of a 96-well PCR plate for each target cytokine and housekeeping gene, for both standards and samples of interest. This was performed in duplicate. The PCR plate was placed into the ABI 7000 sequence detection system for 1 hour and 48 minutes and then the results were analysed.

**IFN-γ**

**F464: CTAATTATTCGGTAACTGACTTGA**

**R538: ACAGTTCAGCCATCACTTGGA**

**P491: 6Fam-TCCSSCGCAAAGCAATACATGAAC-Tamra-p**


**β-actin**

**F976: GGATGCAGAAGGAGATCACTG**

**R1065: CGATCCACACGGAGTACTTG**

**P997: 6Fam-CCCTGGCACCCAGCACAATG-Tamra-p**


**Figure 2.6: Primer and probe sequences for IFN-γ and β-actin. F** and **R** refer to the forward and reverse primer sequences with sequence position indicated by the adjoined number. **P** refers to the probe which is dually labelled with a reporter dye (FAM, 6-carboxyfluorescein) covalently attached at the 5' end and a quencher dye (TAMRA, 6-carboxytetramethylrhodamine) covalently attached at the 3' end. The nuclease activity of the Taqman DNA polymerase separates the quencher from the reporter and the increase in fluorescence emission of the reporter dye is quantitative for the initial amount of template.


### 2.5.3.5 Controls used for RT-PCR

Several experimental controls were used to ensure that the results of qRT-PCR were both accurate and true. The first experimental control was the housekeeping gene, β-actin. It was used as a marker of cDNA quality i.e. if the cDNA was of poor quality then the cycle threshold ($C_t$) values for the housekeeping gene would be out of range i.e. significantly less than 15 or greater than 18. The second control was the standard curve which, was created for each target cytokine and housekeeping gene by carrying out serial dilutions of target cytokine or housekeeping gene standard cDNA ranging from $10^3$ to $10^8$. The slope of the standard curve must be between -3.2 and -3.6 to facilitate successful extrapolation of copy numbers from the $C_t$ values for the samples of interest. The standard curve also played a dual role as an indicator of primer and probe function. The third control was the non-template control (NTC). It contains the PCR reaction mixture for each cytokine, with RNase-free water substituting the cDNA. It is used to control for contamination and due to the intentional omission of cDNA, it should yield an undetermined $C_t$ value unless, the PCR reaction mixture is contaminated. The final

control was the assignment of duplicate wells for all standards and samples of interest. This reduces the potential for experimental error and maximises accuracy by ensuring that the $C_t$ values for each duplicate are within 1-2 cycles of each other.

### 2.5.3.6 Analysis of PCR data

The $C_t$ values are the cycle at which fluorescence from a sample crosses the threshold of background. The quantity of DNA doubles every cycle during the exponential phase and relative amounts of DNA can be calculated. For example, a sample whose Ct value is 3 cycles earlier than that of another sample has $2^3$ times more template.

After completion of PCR, the standard curve for each target cytokine and house-keeping gene was plotted with $C_t$ values on the Y axis and the copy numbers (CN) on the X axis to determine if the slope of the line was between -3.2 and -3.6 (Figure 2.7a). The $C_t$ values were then plotted on the X axis and the CN values on the y axis and, the CN of all samples could be extrapolated from the standard curve by using the equation of the line; $y = mx + C$ where m and C were given, x is the $C_t$ value of each sample and y is the CN (Figure 2.7b).

The target cytokine CN of each sample was then corrected by normalisation against the transcripts of the housekeeping gene. Normalisation permits accurate comparison of expression of the gene of interest between different samples, provided that the expression of the housekeeping gene is very similar against all samples. Since β-actin is a protein constitute of the microfilaments of the cytoskeleton, it definitely fulfils this criterion. The corrected copy number (CCN) of the cytokine mRNA was calculated by dividing the cytokine mRNA CN of the sample by the β-actin mRNA CN of the sample;

*CCN = (cytokine mRNA CN / β-actin mRNA CN)\* (β-actin mRNA CN at time zero)*
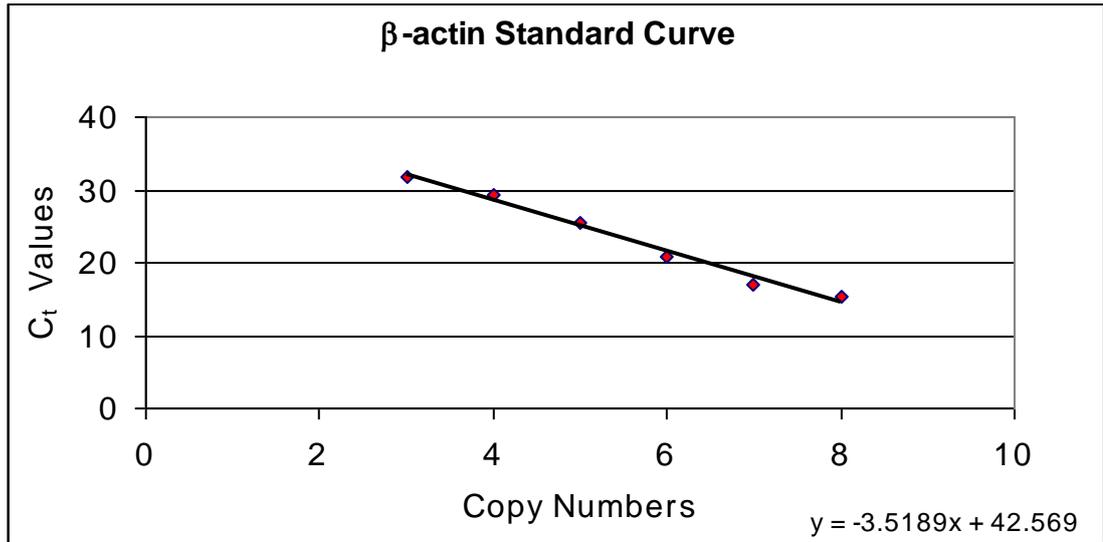
**Figure 2.7a Typical β-actin standard curve:** The scatter plot shows typical $C_t$ values for the β-actin standards on the Y axis and β-actin mRNA copy numbers on the X-axis. A trendline was drawn through the points and the equation of this line is shown in the bottom right corner. The slope m of this line is between -3.2 and -3.6 thus, showing that this is an accurate standard curve.
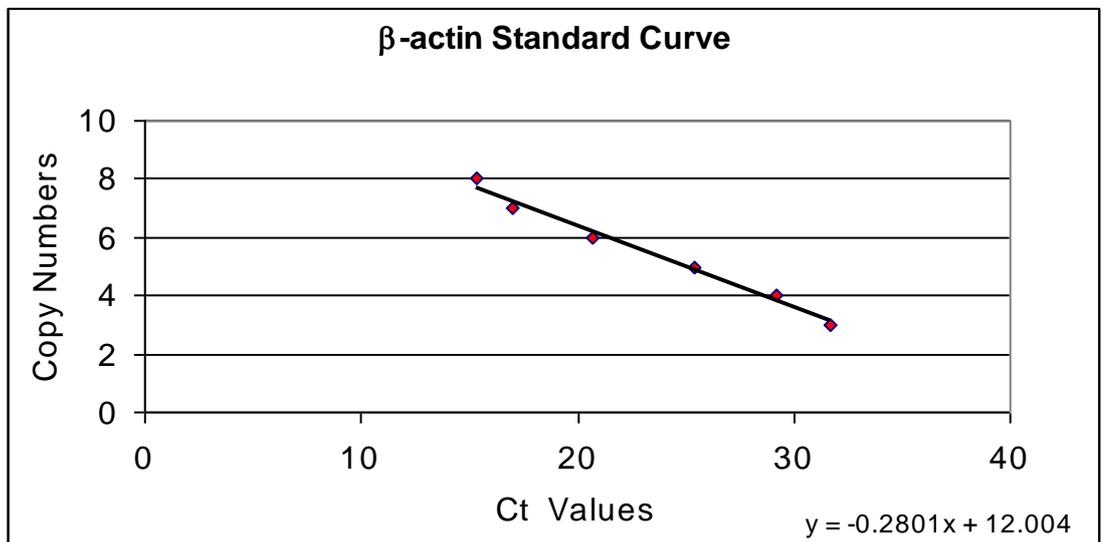


**Figure 2.7b Typical β-actin standard curve with axes adjusted for extrapolating data:** The scatter plot shows the β-actin mRNA copy numbers on the Y axis and the $C_t$ values for the β-actin standards on the X axis. A trendline was drawn through the points and the equation of this line (shown in the bottom right corner) was used to extrapolate the sample data. The β-actin mRNA copy number y was evaluated by substituting the $C_t$ value of β-actin standard for x.

## 2.5.4 Cytokine analysis using the BD$^{TM}$ Cytometric Bead Array

The BD$^{TM}$ Cytometric Bead Array (CBA) is a flow cytometry application which involves the use of antibody-coated beads to capture and quantify analytes in solution. In the present study, this kit was adapted for measurement of the cytokines and growth factors; IL-4, IL-6, IL-10, IL-12, IL-13, IFN-γ and TGF-β1. The kit consisted of the Cytometric Bead Array Human Soluble Protein Master Buffer Kit which comprised instrument set up reagents, wash buffer, assay diluent, detection reagent diluent, capture bead diluent and sets of single plex flex kits containing human cytokine capture beads (e.g. Human TGF-β1 Capture bead), human cytokine PE detection reagent (e.g. Human TGF-β1 PE detection reagent) and human cytokine standards (e.g. Human TGF-β1 Standard). The capture beads for each cytokine or growth factor have unique fluorescence intensities and this facilitates the use of several beads in a single tube. Using this application, 6 cytokines were analysed simultaneously in a sample of 50 μl of supernatant.

## 2.5.4.1 Preparation of samples and standards for CBA

The protocol was carried out as per the BD Cytometric Bead Array Human Soluble Protein Master Buffer Kit Instruction Manual. The BD CBA Human Soluble Protein Flex Set Standards were first prepared by pooling all lyophilized standards into one flow cytometry tube and then reconstituting them with 4 ml of Assay Diluent. This is known as the 'Top Standard' tube and contains 2500 pg/ml of protein. Serial dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256 were then performed using Assay Diluent and, the approximate corresponding protein concentrations in these tubes are shown in Table 2.14.

**Table 2.14:**

**Preparation for Standard Curve in CBA:** The serial dilutions required to construct standard curve for calculation of cytokine levels.

| Serial Dilution Tube | Top | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | Bottom |
|---|---|---|---|---|---|---|---|---|---|---|
| Protein (pg/ml) | 2500 | 1250 | 625 | 312.5 | 156 | 80 | 40 | 20 | 10 | 0 |

**\* Bottom refers to the tube containing only Assay Diluent. This contains the 0 pg/ml of protein and is regarded as the negative control.**

Multiplex assays were carried out for IL-4, IL-6, IL-10, IL-12, IL-13 and IFN-γ, while a single bead assay was performed for TGF-β1 because it required different sample preparation.

The Capture Beads for each cytokine were diluted 1/50 with Capture Bead Diluent and pooled in a tube labelled 'Mixed Capture Beads'. Similarly, the total amount of PE Detection Reagent for each cytokine was diluted 1/50 with Detection Reagent Diluent and pooled in a tube labelled 'Mixed PE Detection Reagents'. Fifty micro litres of the 'Mixed Capture Beads' were added to each of the sample and standard tubes, followed by 50 μl of sample or standard, respectively. Each tube was mixed gently and incubated for 1 hour at room temperature. Fifty micro litres of the 'Mixed PE Detection Reagents' were then added to each tube. Each tube was mixed gently and incubated for 2 hours at room temperature. One millilitre of Wash Buffer was added to each assay tube and all tubes were centrifuged at 20$0$ $x$ $g$ for 5 min. The supernatant was carefully aspirated and discarded and 300 μl of Wash Buffer was added to each tube. The tubes were vortexed to re-suspend the beads for subsequent acquisition on the FACS Calibur.

The instrument set up was carried out as per the BD FACS Calibur Flow Cytometer BD CBA Flex Sets: Instrument Setup, Data Acquisition, and Analysis Instruction Manual.

**2.5.4.2 Analysis of samples for CBA**

The FSC -v- SSC dot plot of each sample tube had a characteristic profile (Figure 2.8A). All debris and unbound proteins were excluded by drawing an electronic gate R1 around the antibody-coated beads. A second dot plot with FL4 -v- FL3 was created and by gating appropriately, only the antibody coated beads in R1 were shown on this plot (Figure 2.8B). The characteristic profile of the second dot plot allowed the discrimination between different antibody-coated beads based on their fluorescence intensity in the FL3 and FL4 channels (Figure 2.8B). For example, the gate R6 was drawn around the IL-12-coated beads (Figure 2.8B). The region where each cluster of anti-cytokine-coated beads was positioned could be determined by using a control tube containing only those anti-cytokine-coated beads. Once, each population of anti-cytokine-coated beads had been identified and gated in the second dot plot, a third dot plot with FL2 -v- FL3 was then created. By gating appropriately, only a single population of antibody-coated beads were shown at a time. For example, only IL-12-coated beads are shown in Figures 2.8D-M because the plots are gated in R6. The mean fluorescence intensity (MFI) in the FL2 channel was used to measure the concentration of protein in the sample and therefore, the higher the MFI, the higher the protein concentration. Exact concentrations were calculated by mapping the MFIs from each standard tube to the known concentrations in each tube. For example, if the MFI of IL-12 was 250 in the top standard tube, then an MFI of 125 would equate to an IL-12 concentration of 1250 pg/ml in a sample tube. The dot plots representing the standards gated in R6 are shown in Figures 2.8D-M. They show the increase in IL-12 concentration (represented by the MFI in FL2) increases from the bottom (Figure 2.8D) through to the top standard (Figure 2.8M). Once the standard curve for each cytokine had been constructed, the protein concentration in each tube could be calculated (Figure 2.8C). Figures 2.9A-H show the gating method for each cytokine in a number of sample tubes.

**Figure 2.8**

**Figure 2.9**

## 2.6 Measurement of cell cytotoxicity

### 2.6.1 Chromium release assay

Assessment of the cytotoxic capabilities of PBMC was carried out by analysis of chromium ($^{51}$Cr) release by labelled target cells. $^{51}$Cr is a radioactive label that emits β-rays and is given to target cells in the form of non-toxic sodium chromate ($Na_2$$^{51}$$CrO_4$). Chromium is readily taken up by cells and is only released in significant quantities upon cell lysis or disruption. Labelled target cells were co-incubated with effectors, at a range of effector / target (E/T) ratios, for 4 hours. Some target cells were treated with medium only or with a detergent (Triton X-100) which disrupts all cells, thus providing negative and positive controls, respectively. The cell supernatant was then harvested and the amount of $^{51}$Cr released was determined by scintillation counting. This involved the use of a scintillant, which emits light when exposed to ionising radiation. A scintillation counter was used to measure the level of radioactivity present in samples. The amount of $^{51}$Cr detected in the supernatant reflects the killing capacity of the effector cells.

Target cells used in this assay were derived from the human cancer cell line K562, which is derived from a chronic myeloid erythroleukaemia. These cells are deficient in MHC molecule expression and are commonly used as target cells in NK cell cytotoxicity assays

### 2.6.2 General procedures for use of radioisotopes

All work involving radioactive material was carried out in a designated area of the radiation suite at the National University of Ireland, Maynooth. All users were fully trained and approved prior to commencing work in the radiation area. User attendance, background radiation levels and amount of isotope used were recorded in logbooks for each work session. All work involving $^{51}$Cr isotopes was performed behind a lead-impregnated perspex screen, and lead coats were worn over lab coats throughout. Two pairs of disposable nitrile gloves were also worn. Cotton buds soaked in detergent (2% Decon 90) were used to swab various surfaces in the work area before and after radioisotope work. Swabs were then placed in scintillation fluid and background radiation levels were evaluated by β-counting. Swab counts were then signed by user, dated and added to a logbook. In the event of radiation counts that exceed background levels (50 counts per minute (CPM)), the entire workspace was thoroughly cleaned

using 2% Decon detergent and water and swabs were re-taken. A Geiger counter was used to monitor radiation levels throughout procedure. The chromium isotope has a half-life of 27.7 days. Liquid waste was stored in designated Winchester bottles, labelled with the date of last addition and stored until radiation levels were undetectable. Solid waste was sealed in bags, labelled with date and stored in lead containers until radiation was no longer detectable, as per Radiation Protection Institute of Ireland (RPII) guidelines.

### 2.6.3 Cytotoxicity assay using PBMC from chronic HBV patients and healthy controls

K562 cells were re-suspended at $2 \times 10^6$ cells/ml in RPMI containing 10% human serum. The cells were then treated with 150 μCi $^{51}$Cr/0.5ml and were incubated at $37^o$C for 4 hours in a lead box. Cells were then washed three times in 10ml warm cRPMI to remove excess $^{51}$Cr, centrifuging gently at 45 x g for 5 min each time with the brake off. Supernatants were carefully removed each time using a Pasteur pipette and cells were not re-suspended until after the last wash. Cells were handled gently in order to minimise damage, which would increase background $^{51}$Cr release. Labelled target cells were then counted and re-suspended at $4 \times 10^4$ cells/ml. Cells were then added to wells of a 96-well round-bottomed microtitre plate at 50 μl (i.e. $2 \times 10^3$ cells) per well.

PBMC which had been previously incubated in medium alone or medium containing 100 U/ml of IL-2 or IFN-α, were re-suspended at $4 \times 10^6$ cells/ml, but were plated in varying amounts, according to the desired E/T ratios. E/T ratios tested in this experiment were 50, 25, 5 and 1. Each well was then topped up to 100 μl with cRPMI where necessary. Medium only or Triton X-100 (0.1%) was added to reserved triplicates of labelled target cells for negative and positive controls, respectively. Plates were centrifuged at 15*0 x g* for 1 min (brake off) to ensure interaction of effector and target cells. Plates were then incubated for 4 hrs at $37^o$C in a lead box. Plates were centrifuged again as before and 25 μl supernatant was taken from each well into a 96 well polyethylene sample plate containing 150 μl Optiphase® scintillation cocktail. The plates were gently shaken on a vortex for 5 min to ensure thorough mixing of supernatant with scintillant (turned cloudy). Scintillation counting was then performed on a Trilux 1450 Microbeta Liquid Scintillation Counter. Cytotoxic capability of

effector cells was then calculated and expressed as a percentage of maximum and background counts per minute (cpm) using the following formula:

% specific lysis = $\dfrac{\text{cpm of sample} - \text{cpm of spontaneous release}}{\text{cpm of maximum} - \text{cpm of spontaneous release}}$ X 100

## 2.7 Functional studies on γδ T cells

### 2.7.1 Analysis of γδ T cell subsets

The frequencies of circulating Vδ2 and Vδ1 T cells, and the memory phenotypes of these cells was investigated using flow cytometry, as described in section 2.4.

### 2.7.2 Expansion of Vγ9Vδ2 T cells in response to the pyrophosphate antigen HMBPP

Following PBMC preparation and cell counts, $0.6 \times 10^6$ PBMC were removed for flow cytometric analysis as detailed below. The rest of the PBMC were placed in cRPMI at a density of $1 \times 10^6$ cells/ml and 1 ml was transferred to separate wells of a 24-well tissue culture plate. Stimulation of the cells in each well was performed as listed in Table 2.17.

**Table 2.17**

**Expansion of γδ T cells in vitro:** An outline of the well numbers of the 24-well tissue culture plate and the stimulations designated to each well.

| No. | Stimulation |
|-----|-------------|
| 1 | Medium alone |
| 2 | 100 nM HMBPP and 50 U/ml IL-2 |
| 3 | 5 µg/ml PHA and 50 U/ml IL-2 |

Row No. 1 represents the negative control which would induce no cell expansion and it was expected that the majority of PBMC would be dead after 14 days. Row No. 2 represents the stimulation which would facilitate the enrichment of Vδ2 T cells and these cells were expected to be the predominant cell population on day 14. Row No. 3 represents the stimulation which would induce all T cells to proliferate and αβ T cells were expected to be the predominant T cell population after 14 days.

Cells were incubated in a $CO_2$ Incubator at 37ºC for 5 days. Following 5 days of incubation, the medium was replenished by removing 800 µl of medium from each well and replacing it with 1 ml cRPMI. The 1 ml cRPMI added to wells 2 and 3 contained 50 U IL-2. The medium was replenished in this manner every 5 days.

On days 1, 7 and 14, the number of cells in each well was counted as described in section 2.3.2. Then, $0.6 \times 10^6$ PBMC from each well were surface stained with FITC-labelled anti-Vγ9, PE-labelled anti-Vδ2 and PerCP- or PE-Cy5-labelled anti-CD3, as detailed in section 2.4. The percentages of lymphocytes that expressed γδ TCRs were quantified and the absolute numbers of γδ T cells per well could be calculated.

The use of cell counting and flow cytometry ensured that any higher frequencies of γδ T cells among total PBMC in response to HMBPP and IL-2 was not only due to the death of other PBMC but, was also due to the selective expansion of γδ T cells

On day 1, the frequencies of Vγ9Vδ2 T cells among the PBMC used in these experiments, ranged from to 0.3% to 7.5%, as a percentage of T cells.

On day 7, the purity of HMBPP and IL-2-expanded Vγ9Vδ2 T cells ranged from less than 1% to 29% of viable PBMC and, Vγ9Vδ2 T cell numbers per well ranged from 3,000 to 800,000 cells.

On day 14, percentages of HMBPP and IL-2-expanded Vγ9Vδ2 T cells ranged from less than 1% to 91.9% and cell numbers ranged from 7,000 to $1.9 \times 10^6$ cells. On day 14, only Vγ9Vδ2 T cell populations with purities greater than 80% were used for subsequent experiments. The mean percentage of PHA and IL-2-expanded total CD3$^+$ T cells was 91.9+/-9.2%. While the majority of PHA and IL-2-expanded cells were αβ T cells, a significant expansion of Vγ9Vδ2 T cells was observed also with an average of 20.7+/-7.6% of total CD3$^+$ T cells on day 14. PHA and IL-2-expanded αβ T cells were used to control for HMBPP and IL-2-expanded Vγ9Vδ2 T in the subsequent co-culture experiments with epithelial cells.

**2.7.3 Interactions between Vγ9Vδ2 T cells and epithelial cells**

All epithelial cells were suspended at a cell density of $1 \times 10^6$ cells per ml of medium and 500 µL of this was added to the wells of a 24-well tissue culture plate and allowed to adhere for 24 hours. Following this 24 hour incubation, each well was supplemented with the appropriate medium and cells, as listed in Table 2.18. The co-culture was then incubated for a further 24 hours.

Following 24 hours of co-culture, the plates were centrifuged at 45 x g for 3 minutes. The supernatants were removed and stored at -20$^o$C for subsequent analysis of cytokine release by ELISA. Two hundred micro litres of cell dissociation solution was added to all wells for 1 min and all cells were removed, centrifuged at 400 x g for 8 minutes and, pellets were re-suspended in PBA. Surface staining for subsequent phenotypic flow cytometric analysis was then carried out as in section 2.4, using the fluorochrome-conjugated mAbs listed in Table 2.19.

**Table 2.19**

**Analysis of interactions between epithelial cells and γδ T cells:** The fluorescence-labelled mAbs used for cell surface staining of γδ T cells and epithelial cell lines, for analysis of their reciprocal effects on each other.

| Tube | FITC | PE | PerCP | APC |
|------|------|------|-------|------|
| 1 | IgG1 | IgG1 | IgG1 | IgG1 |
| 2 | BerEP4* | HLA-E | CD3* | CD54 |
| 3 | BerEP4 | | CD3 | MICA/B |
| 2 | Vγ9 | CD69 | | NKG2D |
| 4 | HLA-DR | Vδ2 | | NKG2A |

*CD3 and BerEP4 were the key surface markers used to distinguish between epithelial and T cell populations after co-culture.

## 2.8 Statistical calculations:

### 2.8.1 The Mann-Whitney U test

The Mann-Whitney U test was used to ascertain whether there were significant differences between the frequencies of cells in patients and control subjects. P-values lower than 0.05 were considered as significant, while those less than 0.005 and 0.0005 were considered to be highly significant. While many studies would prefer the use of medians and inter-quartile ranges for non-parametric data, means and standard errors were calculated in the present study.

### 2.8.2 The correlation function

The correlation function was used to determine whether the frequencies of cells changed with clinical parameters such as ALT level, viral load, age or gender. The correlation coefficient ranges from +1, indicating a perfect positive linear relationship, to -1, indicating a perfectly negative linear relationship. However, this test does not reveal if there is a slight causation. In order to determine this, further statistical analysis would be required using regression. However, regression is only recommended when a strong correlation exists and such a relationship was not found between any of the cell frequencies and clinical parameters.

### 2.8.3 Bonferroni correction method

Many comparisons were made in the study and many observations were deemed statistically significant by the Mann-Whitney U test. However, the number of variables tested increases the likelihood of a false positive statistic and such statistics can be corrected using the Bonferroni correction method – a correction method derived from Boole's inequality by Carlo Emilio Bonferroni.

$$p\text{-}value = \alpha/n, \ p_c\text{-}value = \alpha$$

It assumes that the occurrence of such false positive significant results increase with the number of tests. Therefore, the p-value ($\alpha/n$) is multiplied by the number of tests (n) to give the corrected p-value or $p_c$-value ($\alpha$). If the $p_c$-value is still within our chosen confidence interval i.e. below 0.05, then the finding is considered to be really statistically significant (Shaffer 1995). This method was used to distinguish between real significant statistics and to exclude the more spurious positives.

# Chapter 3

# Phenotypic analysis of innate lymphocytes in HBV infection

**3.1 Introduction**

Innate lymphocytes comprise a group of MHC-unrestricted, early responding lymphocytes that are characterized by potent cytokine production and cytotoxic activity. They include NK, NT, iNKT and γδ T cells and are believed to be crucial in the early immune responses to viral infection (Biron *et al.* 1999; Fisicara *et al.* 2009; Ismaili *et al.* 2002; Dieli *et al.* 2004; Devilder *et al.* 2006; Orange *et al.* 1996). Through cytokine production and direct contact with other cells, innate lymphocytes can also stimulate and modulate adaptive immune responses and are therefore, major players in determining the overall outcome of infection (Kakimi *et al.* 2000; Liu *et al.* 2000; Guidotti & Chisari 2001; Nishimura *et al.* 2000). In particular, innate lymphocytes can induce maturation of dendritic cells and B cells into antigen-presenting cells and therefore, deficiencies of such cells could lead to an impaired adaptive response which could result in an unfavorable outcome for the host (Vincent *et al.* 2002; Ismaili *et al.* 2002; Dieli *et al.* 2004; Devilder *et al.* 2006; Ing & Stevenson 2009; Martino *et al.* 2002; Cooper *et al.* 2001; Dunne, unpublished data).

It is believed that NK and NT cells play a crucial role in the early defence against HBV infection and these potent cytotoxic cells have also been implicated in the pathogenesis of disease (Kimura *et al.* 2002; Pasquetto *et al.* 2000; Kakimi *et al.* 2000; Echevarria *et al.* 1991.; Guidotti & Chisari 2001; Zou *et al.* 2010; Sitia *et al.* 2002; Albarran *et al.* 2005; Fisicaro *et al.* 2009). Kimura *et al.* (2002) found that IL-12 induced IFN-γ production by NK cells was important in the inhibition of HBV replication in transgenic mice while Kakimi *et al.* (2000) have shown that the NKT-mediated recruitment of NK cells into the liver facilitates inhibition of HBV replication via the production of antiviral cytokines. Pasquetto *et al.* (2000) found that liver inflammation corresponded to the intrahepatic infiltration of NK cells among other cells in HBV transgenic mice while Zou *et al.* (2010) found that NK cells were involved in the development of hepatocyte necrosis in a mouse model of virus induced hepatitis and significantly contributed to fulminant hepatic failure and HBV-associated acute-on-chronic liver failure in such mice via cytotoxicity and IFN-γ and TNF-α production. Enhanced NK cell cytotoxicity has also been observed in the early phase of acute HBV infection (Echevarria *et al.* 1991). Involvement of NK and NT cells in the immune responses against HBV is further evident in the findings of Albarran *et al.* (2005) who have found that the frequencies of NK and NT cells are Higher in responders to

vaccination with HBsAg, along with their IFN-γ expression. Furthermore, deficiencies of such cells could be important in HBV infection where persistence is associated with weakened adaptive immune responses whereas strong and multi-specific CTL responses are associated with resolution of infection (Penna *et al.* 1991; Missale et al; 1992; Bertoletti *et al.* 1991; Nayersina *et al.* 1993; Rehermann *et al.* 1995; Thimme *et al.* 2003; Maini *et al.* 1999). Deficiencies of circulating NK cells have previously been identified in subjects persistently infected with HCV while others have found depleted numbers of hepatic iNKT cells and NT cells in the livers of HCV-infected subjects (Golden-Mason *et al.* 2008; Deignan *et al.* 2002).

The two main subsets of NK cells are distinguished based on the density of their CD56 expression. CD56$^{BRIGHT}$ NK cells make up ~10% of the NK cell population. They express high levels of CD56 and are the main producers of IFN-γ while, the majority of NK cells are CD56$^{DIM}$ and they are primarily responsible for natural cytotoxicity (Robertson & Ritz 1990; Cooper *et al.*2001). In our recent work, it was also found that the frequencies of circulating CD56$^{DIM}$ NK cells were depleted in chronic HCV infection, while the frequencies of CD56$^{BRIGHT}$ NK cells were expanded (Golden-Mason *et al.* 2008). Such differences resulted in the overall lower numbers of NK cells and it was aimed to determine if such differences in NK subsets existed in HBV infection. The majority of NT cells express low levels of CD56 and so, they are classified as CD56$^{DIM}$ but it was also found some NT cells expressing higher levels of CD56 and categorized these as CD56$^{BRIGHT}$. Therefore, these divisions were included in our analysis of NT cells, also.

Another interesting group of innate lymphocytes are the γδ T cells. They express a γδ-TCR and unlike their αβ$^+$ counterparts, they are not MHC-restricted and can respond to infection in under an hour (compared to 4 – 96 hours in αβ T cells) (Groh *et al.* 1998; Hayday 2000). They are therefore, crucial in the early responses to infection and their expansion has been observed in several viral infections (De Paolo *et al.*1990; Poccia *et al.*1999; Maccario *et al.*1995; Agrati *et al.* 2001). However, their role in HBV infection is not fully understood. Sing *et al.* (1998) have identified a putative role for γδ T cells in the clearance of HBV infection since the frequencies of CD4$^+$CD8$^+$ T cells bearing the γδ TCR are significantly Higher in patients who have seroconverted. Also, recent work by Chen *et al.* (2008) indicates an association between impaired frequencies and functions of circulating Vδ2 T cells and persistent HBV infection with

the most significant depletions of such cells observed in the patients with the most severe liver disease. Another group who were predominantly interested in HIV found no differences between controls and HBV patients when frequencies of $V\delta1^+$ T cells were investigated (Rossol *et al.* 1998). Since the body of work on the role of $\gamma\delta$ T cells in HBV is not definitive, it was decided to carry out a preliminary investigation of $\gamma\delta$ T cell frequencies in the peripheral blood of HBV patients and controls. The identification of a role for $\gamma\delta$ T cells in immune responses against HBV infection could contribute to the development of improved immunotherapy for HBV-infected patients. These innate T cells are already the centre of several immunotherapeutic studies for a variety of malignancies and could therefore, have great potential in the treatment of HBV (Dieli *et al.* 2007; Bennouna *et al.* 2008).

The invariant NKT (iNKT) cells are a group of innate lymphocytes that elicit potent IFN-$\gamma$ production and cytotoxic activity. They are not MHC-restricted but rather CD1d-restricted because of their recognition of lipid antigens presented by the CD1d glycoprotein. As their name suggests, iNKT cells express both NK and T cell receptors. In humans, they are characterized by the expression of a TCR consisting of a V$\alpha$24J$\alpha$18 $\alpha$-chain, paired with a limited number of $\beta$-chains (Bendelac *et al.* 1997). In this study, a fluorochrome-conjugated antibody specific for both the V$\alpha$24 chain and the V$\beta$11 chain was chosen to identify cells co-expressing both chains. It was also used an antibody called 6B11 to identify the V$\alpha$24J$\alpha$18-TCR. Much of the work on iNKT cells has been performed in murine models and has revealed the massive potential of these cells in anti-tumor, anti-microbial and antiviral immune responses (Kakimi *et al.* 2000). However, while they account for 5% of circulating and 30% of hepatic T cells in mice, they only constitute 0.02-0.8% of circulating T lymphocytes and less than 1% of liver T lymphocytes in humans, therefore, making them a less powerful force in human disease (Kenna *et al.* 2004). So while, iNKT cells can inhibit HBV replication in a mouse model, their involvement in natural HBV infection has not yet been elucidated. Human studies in HCV and HIV-1 have associated deficiencies of iNKT cells with the failure to clear such viruses (Deignan *et al.*2002; van der Vliet 2002). It was hypothesised that such deficiencies in HBV infection could pre-dispose individuals to development of chronic liver disease and therefore, studied the frequencies of circulating iNKT cells in a cohort of HBV patients and a group of healthy control subjects. CD1 expression was also studied so that any observed differences might be associated with differences in

CD1 expression. The immunotherapeutic potential of iNKT cells is already under investigation in cancer and the elucidation of their role in natural HBV infection could lead to the development of improved therapeutics for this disease (Chang *et al.* 2005; Motohashi *et al.* 2009).

CD1 is a MHC-like glycoprotein which is expressed on the surface of antigen presentation cells such as B cells and monocytes and facilitates the presentation of lipid antigens to NKT cells (Porcelli *et al.* 1998). It is believed to play an important antigen presentation role in several bacterial infections, most notably *Mycobacterium tuberculosis* infection (Park & Bendelac 2000; Skold & Behar 2005). However, the role of CD1 lipid antigen presentation in viral infection is less understood but its role in the inhibition of HBV replication in transgenic mice and its downregulation in HSV and HIV infection indicates that it is important in immune responses against viruses (Kakimi *et al.* 2000; Yuan *et al.* 2006; Chen *et al.* 2006). To our knowledge no studies have phenotyped CD1-expressing cells in HBV patients and so, the frequencies of CD1$^+$ monocytes and B cells was investigated in the peripheral blood of HBV patients and healthy controls. Any differences observed may indicate a role of CD1-mediated antigen presentation in HBV and could aid the development of a novel immunotherapy against the virus. The therapeutic potential of CD1-presented lipid antigens is being investigated along with that of iNKT cells and certain studies have involved administration of α-galactosylceramide-loaded CD1d-expressing DCs in an effort to activate iNKT cells *in vivo* in cancer patients (Chang *et al.* 2005). Therefore, the development of therapeutics involving the CD1-restricted iNKT cells may be more focused on manipulating CD1 antigen presentation *in vitro* to facilitate a boosted iNKT cell response *in vivo*.

The identification of deficient numbers or impaired functions of innate lymphocytes in HBV could have potential in the development of therapies that could be used to strengthen immune responses in patients. Innate lymphocytes are ideal targets for immunotherapy as they are easily cultured, do not require specific antigen stimulation in order to carry out effector functions and are already the centre of several immunotherapeutic clinical trials (Chang *et al.* 2005; Motohashi *et al.* 2009; Dieli *et al.* 2009; Bennouna *et al.* 2008; Shi *et al.* 2004).  These trials have shown that intravenous administration of various innate lymphocyte stimulatory ligands and adoptive transfer of *ex vivo* stimulated innate lymphocytes are well-tolerated and can stimulate adaptive immune responses *in vivo.*

To test our hypothesis that innate lymphocytes are required for immune control of HBV, the frequencies of circulating NK, NT, iNKT and γδ T cells were examined in a group of HBV patients and compared them to those in a group of uninfected control subjects. Since these HBV patients have relatively low viral load and mild liver disease, they were considered as a model of immune control rather than a model of uncontrollable HBV replication and severe liver disease. Because of this, it was expected the frequencies of innate lymphocytes to inform us of the role of these cells in an immune response that controls HBV infection without causing liver damage.

Since it is likely that the frequencies and functions of iNKT cells are governed by the expression of CD1d (Lynch *et al.* 2009), CD1d expression on B cells and monocytes from patients and controls was also investigated. Therefore, any alterations observed in the frequencies of iNKT cells might correlate with alterations in CD1d expression. CD1d expression is downregulated by HIV and HSV and since iNKT cells were being examined in this study, its inclusion was an obvious choice (Yuan *et al.* 2006; Chen *et al.* 2006).

This study, therefore, aims to examine the frequencies of all innate lymphocytes listed above and to evaluate their role in the control of HBV infection in the absence of associated liver injury.

**3.2 Innate lymphocytes in HBV infection**

**3.2.1 The frequencies of circulating NK cells and NT cells are higher in HBV-infected subjects than in control subjects**

PBMC were prepared from 62 HBV-infected individuals and from 66 control subjects. Surface staining with PE-labelled anti-CD56 mAb and PE-Cy5-labelled anti-CD3 mAb was performed to identify NK cells and NT cells in the peripheral blood. Lymphocytes were identified based on size and granularity using forward and side scatter parameters and then NK cells were classified as those cells that stained positive for CD56 and negative for CD3 (Figure 3.1A,B), while NT cells were identified as those lymphocytes which stained positive for both CD56 and CD3. The frequencies of circulating $CD56^+CD3^-$ and $CD56^+CD3^+$ cells as a percentage of total lymphocytes were then quantified. Frequencies of NK cells ranged from a minimum of 2.1% to a maximum of 24.3% in controls, and from 0.8% to 28.5% in HBV-infected individuals (HBV). The mean frequencies of circulating NK cells were 8.1% of lymphocytes with a standard error of 0.6% in controls and 10.6% +/- 0.83% in HBV. This higher frequency of circulating NK frequencies in HBV infection was significant (p=0.035, Figure 3.1C). Frequencies of circulating NT cells ranged from 0.5% to 19.1% in controls, and from 0.3% to 38.6% in HBV. The mean frequencies were 3.3% of lymphocytes +/- 0.4% in controls and 5.6+/-0.78% in HBV patients. This higher frequency was also significant (p=0.0002, Figure 3.1D). Frequencies of circulating total $CD56^+$ lymphocytes ranged from 3.9% to 27.7% in controls, and from 1.2% to 42.3% in HBV, with mean frequencies of 11.2+/-0.71% in controls and 16+/-1.1% in HBV. This higher frequency was significant (p=0.0005, Figure 3.1E).

These data show that there are elevated numbers of circulating NK and NT cells, and indeed total $CD56^+$ lymphocytes, in chronic HBV infection.

**FIGURE 3.1**

### 3.2.2 The CD56$^{DIM}$, but not the CD56$^{BRIGHT}$, subset of NK, NT and total CD56$^+$ cells are found at higher frequencies in HBV infection

The frequencies of circulating CD56$^{DIM}$ and CD56$^{BRIGHT}$ NK cells, NT cells and total CD56$^+$ cells were quantified as a percentage of lymphocytes and as a percentage of each CD56$^+$ cell population. CD56$^{DIM}$ and CD56$^{BRIGHT}$ cells were identified as those lymphocytes which expressed low and high fluorescence levels of CD56 staining, respectively (Figure 3.2A).

The mean frequencies of CD56$^{DIM}$ NK cells as a percentage of total NK cells were 91.1+/-1.2% in controls and 92.6+/-1.6% in HBV patients. This higher frequency was significant (p=0.006, Figure 3.2B). As a percentage of total lymphocytes, the frequencies of CD56$^{DIM}$ NK cells ranged from 1.9% to 24.2% in controls, and from 0.24% to 28% in HBV, with mean frequencies of 7.3+/-0.54% and 9.9+/-0.8%, respectively. This higher frequency was also significant (p=0.035, Figure 3.2C). The mean frequencies of CD56$^{DIM}$ NT cells as a percentage of total NT cells were 97.3+/-0.5% in controls and 97.9+/-0.5% in HBV patients. This higher frequency was significant (p=0.009, Figure 3.2B). As a percentage of total lymphocytes, the frequencies of CD56$^{DIM}$ NT cells ranged from 0.49% to 19.1% in controls, and from 0.3% to 38.4% in HBV, with mean frequencies of 3.2+/-0.43% and 5.5+/-0.83%, respectively (p=0.003, Figure 3.2C). The mean frequencies of CD56$^{DIM}$ cells as a percentage of total CD56$^+$ cells were 89.9+/-1.9% in controls and 93.3+/-1.9% in HBV patients. This higher frequency was significant (p=0.0007, Figure 3.2B). As a percentage of lymphocytes, the frequencies of total CD56$^{DIM}$ cells ranged from 2% to 26.6% in controls, and from 0.56% to 40.4% in HBV, with mean frequencies of 9.6+/-0.76% and 13.4+/-1.2%, respectively. This higher frequency in total CD56$^{DIM}$ cells, as a percentage of lymphocytes, in HBV patients was also significant (p=0.02, Figure 3.2C).

The mean frequencies of CD56$^{BRIGHT}$ NK cells as a percentage of total NK cells were 8.9+/-1.2% in controls and 7.4+/-1.6% in HBV patients. These lower frequencies were found to be significant (p=0.006, Figure 3.2D). As a percentage of total lymphocytes, the frequencies of CD56$^{BRIGHT}$ NK cells ranged from 0.1% to 3.1% in controls, and from 0.01% to 1.7% in HBV, with mean frequencies of 0.61+/-0.06% and 0.49+/-0.05%, respectively. These numbers were not significantly different (p=0.3, Figure 3.2E). The mean frequencies of CD56$^{BRIGHT}$ NT cells as a percentage of total NT cells were 2.7+/-0.5% in controls and 2.2+/-0.5% in HBV patients. These lower frequencies were found to be significant (p=0.009, Figure 3.2D). As a percentage of

total lymphocytes, the frequencies of CD56$^{BRIGHT}$ NT cells ranged from 0% to 0.8% in controls, and from 0% to 0.79% in HBV, with mean frequencies of 0.07+/-0.02% and 0.07+/-0.02%, respectively (p=0.8, Figure 3.2E). The mean frequencies of CD56$^{BRIGHT}$ cells as a percentage of total CD56$^+$ cells were 8.5+/-1.2% in controls and 5+/-1% in HBV patients. These lower frequencies were found to be significant (p=0.0007, Figure 3.2D). As a percentage of lymphocytes, the total frequencies of CD56$^{BRIGHT}$ cells ranged from 0.11% to 3.3% in controls, and from 0.03% to 1.9% in HBV, with means of 0.57+/-0.07% and 0.48+/-0.06%, respectively (p=0.55, Figure 3.2E).

These data show that the numbers of circulating CD56$^{DIM}$, but not CD56$^{BRIGHT}$ cells, are higher in HBV patients than in control subjects and are therefore, the major contributing subset to the overall higher frequencies of NK and NT cells. In addition, these data indicate that the expansion of CD56$^{DIM}$ cells in HBV occurs at the expense of the frequencies of CD56$^{BRIGHT}$ cells, which appear to be lower in HBV. This lower frequency proved to be most significant when CD56$^{BRIGHT}$ cells were calculated as a proportion of the total CD56$^+$ populations.

FIGURE 3.2

### 3.2.3 The frequencies of circulating γδ T cells are significantly higher in subjects with HBV infection

PBMC were prepared from 20 HBV-infected subjects and from the buffy coat packs of 23 control subjects. Surface staining with FITC-labelled anti-γδ-TCR mAb and PE-Cy5-labelled anti-CD3 mAb was carried out to identify γδ T cells in the peripheral blood (Figure 3.3A). Frequencies of circulating γδ T cells ranged from 0.36% to 5.86% in controls, and from 0.35% to 30.67% in HBV, with mean frequencies of 2.8+/-0.27% and 6.8+/-1.5%, respectively (p=0.0009, Figure 3.3B).

These data show that circulating γδ T cells are significantly expanded in HBV infection.

### 3.2.4 There are no significant differences in the numbers of circulating invariant NKT cells in HBV infection.

PBMC were prepared from 18 HBV–infected individuals and from the buffy coat packs of 22 control subjects. Surface staining with FITC-labelled anti-Vα24 mAb and PE-labelled anti-Vβ11 mAb, together with PE-Cy5-labelled anti-CD3 mAb, was performed to identify the T cells with the invariant Vα24Vβ11 TCR (Figure 3.4A). PE-labelled anti-6B11 mAb was also used together with PE-Cy5-labelled anti-CD3 mAb as an alternative means of identifying such iNKTs (Figure 3.4C). Frequencies of circulating Vα24$^+$Vβ11$^+$ T cells ranged from 0.01% to 0.81% in controls, and from 0.01% to 0.24% in HBV. The mean frequencies of circulating Vα24$^+$Vβ11$^+$ T cells were 0.11% of T cells +/- 0.04% in controls and 0.09 +/-0.02% in HBV. This difference was not significant (p=0.29, Figure 3.4B). Frequencies of circulating 6B11$^+$ T cells ranged from 0.09% to 1.5% in controls, and from 0.04% to 3.5% in HBV with, mean frequencies of 0.54+/-0.1% and 0.62 +/-0.19%, respectively. This difference was not significant (p=0.21, Figure 3.4D). The two methods used to label iNKT cells yielded slightly different numbers but overall, both sets of mAbs supported the finding that the frequencies of circulating iNKT cells are unchanged in HBV infection.

**FIGURE 3.3**

**FIGURE 3.4**

**3.2.5 The frequencies of B cells and monocytes expressing CD1a, b, c and d cells are similar in the peripheral blood of healthy control subjects and HBV patients.**

PBMC were prepared from the fresh blood of 18 HBV patients and from the buffy coat packs of 18 control subjects. Lymphocytes were identified based on size and granularity using forward and side scatter parameters and then surface staining with APC-labelled anti-CD19 mAb was performed to identify the B cells. Similarly, monocytes were identified based on size and granularity using forward and side scatter parameters and then PE-Cy5-labelled anti-CD14 mAb was used to identify the monocytes. FITC-labelled anti-CD1c mAb or anti-CD1b mAb and PE-labelled anti-CD1d mAb or anti-CD1a mAb were used to identify those B cells and monocytes expressing CD1a, b, c and d (Figure 3.5A and Figure 3.5C). The mean frequencies of B cells expressing CD1a, b, c and d were 0.99+/-0.31%, 10.9+/-3.6%, 27.3+/-4.6% and 65.1+/-4%, respectively, in controls and 0.61+/-0.12%, 3.2+/-0.4%, 22.6+/-1.9 and 53.1+/-4.2%, respectively, in HBV patients (Figure 3.5B). The mean frequencies of monocytes expressing CD1a, b, c and d were 7.5+/-3.9%, 12.7+/-4.3%, 7.6+/-2.9%, 47.3+/-10.8%, respectively, in controls and 7.6+/-1.9%, 10.9+/-2.3%, 4.2+/-1.14% and 52.3+/-8.9%, respectively, in HBV (Figure 3.5D). None of these differences were significant.

These data show that the numbers of iNKT cells and CD1-expressing cells are similar in controls and HBV-infected individuals.

**FIGURE 3.5**

### 3.3 NK, NT, γδ T, iNKT and CD1$^+$ cells in HBV patient groups

It was not sufficient to compare the frequencies of innate lymphocytes in persistently infected HBV patients with those in uninfected control subjects. To gain a better understanding of the role of innate lymphocytes in HBV infection, the patient cohort needed to be carefully examined. Patient data were obtained from the hepatology clinic in St James's hospital and was used to divide the patients into groups based on age, gender, viral load and ALT. Viral load was a measure of HBV replication and control of infection whereas ALT was a measure of liver disease. Since the upper limit of normal (ULN) for ALT is between 25 and 40 international units (IU)/ml, 40 was chosen as our cut off to distinguish between patients with and without liver damage (Rehermann & Nascimbeni 2005). Even though the majority of the patients studies had a viral load below 20,000 copies/ml, 100,000 copies/ml was chosen as the cut-off to distinguish between higher and lower viral load. Since the oldest HBV patient was only 55 years old, a midpoint between youngest and oldest was chosen to distinguish between patients who were relatively younger or older. It was also sought to determine if there were any gender-specific differences between the cell frequencies in our patients.

### 3.3.1 The frequencies of circulating NK cells are not significantly different between HBV patient groups

To determine if there were differences in NK frequencies within the HBV patient cohort, frequencies were compared between HBV-infected subjects of different age, gender, serum ALT level and viral load. Figure 3.6A shows the frequencies of circulating CD56$^+$CD3$^-$ cells as a percentage of total lymphocytes for 41 HBV patients with a low viral load i.e. below 100,000 copies / ml and 8 HBV patients with a high viral load i.e. between 100,000 and $5\times10^8$ copies / ml. The frequencies of circulating NK cells in patients with low viral load ranged from 0.83% to 28.47% of lymphocytes and the mean frequency (+/- SEM) was 9.6+/-0.9%. The frequency of circulating NK cells in patients with high viral load ranged from 6% to 17.3% of lymphocytes with, a mean frequency of 11.1+/-1.6%. This difference was not significant, according to the Mann-Whitney U-test (p=0.2). Furthermore, a Spearman correlation test yielded a correlation coefficient of r = 0.135, suggesting that there is no linear correlation between the frequencies of NK cells and viral load. Figure 3.6B shows the mean frequencies of circulating CD56$^+$CD3$^-$ cells as a percentage of total lymphocytes for 39 HBV patients with an

ALT below 40 IU/ ml and 13 HBV patients with an ALT above 40 IU/ml. For those patients with ALT below 40 IU/ml, the frequencies of NK cells ranged from 0.8% to 28.47% with an average of 9.8+/-0.9%. For those HBV patients with an ALT above 40 IU/ml, the frequencies of NK cells in peripheral blood ranged from 3.7% to 22.11% with an average of 9.8+/-1.4%. This difference was not significant (p=0.9) and moreover, there was no linear correlation between the numbers of NK cell and ALT levels (r = 0.0642). Figure 3.6C shows the frequencies of circulating $CD56^+CD3^-$ cells as a percentage of total lymphocytes in 27 males and 28 females. The numbers ranged from 0.83% to 28.47% in males, with a mean of 10.2+/-0.2% and from 0.91% to 28.27% in females with a mean of 10.4+/-0.2% (p=0.45). Figure 3.6D shows the frequencies of circulating $CD56^+CD3^-$ cells as a percentage of total lymphocytes in 28 HBV patients aged between 19 and 35 years of age and 27 HBV patients aged between 35 and 55 years of age. The frequency of circulating NK cells ranged from 3.14% to 28.27% in the younger group of patients, with a mean of 10.5+/-1.2%. The frequency of circulating NK cells ranged from 0.83% to 28.47% in the older group of HBV patients with a mean of 10+/-1.2%. (p=0.9) and there was no linear correlation between the frequencies of circulating NK cells and the age of the HBV patients (r = 0.0846).

These data suggest that although the frequencies of circulating NK cells are higher in HBV-infected subjects than in control subjects, their numbers do not correlate with viral load, disease severity, gender or age.

### 3.3.2 The frequencies of circulating NT cells are not significantly different between HBV patient groups

Frequencies were compared between HBV-infected subjects of different age, gender, serum ALT level and viral load to determine if there were differences in NT frequencies within the HBV patient cohort. Figure 3.7A shows the frequencies of circulating $CD56^+CD3^+$ cells as a percentage of total lymphocytes for 40 HBV patients with a low viral load i.e. below 100,000 copies / ml and 9 HBV patients with a high viral load i.e. between 100,000 and $5x10^8$ copies / ml. The frequencies of circulating NT cells in patients with low viral load ranged from 0.3% to 38.6% of lymphocytes with a mean of 5.6+/-1.2%. The frequencies of circulating NT cells were similar in patients with high viral load ranging from 1.1% to 10.2% of lymphocytes and with a mean of 5.2+/-0.5% (p=0.42). Furthermore, a Spearman correlation test yielded a correlation coefficient of r = 0.039, suggesting that there is no linear correlation between the frequencies of NT

cells and viral load. Figure 3.7B shows the mean frequencies of circulating $CD56^+CD3^+$ cells as a percentage of total lymphocytes for 38 HBV patients with an ALT below 40 IU/ml and 13 HBV patients with an ALT above 40 IU/ml. For those patients with low ALT, the frequencies of NT cells ranged from 0.32% to 10.43% with a mean of 4.4+/-0.5%. For those HBV patients with high ALT, the frequencies of NT cell ranged from 0.6% to 38.6% with a mean of 8.7+/-3.3%. These differences were not significant (p=0.67) and there was no linear correlation between the numbers of NT cell and ALT levels (r = -0.0088). Figure 3.7C shows the frequencies of circulating $CD56^+CD3^+$ cells as a percentage of total lymphocytes in 26 males and 28 females. The numbers ranged from 0.32% to 7.1% in males (mean 6.9+/-0.3%) and from 1% to 10.4% in females (mean 4.2+/-0.1%). This difference was not significant (p=0.37). Figure 3.7D shows the frequencies of circulating $CD56^+CD3^+$ cells as a percentage of total lymphocytes in 33 HBV patients aged between 19 and 35 years of age and 21 HBV patients aged between 35 and 55 years of age. The frequencies ranged from 1% to 38.6% in the younger group of patients (mean 6.8+/-1.6%) and from 0.3% to 10% in the older group of HBV patients (mean 4+/-0.5%). This difference was not significant (p=0.26). There was no linear correlation between the frequencies of circulating NT cells and the age of the HBV patients (r = -0.16).

These data suggest that although the frequencies of circulating NT cells are higher in HBV-infected subjects than in control subjects, their numbers do not correlate with viral load, disease severity, gender or age.

**Figure 3.6**

**Figure 3.7**

### 3.3.3 The frequencies of circulating γδ T cells are not significantly different between HBV patient groups

The γδ T cell frequencies of the HBV patients within the study cohort were also compared based on clinical parameters. Figure 3.8A shows the frequencies of circulating γδ-TCR$^+$CD3$^+$ cells as a percentage of total lymphocytes for 14 HBV patients with a low viral load i.e. below 100,000 copies / ml and 4 HBV patients with a high viral load i.e. between 100,000 and 5 x 10$^8$ copies / ml. The frequencies of circulating γδ T cells in patients with low viral load ranged from 0.35% to 30.7% (mean 6.2+/-1.9%) and in patients with high viral load, ranged from 1.4% to 15.8% (mean 8.6+/-3.2%). There was no significant difference between these numbers (p=0.31). Furthermore, a Spearman correlation test yielded a correlation coefficient of r = 0.146, suggesting that there is no linear correlation between the frequencies of γδ T cells and viral load. Figure 3.8B shows the mean frequencies of circulating γδ-TCR$^+$CD3$^+$ cells as a percentage of total lymphocytes for 11 HBV patients with a low ALT and 7 HBV patients with a high ALT. For those patients with low ALT, the frequencies of γδ T cells ranged from 0.35% to 10.5% (mean 5+/-0.95%) and for those with a high ALT, the frequencies ranged from 1.2% to 30.7% (mean 10.9+/-3.3%). There were no significant differences (p=0.28) and moreover, there was no linear correlation between the numbers of γδ T cell and ALT levels (r = 0.2). Figure 3.8C shows the frequencies of circulating γδ-TCR$^+$CD3$^+$ cells as a percentage of total lymphocytes in 11 males and 9 females. The frequencies ranged from 0.35% to 30.7% in males (mean 7.8+/-2.5%) and from 1.4% to 15.8% in females (5.5+/-1.4%). There was no significant difference (p=0.76). Figure 3.8D shows the frequencies of circulating γδ-TCR$^+$CD3$^+$ cells as a percentage of total lymphocytes in 10 HBV-infected subjects aged between 19 and 35 years of age and 10 HBV-infected subjects aged between 35 and 55 years of age. The frequency of circulating γδ T cells ranged from 1.4% to 15.8% in the younger group of patients (mean 6.5+/-1.5%) and ranged from 0.35% to 30.7% in the older group of patients (mean 7+/-2.8%). There was no significant difference (p=0.74) and there was no linear correlation between the frequencies of circulating γδ T cells and the age of the HBV patients (r = -0.18).

These data suggest that although the frequencies of circulating γδ T cells are higher in HBV-infected subjects than in control subjects, their numbers do not correlate with viral load, disease severity, gender or age.

**Figure 3.8**

### 3.3.4 The frequencies of circulating iNKT cells are not significantly different between HBV patient groups

The iNKT cell frequencies of the HBV patients within the study cohort were also compared based on clinical parameters. Both the frequencies of circulating $V\alpha24^+V\beta11^+CD3^+$ and $6B11^+CD3^+$ cells were quantified as a percentage of total lymphocytes within different patient groups. The minimum, maximum and mean frequencies for circulating $V\alpha24^+V\beta11^+CD3^+$ and $6B11^+CD3^+$ cells are shown in Table 3.1 and Table 3.2, respectively. The frequencies of $V\alpha24^+V\beta11^+CD3^+$ cells divided based on ALT, viral load, gender and age are shown in Figures 3.9A, C, E and G, respectively. The frequencies of $6B11^+CD3^+$ cells divided based on ALT, viral load, gender and age are shown in Figures 3.9B, D, F and H, respectively. Statistical significance was tested using the Mann-Whitney U-test and the Spearman Correlation test. None of the differences observed in the occurrence of iNKT cells between different patient groups were deemed statistically significant.

### 3.3.5 The frequencies of circulating $CD1^+$ B cells or monocytes are not significantly different between HBV patient groups

The frequencies of $CD1^+CD19^+$ cells were quantified as a percentage of lymphocytes and the numbers of $CD1^+CD14^+$ cells were quantified as a percentage of monocytes, within different HBV patient groups. The minimum, maximum and mean frequencies for circulating $CD1^+CD19^+$ and $CD1^+CD14^+$ cells are shown in Table 3.3 and Table 3.4, respectively. The frequencies of $CD1^+CD19^+$ lymphocytes divided based on ALT, viral load, gender and age are shown in Figures 3.10A, B, C and D, respectively. The frequencies of $CD1^+CD14^+$ cells divided based on ALT, viral load, gender and age are shown in Figures 3.10E, F, G and H, respectively. The performance of Mann-Whitney and Spearman Correlation tests revealed that there were no statistically significant differences in the frequencies of circulating $CD1^+$ cells between HBV patient groups.

These data suggest that the frequencies of iNKT cells and CD1-expressing cells within our HBV patient population do not significantly change with age, gender, viral load or ALT levels.

**Table 3.1**

**The frequencies of circulating iNKT cells in HBV:** The frequencies of Vα24$^+$Vβ11$^+$CD3$^+$ lymphocytes within divisions of the HBV patient study cohort, based on varying ALT, viral load, age and gender.

| | ALT < 40 | ALT >40 | Low Viral Load | High Viral Load | Age < 35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|
| **Min %** | 0.01 | 0.03 | 0.01 | 0.16 | 0.01 | 0.03 | 0.03 | 0.01 |
| **Max %** | 0.18 | 0.24 | 0.18 | 0.24 | 0.24 | 0.18 | 0.24 | 0.18 |
| **Mean %** | 0.09 | 0.09 | 0.07% | 0.2 | 0.11 | 0.07 | 0.08 | 0.1 |
| **SEM %** | 0.02 | 0.05 | 0.02% | 0.04 | 0.03 | 0.02 | 0.03 | 0.02 |
| **N*** | 12 | 5 | 15 | 2 | 7 | 10 | 9 | 8 |
| **p*** | | 0.79 | | 0.07 | | 0.4 | | 0.36 |
| **r*** | | -0.01 | | 0.184 | | -0.183 | | - |

**\* Low viral load** is below 10,000 copies / ml, **high viral load** ranges from 100,000 to $1 \times 10^8$ copies / ml. **N\*** is the number of subjects. The p-value **p\*** is a product of the Mann-Whitney U-test and indicates statistical significance when it falls below 0.05. The correlation coefficient **r\*** is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a linear correlation between two arrays of numbers. The column with header **M\*** comprises the male subjects and **F\*** comprises the female subjects.

**Table 3.2**

**The frequencies of circulating iNKT cells in HBV:** The frequencies of 6B11$^+$CD3$^+$ lymphocytes within divisions of the HBV patient study cohort, based on varying ALT, viral load, age and gender.

|  | ALT < 40 | ALT >40 | Low Viral Load | High Viral Load | Age < 35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|
| **Min %** | 0.14 | 0.04 | 0.04 | 0.25 | 0.25 | 0.04 | 0.04 | 0.14 |
| **Max %** | 3.5 | 1.3 | 3.5 | 1.26 | 1.26 | 3.5 | 3.5 | 1.26 |
| **Mean %** | 0.67 | 0.58 | 0.59 | 0.78 | 0.53 | 0.69 | 0.83 | 0.41 |
| **SEM %** | 0.27 | 0.22 | 0.22 | 0.36 | 0.12 | 0.33 | 0.36 | 0.11 |
| **N*** | 12 | 6 | 15 | 3 | 8 | 10 | 9 | 9 |
| **p*** |  | 0.96 |  | 0.41 |  | 0.49 |  | 0.46 |
| **r*** |  | 0.094 |  | -0.14 |  | -0.18 |  |  |

\* **Low viral load** is below 10,000 copies / ml, **high viral load** ranges from 100,000 to $1x10^8$ copies / ml. **N\*** is the number of subjects. The p-value **p\*** is a product of the Mann-Whitney U-test and indicates statistical significance when it falls below 0.05. The correlation coefficient **r\*** is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a linear correlation between two arrays of numbers. The column with header **M\*** comprises the male subjects and **F\*** comprises the female subjects.

**Table 3.3**

**The frequencies of circulating CD1$^+$ B lymphocytes in HBV:** The frequencies of CD1$^+$CD19$^+$ lymphocytes within divisions of the HBV patient study cohort, based on varying ALT, viral load, age and gender.

| | CD1 isoform | ALT <40 | ALT >40 | Low Viral Load | High Viral Load | Age < 35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|---|
| **Min %** | CD1a | 0.05 | 0.3 | 0.05 | 0.27 | 0.05 | 0.22 | 0.22 | 0.05 |
| | CD1b | 1 | 1 | 1 | 2.1 | 1.7 | 1 | 1 | 1.7 |
| | CD1c | 11.1 | 18.6 | 11.1 | 18.6 | 11.1 | 11.9 | 11.9 | 11.1 |
| | CD1d | 22.6 | 20.7 | 20.7 | 22.6 | 20.7 | 34.1 | 20.7 | 22.6 |
| **Max %** | CD1a | 1.6 | 1.3 | 1.6 | 0.4 | 1.6 | 1.5 | 1.6 | 1.4 |
| | CD1b | 7.2 | 6.7 | 7.2 | 6.7 | 6.7 | 7.2 | 7.2 | 6.7 |
| | CD1c | 31.8 | 44 | 44 | 23.5 | 44 | 31.8 | 31.8 | 44 |
| | CD1d | 74.3 | 91.9 | 91.9 | 60.8 | 91.9 | 74.3 | 64.5 | 91.9 |
| **Mean %** | CD1a | 0.6 | 0.7 | 0.7 | 0.3 | 0.6 | 0.4 | 0.7 | 0.5 |
| | CD1b | 3.1 | 3.3 | 3 | 3.7 | 3.1 | 2.6 | 2.7 | 3.6 |
| | CD1c | 21.6 | 24.5 | 24.3 | 21.4 | 22.9 | 21.2 | 21.4 | 24 |
| | CD1d | 53.6 | 52.2 | 57.2 | 45.5 | 50.7 | 52.8 | 50 | 56.5 |
| **SEM %** | CD1a | 0.2 | 0.2 | 0.1 | 0.03 | 0.2 | 0.2 | 0.2 | 0.15 |
| | CD1b | 0.5 | 0.9 | 0.4 | 1.5 | 0.6 | 0.6 | 0.6 | 0.5 |
| | CD1c | 2.1 | 4 | 2.6 | 1.5 | 3.1 | 2.1 | 1.9 | 3.5 |
| | CD1d | 4.1 | 10 | 5 | 11.7 | 7.2 | 4.1 | 4.8 | 7.1 |
| **N*** | CD1a | 12 | 6 | 15 | 3 | 8 | 10 | 9 | 9 |
| | CD1b | 12 | 6 | 15 | 3 | 8 | 10 | 9 | 9 |
| | CD1c | 11 | 6 | 15 | 3 | 8 | 9 | 9 | 8 |
| | CD1d | 11 | 6 | 15 | 3 | 8 | 9 | 9 | 8 |
| **p*** | CD1a | | 0.3 | | 0.5 | | 0.9 | | 0.6 |
| | CD1b | | 0.9 | | 0.9 | | 0.6 | | 0.1 |
| | CD1c | | 0.8 | | 0.9 | | 0.8 | | 0.7 |
| | CD1d | | 0.6 | | 0.6 | | 0.7 | | 0.7 |
| **r*** | CD1a | | 0.2 | | -0.2 | | -0.02 | | - |
| | CD1b | | 0.004 | | 0.1 | | 0.1 | | - |
| | CD1c | | 0.2 | | -0.2 | | 0.1 | | - |
| | CD1d | | -0.2 | | -0.2 | | 0.1 | | - |

* **Low viral load** is below 10,000 copies / ml, **high viral load** ranges from 100,000 to $1 \times 10^8$ copies / ml. **N*** is the number of subjects. The p-value **p*** is a product of the Mann-Whitney U-test and indicates statistical significance when it falls below 0.05. The correlation coefficient **r*** is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a linear correlation between two arrays of numbers. The column with header **M*** comprises the male subjects and **F*** comprises the female subjects.

**Table 3.4**

**The frequencies of circulating CD1$^+$ monocytes in HBV:** The frequencies of CD1$^+$CD14$^+$ monocytes within divisions of the HBV patient study cohort, based on varying ALT, viral load, age and gender.

| | CD1 isoform | ALT <40 | ALT >40 | Low Viral Load | High Viral Load | Age < 35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|---|
| **Min %** | CD1a | 1.9 | 0.6 | 0.62 | 1.5 | 0.62 | 2.4 | 0.62 | 1.2 |
| | CD1b | 2.6 | 2.2 | 2.3 | 2.2 | 2.2 | 2.6 | 2.2 | 2.6 |
| | CD1c | 0.3 | 0.9 | 0.3 | 1.3 | 0.5 | 0.3 | 0.27 | 0.5 |
| | CD1d | 11.5 | 4.5 | 4.5 | 10.6 | 4.5 | 11.5 | 4.5 | 10.6 |
| **Max %** | CD1a | 25.3 | 17.8 | 25.3 | 2.7 | 25.3 | 17.8 | 17.9 | 25.3 |
| | CD1b | 35.2 | 18.6 | 35.2 | 14.2 | 35.2 | 29.8 | 35.2 | 22.7 |
| | CD1c | 12.9 | 13.7 | 13.7 | 5.9 | 12.9 | 13.7 | 13.7 | 5.9 |
| | CD1d | 98.6 | 91.2 | 98.6 | 60.5 | 98.3 | 57 | 98.6 | 98.3 |
| **Mean %** | CD1a | 10.5 | 4.6 | 8.7 | 2 | 6.7 | 8.3 | 8.1 | 7 |
| | CD1b | 12.1 | 8.3 | 11.7 | 6.9 | 11.3 | 10.5 | 12.5 | 9.3 |
| | CD1c | 4.8 | 4.5 | 4.3 | 3.6 | 4 | 4.4 | 5.1 | 3.1 |
| | CD1d | 65.1 | 36.3 | 54.6 | 35.5 | 46.2 | 57 | 54.9 | 48.8 |
| **SEM %** | CD1a | 2.6 | 2.7 | 2.1 | 0.4 | 3.3 | 2 | 2.4 | 2.9 |
| | CD1b | 3.2 | 2.7 | 2.7 | 3.7 | 4.2 | 2.6 | 4.1 | 2.2 |
| | CD1c | 1.5 | 2 | 1.2 | 1.9 | 1.6 | 1.6 | 1.9 | 0.8 |
| | CD1d | 10.2 | 13.8 | 9.5 | 20.4 | 13.2 | 11.4 | 11 | 13.9 |
| **N*** | CD1a | 12 | 6 | 15 | 3 | 8 | 10 | 9 | 9 |
| | CD1b | 12 | 6 | 15 | 3 | 8 | 10 | 9 | 9 |
| | CD1c | 11 | 6 | 15 | 3 | 8 | 9 | 9 | 8 |
| | CD1d | 11 | 6 | 15 | 2 | 8 | 9 | 9 | 8 |
| **p*** | CD1a | | 0.1 | | 0.1 | | 0.1 | | .6 |
| | CD1b | | 0.5 | | 0.4 | | 0.7 | | 0.9 |
| | CD1c | | 0.9 | | 0.9 | | 0.9 | | 0.9 |
| | CD1d | | 0.1 | | $ | | 0.2 | | 0.7 |
| **r*** | CD1a | | -0.4 | | -0.3 | | 0.5 | - | - |
| | CD1b | | -0.09 | | -0.3 | | 0.2 | - | - |
| | CD1c | | 0.1 | | -0.09 | | 0.06 | - | - |
| | CD1d | | -0.5 | | -0.1 | | 0.1 | - | - |

* **Low viral load** is below 10,000 copies / ml, **high viral load** ranges from 100,000 to $1 \times 10^8$ copies / ml. **N*** is the number of subjects. The p-value **p*** is a product of the Mann-Whitney U-test and indicates statistical significance when it falls below 0.05. The correlation coefficient **r*** is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a linear correlation between two arrays of numbers. The column with header **M*** comprises the male subjects and **F*** comprises the female subjects.

**Figure 3.9 plots**

**<u>Figures 3.9 legend</u>**

**Figure 3.10**

## 3.4 NK, NT and iNKT cell frequencies in demographically-matched healthy controls

As mentioned in Chapter 2, our patient cohort was one of mixed race; 38% of the patients were African, while 45% were Caucasian and 17% were Asian. The control group, however, comprised buffy coat packs obtained form the IBTS. Since the knowledge of our healthy controls was limited, some demographically-matched samples were obtained as a control measure. This would minimise the risk of false-positive results based on race and not HBV infection. The demographically-matched control blood samples were taken from 15 African, 15 Caucasian and 10 Asian healthy donors. PBMC preparation and surface staining was performed in the same manner as for all other subjects and the frequencies of NK, NT, iNKT and $\gamma\delta$ T cells were then quantified.

### 3.4.1 NK cell frequencies in demographically-matched healthy controls

The mean frequencies of NK cells, as a percentage of lymphocytes, were 5.6+/-0.9% in the African controls, 4.7+/-0.7% in the Caucasian controls and 6.4+/-1.6% in the Asian controls. These frequencies were not significantly different with no p-values falling below 0.05 (Figure 3.11A).

The mean frequencies of the CD56$^{DIM}$ and CD56$^{BRIGHT}$ subsets of NK cells were calculated as a percentage of NK cells and as a percentage of lymphocytes. The mean frequencies of CD56$^{DIM}$ NK cells, as a proportion of NK cells, were 94.1+/-1.2%, 89.5+/-1.7% and 93.6+/-1.7% in Africans, Caucasians and Asians, while the frequencies of CD56$^{BRIGHT}$ NK cells were 5.9+/-1.2%, 10.5+/-1.7% and 6.5+/-1.7% (Figure 3.11B, D). When calculated as a percentage of lymphocytes, the mean frequencies of CD56$^{DIM}$ NK cells were 4.9+/-0.8%, 4+/-0.6% and 5.9+/-1.6% in Africans, Caucasians and Asians, respectively, while the frequencies of CD56$^{BRIGHT}$ NK cells were 0.5+/-0.1%, 0.3+/-0.1% and 0.4+/-0.1% (Figure 3.11C, E). Although the observations suggest that there may be expansions of CD56$^{DIM}$ NK cells in the African and Asian populations, compared to the Caucasians, our statistical analysis revealed that such differences are not statistically different with all p-values falling outside our confidence interval of 0.05.

These data suggest that there are no significant differences in NK cell frequencies between African, Caucasian and Asian control populations and that the differences observed between HBV patients and controls are not significantly influenced by the race of the patients.

**3.4.2 NT cell frequencies in demographically-matched healthy controls**

The mean frequencies of NT cells, as a percentage of lymphocytes, were 2.1+/-0.3% in the African controls, 0.8+/-0.1% in the Caucasian controls and 1.6+/-0.3% in the Asian controls (Figure 3.12A). Performance of the Mann-Whitney U-test on these data sets revealed that the frequencies of circulating NT cells in healthy donors of African race were significantly higher than those of their Caucasian counterparts (p=0.02). Due to this striking result, it was decided to compare the NT cell frequencies within our patient cohort and found that no significant differences existed in our HBV group (Figure 3.12B). Therefore, although these data suggest the NT cells are expanded in the peripheral blood of African persons compared to Caucasians, our subsequent analysis revealed that this does not represent our patient group. Since the mean frequency of circulating NT cells was 3.1% in our initial patient group, it can be hypothesised that the NT cell numbers observed in our demographically-matched caucasian population may be lower than the normal average.

The mean frequencies of the CD56$^{DIM}$ and CD56$^{BRIGHT}$ subsets of NT cells were calculated as a percentage of NT cells and as a percentage of lymphocytes. The mean frequencies of CD56$^{DIM}$ NT cells, as a proportion of NT cells, were 98.7+/-0.4%, 99+/-0.5% and 99.6+/-0.3% in Africans, Caucasians and Asians, while the frequencies of CD56$^{BRIGHT}$ NT cells were 1.3+/-0.4%, 0.9+/-0.5% and 0.4+/-0.3% (Figure 3.12 B, D). When calculated as a percentage of lymphocytes, the mean frequencies of CD56$^{DIM}$ NT cells were 2.1+/-0.4%, 0.7+/-0.1% and 1.6+/-0.3% in Africans, Caucasians and Asians, respectively, while the frequencies of CD56$^{BRIGHT}$ NT cells were 0.03+/-0.01%, 0.01+/-0.004% and 0.005+/-0.003% (Figure 3.12C, E). These data show that the differences in the frequencies of NT cells between African and Caucasian populations are due to differences in the CD56$^{DIM}$ subset (p=0.008).

These data suggest that NT cell frequencies may differ between African and Caucasian populations, although careful examination of our patient cohort revealed that such a hypothesis is not indicative of our HBV patients. Therefore, it is likely that the expansions observed in HBV patients were not skewed by the demographics of the patients.

**3.4.3 Total CD56$^{+}$ cell frequencies in demographically-matched healthy controls**

The mean frequencies of total $CD56^+$ cells, as a percentage of lymphocytes, were 7.3+/-1% in the African controls, 5.5+/-0.8% in the Caucasian controls and 7.9+/-1.8% in the Asian controls (Figure 3.13A). These frequencies were not significantly different with no p-values falling below 0.05.

The mean frequencies of the $CD56^{DIM}$ and $CD56^{BRIGHT}$ subsets of total $CD56^+$ cells were calculated as a percentage of NK cells and as a percentage of lymphocytes. The mean frequencies of $CD56^{DIM}$ $CD56^+$ cells, as a proportion of total $CD56^+$ cells, were 88.6+/-1.7%, 92.8+/-1.3% and 93.1+/-1.6% in Africans, Caucasians and Asians, while the frequencies of total $CD56^{BRIGHT}$ cells were 7.2+/-1.3%, 11.4+/-1.7% and 6.9+/-1.6% (Figure 3.13 B, D). When calculated as a percentage of lymphocytes, the mean frequencies of $CD56^{DIM}$ cells were 6.1+/-1.1%, 4.8+/-0.7% and 7.5+/-1.7% in Africans, Caucasians and Asians, respectively, while the frequencies of $CD56^{BRIGHT}$ cells were 0.2+/-0.1%, 0.5+/-0.1% and 0.4+/-0.1% (Figure 3.13C, E). Although the observations suggest that there may be a trend towards higher levels of $CD56^{DIM}$ NK cells in the African and Asian populations, compared to the Caucasians, our statistical analysis revealed that such differences are not statistically different with all p-values falling outside our confidence interval of 0.05.

These data suggest that there are no significant differences in total $CD56^+$ cell frequencies between African, Caucasian and Asian populations, despite the apparent expansions of circulating NT cells in Africans, and that the significant higher frequencies of total $CD56^+$ cells observed in HBV patients are not significantly influenced by the race of the patients.

### 3.4.4 Invariant NKT cells frequencies in demographically-matched healthy controls

The mean frequencies of $6B11^+$ cells, as a percentage of total T cells, were 0.5+/-0.2% in the African controls, 0.4+/-0.1% in the Caucasian controls and 0.3+/-0.1% in the Asian controls (Figure 3.14). These frequencies were not significantly different with no p-values falling below 0.05.

These data suggest that there are no differences in the frequencies of circulating iNKT cell frequencies between individuals of different race.

Overall, these data show that, although there may be some differences in the proportions of circulating innate lymphocytes between persons of different race, there is no

significant evidence to suggest that such differences have influenced the alterations observed in HBV patients.

**Figure 3.11**

**Figure 3.12**

**Figure 3.13**

**Figure 3.14**

**3.5 Cytotoxicity of PBMC in HBV infection**

A preliminary study examining the cytotoxic capabilities of PBMC from HBV patients and uninfected controls was performed by Ross McNicholas for several reasons. Firstly, since HBV is a non-cytopathic virus, the liver damage associated with chronic infection is believed to be immune-mediated. Secondly, the control and elimination of HBV may be dependent on the killing activity of innate lymphocytes which may reduce viral load to a manageable level before adaptive responses come into play. Therefore, if the cytotoxic capabilities of PBMC were enhanced in the individuals persistently-infected with HBV then, this may indicate a mechanism of immune control by innate lymphocytes in HBV. Such findings would have to be followed by cytotoxic assays using enriched populations of innate lymphocytes to confirm which cells were the most potent killers.

Assessment of the cytotoxic capabilities of PBMC in 7–55 healthy control subjects and 15 HBV patients was carried out by analysis of chromium ($^{51}$Cr) release by the labelled myeloid erythroleukaemia-derived K562 cells. The cytotoxic capabilities were evaluated at E:T ratios of 1, 5, 25 and 50. Natural cytotoxicity of PBMC was examined while treatment with IFN-α or IL-2 induced the cytotoxic activity of NK cells and T cells.

**3.5.1 There are no significant changes in the natural cytotoxicity of PBMC in HBV infection**

At E:T ratios of 5:1, the specific lysis ranged from 0-1% in healthy control subjects and from 0-10.2% in HBV patients (Figure 3.14A and Figure 3.14B), with means of 0.13+/-0.13% and 1.6+/-1.1% (Figure 3.14G). This difference was not significant (p=0.15). At E:T ratios of 25:1, the specific lysis ranged from 0-4.25% in healthy control subjects and from 0-21.44% in HBV patients (Figure 3.14A and Figure 3.14B), with mean specific lyses of 1.6+/-0.5% and 4.6+/-2.7%, respectively (Figure 3.14H). This difference was not significant (p=0.80). At E:T ratios of 50:1, the specific lysis ranged from 0-39.9% in healthy control subjects and from 0-28.7% in HBV patients (Figure 3.14A and Figure 3.14B), with means of 1.4+/-1.1% and 7.1+/-2.3% (Figure 3.14I, p=0.18; not significant).

Although, natural cytotoxicity of PBMC in HBV patients appears to be higher at all E:T ratios, these data do not show any statistically significant differences between HBV patients and healthy controls.

### 3.5.2 Higher IL-2-induced cytotoxicity of PBMC in HBV infection

At E:T ratios of 5:1, the specific lysis ranged from 0-2.9% in healthy control subjects and from 0-18.3% in HBV patients (Figure 3.14C and Figure 3.14D), with means of 1.1+/-0.3% and 4.4+/-1.3%, respectively (Figure 3.14G). This higher frequency was significant (p=0.04). At E:T ratios of 25:1, the specific lysis ranged from 0.6-20.3% in healthy control subjects and from 0-44.4% in HBV patients (Figure 3.14C and Figure 3.14D), with means of 12.4+/-1.8% and 15.7+/-3.3%, respectively (Figure 3.14H, p=0.79; not significant). At E:T ratios of 50:1, the specific lysis ranged from 1-53.6% in healthy control subjects and from 1.7-47.4% in HBV patients (Figure 3.14C and Figure 3.14D). The mean specific lyses were 15.9+/-1.5% and 19.4+/-3.4%, respectively (Figure 3.14I) with no statistically significant difference between subject groups (p=0.39).

These data show that IL-2-induced cytotoxicity of PBMC is significantly Higher in HBV patients, compared with control subjects, at an E:T ratio of 5:1.

### 3.5.3 There are no significant differences in the IFN-α-induced cytotoxicity of PBMC in HBV infection

At E:T ratios of 5:1, the specific lysis was 0% for all healthy control subjects and ranged from 0-2.6% in HBV patients (Figure 3.14E and Figure 3.14F), with means of 0% and 0.64+/-0.25% (Figure 3.14G). No statistical comparison between HBV patients and control subjects was carried out due to the lack of response to IFN-α in control subjects, at this E:T ratio. At E:T ratios of 25:1, the specific lysis ranged from 0-6.4% in healthy control subjects and from 0-22.1% in HBV patients (Figure 3.14E and Figure 3.14F), with means of 2.2+/-0.55% and 5+/-1.6% (Figure 3.14H, p=0.40; not significant). At E:T ratios of 50:1, the specific lysis ranged from 0-10.5% in healthy control subjects and from 0-26.6% in HBV patients (Figure 3.14E and Figure 3.14F). The mean specific lyses were 3.9+/-1.4% and 6.9+/-1.9%, respectively (Figure 3.14I, p=0.57; not significant).

Although, PBMC isolated from HBV patients appear to be more responsive to IFN-α treatment, these data do not show any statistically significant differences between HBV patients and healthy controls.

**Figure 3.15**

**Figure 3.15**

**Figure 3.15**

## 3.6 Bonferroni correction method

Since the present study involved the analysis of the frequencies of multiple cell populations in patient and control subject groups, it is possible that statistically-significant differences in the two subject groups will be identified by chance alone. The Bonferroni correction method was used to attempt to distinguish between real and false-positive results. The method assumes that spurious positives will arise when a large number of tests are performed. Therefore, the p-value is multiplied by the number of tests and if the corrected p-value ($p_c$) is still within our chosen confidence interval i.e. below 0.05, then the finding is considered to be statistically significant (Shaffer 1995).

For the phenotypical study of innate lymphocytes, 26 tests were performed and 13 of these yielded significant p-values, as shown in Table 3.5. By applying the Bonferroni correction method to the p-values obtained from the Mann-Whitney U tests, the most substantial differences could be filtered out. Both the p and $p_c$ values for all tests are listed in Table 3.5. Following this correction, the differences in the frequencies of NT cells and γδ T cells, only, were shown to be statistically sound with $p_c$-values of 0.005 and 0.02, respectively (Table 3.5).

These data provide strong evidence that the frequencies of circulating NT and γδ T cells are Higher in HBV infection.

**Table 3.5**

**Statistical analysis of the significant phenotypical differences observed in innate lymphocyte populations in HBV infection:** The p-values and corrected p-values for 13 of 26 statistical tests performed in the phenotypical study of innate lymphocytes in HBV infection.

| TEST | *Change in HBV | p value | $p_c$ value |
|---|---|---|---|
| NK cells | Higher | 0.04 | 1.04 |
| CD56$^{DIM}$ NK cells (% NK cells) | Higher | 0.006 | 0.2 |
| CD56$^{DIM}$ NK cells (% lymphocytes) | Higher | 0.04 | 1.04 |
| CD56$^{BRIGHT}$ NK cells (% NK cells) | Lower | 0.006 | 0.2 |
| NT cells | Higher | 0.0002 | *0.005* |
| CD56$^{DIM}$ NT cells (% NT cells) | Higher | 0.009 | 0.2 |
| CD56$^{DIM}$ NT cells (% lymphocytes) | Higher | 0.003 | 0.08 |
| CD56$^{BRIGHT}$ NT cells (% NT cells) | Lower | 0.009 | 0.2 |
| Total CD56$^+$ cells | Higher | 0.005 | 0.1 |
| CD56$^{DIM}$ cells (% total CD56$^+$ cells) | Higher | 0.007 | 0.2 |
| CD56$^{DIM}$ cells (% lymphocytes) | Higher | 0.02 | 0.5 |
| CD56$^{BRIGHT}$ cells (% total CD56$^+$ cells) | Lower | 0.007 | 0.2 |
| γδ T cells | Higher | 0.0009 | *0.02* |

**\*Change in HBV** indicates whether the frequencies of cells are higher or lower in HBV-infected subjects, compared to control subjects.

CD56$^{BRIGHT}$ cells are those cells that express high levels of CD56 while CD56$^{DIM}$ cells are those that express lower levels of CD56. Vα24$^+$Vβ11$^+$ T cells are those iNKT cells that co-express both the Vα24 α-chain and the Vβ11$^+$ β-chain of the αβ-TCR. 6B11$^+$ T cells are those iNKT cells which express the Vα24 α-chain.

Significant results after Bonferroni correction ($p_c$ values) are highlighted in italics.

**3.7 Discussion**

This study investigated the frequencies of circulating innate lymphocytes in HBV infection in an effort to elucidate the early immune response to the virus. Since the majority of the patients had a low viral load and an ALT below 40, the group of patients were considered to have minimal or no liver disease and a low viral burden. Therefore, despite the presence of HBsAg, the group represented an effective antiviral immune response and the frequencies of innate lymphocytes found in such subjects were considered to reflect the responses needed to control HBV infection. The frequencies of circulating NK, NT and total $CD56^+$ cells were quantified in this study along with the frequencies of $CD56^{DIM}$ and $CD56^{BRIGHT}$ populations of these cells. The $CD56^{DIM}$ and $CD56^{BRIGHT}$ subsets were quantified as a percentage of NK, NT and total CD56 cells and as a percentage of lymphocytes. These subsets were identified based on the cell surface density (mean fluorescence intensity) of CD56 and since isotype controls could not be used to distinguish them, it must be acknowledged that their identification was influenced by the operator of the flow cytometer. The frequencies of $\gamma\delta$ T cells, $V\alpha24^+V\beta11^+$ T cells and $6B11^+$ T cells were also quantified along with the frequencies of B cells expressing CD1a, b, c and d and monocytes expressing CD1a, b, c and d.

To date, several studies have revealed a requirement for innate lymphocytes in the clearance of HBV infection. NK cytotoxicity and IL-2 production is Higher in acute HBV infection and returns to normal in the recovery phase thus suggesting that these cells are involved in the resolution of infection (Echevarria *et al.* 1991). The NK and NKT cell-mediated inhibition of HBV replication in transgenic mice also indicates that these cells are important in the fight against HBV (Kimura *et al.* 2002; Pasquetto *et al.* 2000; Kakimi *et al.* 2000). Furthermore, responders to vaccination with HBsAg possess higher frequencies of circulating NK and NT cells and these cells elicit superior cytokine-producing capacities in such subjects (Albarran *et al.* 2005). Since these studies have highlighted the importance of innate lymphocytes in HBV, it was believed that knowledge of the abundance and functions of these cells in controlled HBV infection would facilitate a better understanding of successful immune responses against HBV. Depleted numbers of circulating NK cells in chronically infected HCV patients have previously been found by our research group, while others have found reduced frequencies of iNKT cells and NT cells in HCV-infected livers (Golden-Mason *et al.*

2008; Deignan *et al.* 2002) and in HCC (Kawarabayashi *et al.* 2000). It appears that lower numbers of innate lymphocytes can predispose an individual to viral persistence in HCV and it was proposed the same for HBV. Alternatively, the virus might infect, activate or kill innate lymphocytes and be the cause of the numerical change. It was proposed that impaired innate lymphocyte responses lead to uncontrollable viral replication and inadequate adaptive responses and that, the subsequent immune responses cause liver damage without eliminating the virus. Since HBV is non-cytopathic, such immune responses might be responsible for the liver disease associated with HBV infection. In this study however, patients who were persistently infected with a low viral burden but were not diseased were examined and therefore, exhibited an effective but apparently non-pathological immune response. The response found in such persons might be required to prevent high viral burden and liver cirrhosis in other persistently-infected patients.

Expansions of circulating innate lymphocytes have been found in our patient cohort, compared to a group of uninfected controls, which this might be representative of an active antiviral response. NK, NT and γδ T cells, but not iNKT cells, were more abundant in the peripheral blood of HBV-infected patients than in that of uninfected control subjects. This suggests that these cells are important players in the control of HBV replication.

After using the Bonferroni correction method to eliminate significant differences that may arise solely as a result of multiple testing, the higher frequencies of circulating NT cells and γδ T cells in HBV infection remained statistically significant. Therefore, it is proposed that these expansions were representative of an active but regulated hepatic immune response that kept HBV replication under control without causing liver damage.

Expansions of γδ T cells have been identified in several viral infections and may enhance immune responses via cytokine production as well as eliciting both cytolytic and non-cytolytic inhibition of viral replication. However, a similar study to this has revealed depletions of γδ T cells in both asymptomatic HBV-infected patients and those with liver disease, compared to controls (Chen *et al.* 2008). However, the study population examined by that group was of Asian origin whereas ours is a group of mixed race comprising Caucasians, Africans and Asians. Furthermore, Chen *et al.* (2008) found the lower frequencies in the Vδ2 T cell subset as opposed to the total γδ T

cell population. Both of these TCR-chain recognition and demographic issue have been addressed in Chapter 5 to determine if our data still disagree with Chen et al (2008).

The NT cells may also elicit both cytolytic and non-cytolytic inhibition of viral replication and it is possible that these cells have been derived from $CD8^+$ T cells which could not clear the virus due to weakened recognition capacity caused by antigenic drift (Kelly-Rogers *et al.* 2006). This may, therefore, represent an evolutionary mechanism of the immune system to facilitate a switch from an inefficient specific response to an efficient non-specific response. IL-2 is a potent stimulator of T cell proliferation and is commonly used as an adjuvant in immunotherapeutics (Dieli *et al.* 2007; Bennouna *et al.* 2008). It has also been shown to be a powerful inducer of NT cell-mediated cytotoxicity (Kelly Rogers *et al.* 2006; Jin *et al.* 1998; Zoll *et al.* 1998) and these cells may contribute to the Higher IL-2 induced cytotoxicity observed in HBV. However, it cannot be concluded whether cytotoxicity is a major contributor to immunity against HBV in our patient cohort or whether the higher levels of in cytolytic activity are a by-product of an active antiviral response. Since liver damage is not evident in the majority of our study patients, it is likely that non-cytolytic mechanisms are primarily responsible for the control of HBV infection in these individuals.

Overall, this study yields strong evidence that NT and γδ T cells are involved in the control of HBV replication. Furthermore, the frequencies of these innate T cells do not strongly correlate with differences in gender, age, viral load or disease severity but, although not statistically significant, higher frequencies of both cells are observed in patients with elevated ALT levels. This suggests that if not regulated, the expansions of these potent cytotoxic cells and inflammatory cytokine producers may lead to liver damage. Examination of demographically-matched controls also ensured that any differences observed between the control and patient group were not predominantly influenced by the race of the patients.

Therefore, it is proposed that NK, NT and γδ T cells are important for the control of HBV infection. Our next aims are to determine if the cytokine profiles of innate lymphocytes reveal more about their antiviral functions in HBV and to discover which innate T cell subsets are expanded in this persistent infection.

# Chapter 4

# Cytokine production by innate lymphocytes in HBV infection

**4.1 Introduction**

In Chapter 3, it was shown that circulating innate lymphocytes are expanded in our patient cohort suggesting that these higher frequencies are indicative of a good immune response against HBV since the majority of the patients studied have low viral load and normal ALT levels. Although, viral clearance is not achieved, the majority of these patients appear to represent the best scenario for persistent HBV infection because viral load is maintained at a low level and there is no evidence of liver disease. Such immune control is found in other persistent infections such as CMV and EBV infection. Here, the cytokine profiles of these cells in HBV infection were investigated in order to gain knowledge of their functional capacity in immune responses against the virus. The frequencies of IFN-γ-, IL-10-, IL-13- and IL-17-producing NK cells, NT cells and conventional T cells were investigated. This may inform us on the influence of innate lymphocytes on adaptive T cell responses in HBV infection. The cytokine profile of the total lymphocyte population was also examined to learn more about the overall immune response in these HBV-infected patients.

IFN-γ is the classical antiviral cytokine and can elicit a variety of functions that promote viral clearance (Guidotti & Chisari 2001). These include the recruitment and activation of macrophages, NK cells and T cells, the polarisation of T cell responses, the upregulation of antigen processing, transport and MHC Class I expression in infected cells and the disruption of viral replication. IFN-γ is very important in the non-cytolytic control of virus infection which is often the preferred mechanism when large numbers of cells in large vital organs are infected (Guidotti & Chisari 2001). During infection by hepatotropic viruses such as HBV, strong cytolytic antiviral mechanisms could cause more harm than good and may contribute to the development of liver disease (Maini *et al.* 2000). One study in HBV-infected chimpanzees revealed that the clearance of HBV DNA from liver occurred at the same time as the appearance of IFN-γ and before the peak of T cell infiltration thus implying that viral clearance was mediated non-cytolytically and that conventional T cells may not have been the source. Since NK and NKT cells are potent producers of the cytokine, they were proposed as the source of IFN-γ in this model (Guidotti *et al.* 1999). Therefore, a better approach to clearing HBV without causing significant liver damage might be a non-cytolytic mechanism mediated by cytokines such as IFN-γ and TNF-α. It was hypothesised that IFN-γ would be the predominant cytokine produced by innate lymphocytes in the successful immune

control of HBV. IFN-γ is released by NK cells, NT cells, iNKT cells and several subsets of γδ T cells from mice and humans (Orange *et al.* 1996; Biron *et al.* 1999; Spada *et al.* 2000; Kakimi *et al.* 2000; Kelly-Rogers *et al.* 2006; Eberl *et al.* 2009). It is representative of an active antiviral response and its expression by such cells is substantially higher in responders to HBV vaccination (Albarran *et al.* 2005). Therefore, the frequencies of IFN-γ-expressing NK and NT cells in HBV-infected individuals and uninfected controls were quantified.

IL-10 is an immunomodulatory cytokine produced by T cells, monocytes and NK cells and it is crucial in the regulation of inflammatory responses and the prevention of immune-mediated damage. However, its ability to suppress the differentiation of cells that produce antiviral cytokines has been manipulated by several viruses in order to evade antiviral immune responses (Taoufik *et al.* 1997; Stockl *et al.* 1999; Brady *et al.* 2003). For instance HIV gp120 induces IL-10 production and inhibits IL-12 production by monocytes while CMV produces an IL-10 homologue that can elicit the same biological effects as human IL-10 (Taoufik *et al.* 1997; Kotenko *et al.* 2000). Also, HCV protein NS4 has been shown to induce IL-10 expression and in turn, dampen IFN-γ, IL-12 and IL-17 production (Brady *et al.* 2003; Rowan *et al.* 2008). Therefore, an overriding regulatory response mediated by the production of IL-10 may promote viral persistence in HCV, HIV and CMV, and perhaps the same is true for HBV. The early stage of acute HBV infection is actually marked by a transient inhibition of NK and T cell responses that coincide with an increase in IL-10 at the time of HBV viremia (Dunn *et al.* 2009). This suggests that in persistent HBV infection, the virus may induce IL-10 at an early stage in infection leading to inadequate antiviral immune responses and uncontrollable HBV replication. Furthermore, the higher expression of IL-10 in HCV infection is not only seen in Treg cells and monocytes, but also in NK cells, thus identifying an immunomodulatory role for innate lymphocytes in chronic HCV infection (De Maria *et al.* 2007). The frequencies of IL-10-expressing NK and NT cells were quantified in a cohort of relatively healthy HBV-infected patients compared to a group of uninfected controls to determine the immunomodulatory capacity of innate lymphocytes in a model of immune control.

IL-13 is as a Th2 cytokine that promotes the humoral response while suppressing important components of the antiviral response i.e. macrophage inflammatory cytokine production and Th1 responses. Although IL-13 is predominantly

produced by T cells, NK and iNKT cells have also been shown to express it (Takahashi *et al.* 2002; Hepworth & Grencis 2009). Furthermore, iNKT cells isolated from individuals chronically-infected with HCV secrete higher levels of IL-13 than those taken from uninfected persons. It is possible that HCV induces this IL-13 production by iNKT cells as an immune subversion strategy in which Th2-biased iNKT cells can suppress antiviral responses and contribute to the persistence of HCV infection (Inoue *et al.* 2006). Increases in iNKT-derived IL-13 have also been shown to correlate with the development of liver cirrhosis in viral hepatitis, thus supporting the hypothesis that innate lymphocytes exhibiting Th2 cytokine profiles may impede HBV clearance and promote disease progression (De Lalla 2004). To test this hypothesis, the frequencies of IL-13-producing innate lymphocytes and conventional T cells were examined in persons with controlled HBV infection, expecting that a Th2 bias would not be evident in such individuals.

IL-17 is a pro-inflammatory cytokine whose importance is still not fully understood in the setting of viral infection. The inhibition of IL-17 production by the HCV protein NS4 suggests that the cytokine plays an active role in immunity against the virus (Rowan *et al.* 2008). Several studies in HBV have indicated that the cytokine is a prominent feature in chronic HBV and HBV-related disease. For instance, Zhang *et al.* (2010) have recently found expansions of circulating and intrahepatic IL-17-producing $CD4^+$ T cells in chronic HBV with increases positively correlating with disease progression. Similarly, Ge *et al.* (2010) have reported higher frequencies of circulating IL-17-producing with frequencies negatively correlating with frequencies of circulating IFN-γ-producing T cells. Furthermore, IL-17 has also been implicated in HBV-related liver fibrosis (Xu *et al.*2009). These studies imply that IL-17 promotes liver disease but not necessarily viral clearance in HBV infection. The frequencies of IL-17-expressing NK, NT and conventional T cells were investigated in HBV-infected with low viral burden and with little or no evidence of liver disease to determine the abundance of the cytokine in what seems to be controlled HBV infection.

RT-PCR was used to determine if the gene expression profiles of IFN-γ, IL-10 and IL-17 were altered in PBMC from HBV-infected subjects, compared to control subjects. This would indicate whether the control of such responses was being performed at a protein level or a genetic level. It may also serve to support our results on cytokine expression by lymphocyte subsets.

**4.2 IFN-γ expression by stimulated NK, NT, T and total lymphocytes from HBV patients and healthy controls**

**4.2.1 IFN-γ-producing NK cells in HBV patients and healthy control subjects**

Freshly isolated PBMC from 32 HBV-infected or 23 control subjects were incubated for 4 hours in medium alone or with PMA/I or plate-bound anti-CD3 mAb and anti-CD28 mAb, in the presence of Brefeldin A. FITC-labelled anti-IFN-γ mAb was used in combination with PE-labelled anti-CD56 mAb and PE-Cy5-labelled anti-CD3 mAb to identify IFN-γ-producing NK (CD56$^+$CD3$^-$) cells (Figure 4.1B, C). It was observed that the MFI of CD56 expression was always decreased following PMA/I stimulation. Such findings are consistent with the findings of Kelly-Rogers *et al.* (2006). The frequencies of IFN-γ-producing NK cells were calculated as a percentage of total NK cells (Figure 4.2A) and as a percentage of lymphocytes (Figure 4.2B).

In the absence of *ex vivo* stimulation, the mean frequencies of NK cells expressing IFN-γ were 3.5+/-0.3% and 6.4+/-0.8% of all NK cells in controls and HBV patients, respectively. As expected, these frequencies were not higher when PBMC were stimulated with the T cell mitogen, anti-CD3 and anti-CD28 mAb (2.4+/-0.2% and 3.8+/-0.4% of NK cells). After PMA/I stimulation, means of 18.1+/-1.1% and 22.3+/-1.8% of NK cells from controls and HBV patients expressed IFN-γ. This frequencies of IFN-γ-expressing NK cells were slightly higher in HBV patients in the absence and presence of *in vitro* stimulation but none of these differences were statistically significant (Figure 4.2A, p=0.4, 0.2, 0.2). When calculated as a percentage of total lymphocytes, only 0.4+/-0.04% of lymphocytes from controls were CD56$^+$CD3$^-$ IFN-γ$^+$ lymphocytes, but this was significantly higher in PBMC from HBV patients (0.9+/-0.1%; p = 0.01). These percentages were not higher when anti-CD3 and anti-CD28 mAb treatment was used and they remained significantly higher in HBV patients compared to controls (0.4+/-0.07% and 0.8+/-0.2%; p=0.03). After PMA/I treatment, the percentages of IFN-γ-expressing NK cells were increased to 2.1+/-0.2% and 2.6+/-0.3% in controls and HBV patients, respectively (Figure 4.2B).

These data indicate that NK cells from patients with HBV infection are not more likely to produce IFN-γ than those NK cells from control subjects but that the overall number of IFN-γ-expressing NK cells in PBMC are greater in HBV patients.

**4.2.2 IFN-γ-producing CD56$^{DIM}$ and CD56$^{BRIGHT}$ NK cells in HBV patients and healthy control subjects**

IFN-γ-production was also examined among the CD56$^{DIM}$ and CD56$^{BRIGHT}$ subsets of NK cells and the frequencies of such cells were calculated as a percentage of the CD56$^{DIM}$ and CD56$^{BRIGHT}$ subsets and as a percentage of total lymphocytes (Figure 4.1C).

In the absence of *ex vivo* stimulation, the mean frequencies of CD56$^{DIM}$ NK cells expressing IFN-γ were 4.4+/-0.3% and 8.3+/-1.3% of all CD56$^{DIM}$ NK cells in controls and HBV patients, respectively. As expected, these frequencies were not increased when PBMC were stimulated with anti-CD3 and anti-CD28 mAb (3.1+/-0.3% and 8.5+/-1.4% of CD56$^{DIM}$ NK cells). After PMA/I stimulation, 17.7+/-1.2% of CD56$^{DIM}$ NK cells from controls expressed IFN-γ and this was significantly higher in HBV patients (24+/-1.6%; p=0.006, Figure 4.2C). When calculated as a percentage of total lymphocytes, 0.4+/-0.04% and 0.9+/-0.1% of resting lymphocytes in controls and HBV patients, respectively, were CD56$^{DIM}$ NK cells expressing IFN-γ. This difference was significant (p=0.01). These percentages were not increased by anti-CD3 and anti-CD28 mAb treatment (0.4+/-0.07% and 0.7+/-0.1%). However, after PMA/I treatment, 2+/-0.2% and 2.4+/-0.3% of lymphocytes from controls and HBV patients, respectively, were IFN-γ-expressing CD56$^{DIM}$ NK cells (Figure 4.2D). The overall frequencies of stimulated lymphocytes that were IFN-γ-expressing CD56$^{DIM}$ NK cells were similar in HBV patients and controls.

In unstimulated PBMC, the mean frequencies of CD56$^{BRIGHT}$ NK cells expressing IFN-γ were 36.4+/-4.8% and 38.6+/-4.4% of all CD56$^{BRIGHT}$ NK cells in controls and HBV patients, respectively (Figure 4.2E). These frequencies were not increased when PBMC were stimulated with anti-CD3 and anti-CD28 mAb (6.8+/-1.2% and 14.9+/-1.5% of CD56$^{BRIGHT}$ NK cells, p=0.01). After PMA/I stimulation, the numbers of CD56$^{BRIGHT}$ NK cells shrunk considerably, due to the suspected downregulation of CD56 on the surface of the activated NK cells 22+/-4.1% of CD56$^{BRIGHT}$ NK cells from controls expressed IFN-γ and this was significantly higher in HBV patients (38.2+/-4.5%; p=0.02). When calculated as a percentage of total lymphocytes, 0.04+/-0.01% and 0.05+/-0.01% of resting lymphocytes in controls and HBV patients, respectively, were CD56$^{BRIGHT}$ NK cells expressing IFN-γ (Figure 4.2F). These percentages were not increased by anti-CD3 and anti-CD28 mAb treatment

(0.05+/-0.01% and 0.04+/-0.01%), however, after PMA/I treatment, 0.2+/-0.02% and 0.2+/-0.04% of lymphocytes from controls and HBV patients, respectively, were IFN-γ-expressing CD56$^{BRIGHT}$ NK cells. The overall frequencies of IFN-γ-expressing CD56$^{BRIGHT}$ NK cells were similar in both subject groups.

These data suggest that both CD56$^{DIM}$ and CD56$^{BRIGHT}$ NK cells from HBV patients are more likely to produce IFN-γ than those from healthy control subjects.

**Figure 4.1**

**Figure 4.2**

**4.2.3 The frequencies of IFN-γ-producing NT cells are higher in HBV patients than in healthy control subjects**

FITC-labelled anti-IFN-γ mAb was used in combination with PE-labelled anti-CD56 mAb and PE-Cy5-labelled anti-CD3 mAb to identify IFN-γ-producing NT cells. IFN-γ-producing NT cells were identified as the $CD56^+CD3^+$ cells (Figure 4.1B) which also stained positively for IFN-γ (Figure 4.1D). The frequencies of $IFN-γ^+$ NT cells were calculated as a percentage of the total NT cell population (Figure 4.3A) and as a percentage of total lymphocytes (Figure 4.3B).

In the absence of *ex vivo* stimulation, the mean frequencies of NT cells expressing IFN-γ were 2.8+/-0.6% and 2.1+/-0.3% of all NT cells in controls and HBV patients, respectively. These frequencies were increased when PBMC were stimulated with anti-CD3 and anti-CD28 mAb, (3.2+/-0.5% and 7.6+/-0.9% of NT cells; p<0.005) or with PMA/I (15.7+/-1.4% in controls and 45.2+/-4.2% of NT cells in HBV patients (Figure 4.3A, p<0.0001)). These data suggest that NT cells in chronic HBV patients respond more effectively to stimulation *in vitro* than their counterparts in healthy control subjects. When calculated as a percentage of total lymphocytes, the frequencies of IFN-γ-producing NT cells were less than 0.3% in all subjects. These percentages were slightly increased by anti-CD3 and anti-CD28 mAb treatment and again, the frequencies were significantly higher in HBV patients (0.1+/-0.02% and 0.4+/-0.05%; p<0.0001). Following PMA/I treatment, 0.5+/-0.05% of NT cells from controls expressed IFN-γ, and this was significantly higher in PBMC from HBV patients (2.2+/-0.19% of lymphocytes; Figure 4.3B, p<0.001).

These data indicate that NT cells from patients with HBV infection are more likely to produce IFN-γ in response to *ex vivo* stimulation and, therefore, along with NK cells, they make a significant contribution to the numbers of lymphocytes that produce IFN-γ.

**4.2.4 The frequencies of circulating IFN-γ-producing conventional T cells are higher in HBV patients than in healthy control subjects**

FITC-labelled anti-IFN-γ mAb was used in combination with PE-labelled anti-CD56 mAb and PE-Cy5-labelled anti-CD3 mAb to identify IFN-γ-producing classical T cells i.e. $CD56^-CD3^+$ lymphocytes (Figure 4.1B, 4.1D). The frequencies of T cells were

calculated as percentages of the total CD56⁻ T cell population (Figure 4.3C) and as a percentage of lymphocytes (Figure 4.3D).

As a percentage of the total T cell population, the means frequencies of IFN-γ-producing T cells were 1.1+/-0.1%, 3.7+/-0.53% and 1.7+/-0.2% in controls and 1.7+/-0.3%, 10.8+/-1.1%, and 2.2+/-0.3%, in HBV patients for unstimulated, PMA/I-stimulated and anti-CD3 and anti-CD28-stimulated PBMC, respectively (Figure 4.3C). The frequencies of IFN-γ-producing T cells were consistently higher in HBV patients, with statistical significance when PMA/I-stimulated PBMC were used (p<0.0001). As a percentage of total lymphocytes, the mean frequencies of IFN-γ-producing T cells were 0.5+/-0.1% 2.4+/-0.4% and 0.9+/-0.2% in controls and 0.6+/-0.1%, 4.8+/-0.4%, and 1.1+/-0.1%, in HBV patients for unstimulated, PMA/I-stimulated and anti-CD3 and anti-CD28-stimulated PBMC, respectively. Again, the frequencies of IFN-γ-producing T cells were consistently higher in HBV patients, with statistical significance when PMA/I-stimulated PBMC were used (Figure 4.3D, p<0.0001).

These data suggest that the occurrence of IFN-γ-producing T cells is higher in chronic HBV patients than in healthy control subjects, upon stimulation *in vitro*. Furthermore, as both T and NT cell populations showed higher levels of IFN-γ production, it appears that there is a general overproduction of IFN-γ associated with HBV infection.

### 4.2.5 The frequencies of circulating IFN-γ-producing total lymphocytes are higher in HBV patients than in healthy control subjects

FITC-labelled anti-IFN-γ mAb was used to identify IFN-γ-producing lymphocytes. In the absence of stimulation, the frequencies of IFN-γ-producing lymphocytes as a percentage of total lymphocytes ranged from 0.02% to 5.8% in control subjects and from 0.05% to 37.8% in HBV patients with mean frequencies of 1+/-0.2% and 4.8+/-1.6%, respectively (Figure 4.3E). After PMA/I stimulation, the frequencies of total IFN-γ-producing lymphocytes ranged from 0.47% to 34.2% in control subjects and from 2.4% to 32.3% in HBV patients (Figure 4.3E), with means of 10+/-1.5% and 12.9+/-1.3%, respectively. The frequencies of circulating IFN-γ-producing lymphocytes were significantly higher in chronic HBV patients than in healthy control subjects both in the absence and presence of stimulation (p=0.01, p=0.05).

These data show that all T and NK cell subsets show higher IFN-γ expression which contributes to an overall higher level of IFN-γ production by the total lymphocyte population in the peripheral blood of HBV patients.

**Figure 4.3**

## 4.3 IL-10 expression by stimulated NK, NT, T and total lymphocytes from HBV patients and healthy controls

Freshly isolated PBMC from 28 HBV-infected or 23 control subjects were incubated for 4 hours in medium alone or with PMA/I or plate-bound anti-CD3 mAb and anti-CD28 mAb, in the presence of brefeldin A. PE-labelled anti-IL-10 mAb was used in combination with FITC-labelled anti-CD56 mAb and PE-Cy5-labelled anti-CD3 mAb to identify IL-10-producing NK cells, $CD56^{DIM}$ and $CD56^{BRIGHT}$ subsets of NK cells, NT cells, conventional T cells and total lymphocytes (Figure 4.4A, B).

### 4.3.1 The frequencies of circulating IL-10-producing NK cells are slightly higher in HBV patients than in healthy control subjects

The frequencies of IL-10-producing NK cells were 0.5+/-0.2% and 1.4+/-0.4% of all NK cells in controls and HBV patients, respectively. These frequencies were not increased when PBMC were stimulated with anti-CD3 and anti-CD28 mAb (0.6+/-0.2% and 1.8+/-0.5% of NK cells). After PMA/I stimulation, 0.7+/-0.2% of NK cells from controls expressed IL-10, but this was significantly higher in PBMC from HBV patients (2+/-0.5%; p=0.04, Figure 4.5A). When calculated as a percentage of total lymphocytes, the frequencies of IL-10-producing NK cells were consistently higher in HBV patients but constituted less than 0.5% of lymphocytes in all subjects. *In vitro* stimulation did not have a significant impact on these frequencies (Figure 4.5B).

These data indicate that NK cells from HBV patients are more likely to produce IL-10 than their counterparts in healthy controls. However, their low occurrence suggests that they may have minimal impact on the regulation of inflammatory cytokine production in HBV infection.

### 4.3.2 The frequencies of IL-10-producing $CD56^{DIM}$ and $CD56^{BRIGHT}$ NK cells are higher in the peripheral blood of HBV patients, compared to healthy control subjects

IL-10-production was also examined among the $CD56^{DIM}$ and $CD56^{BRIGHT}$ subsets of NK cells.

In the absence of *ex vivo* stimulation, the mean frequencies of $CD56^{DIM}$ NK cells expressing IL-10 were 0.6+/-0.1% and 1.7+/-0.4% of all $CD56^{DIM}$ NK cells in controls and HBV patients, respectively. As expected, these frequencies were similar when

PBMC were stimulated with anti-CD3 and anti-CD28 mAb (0.7+/-0.1% and 1.9+/-0.5% of CD56$^{DIM}$ NK cells). After PMA/I stimulation, 1+/-0.2% of CD56$^{DIM}$ NK cells from controls expressed IL-10 but this was significantly higher in HBV patients (2.7+/-0.4%; p=0.002, Figure 4.5C). When calculated as a percentage of total lymphocytes, 0.04+/-0.02% and 0.09+/-0.02% of lymphocytes in controls and HBV patients, respectively, were CD56$^{DIM}$ NK cells expressing IL-10. These percentages were not significantly increased after anti-CD3 and anti-CD28 mAb treatment (0.04+/-0.02% and 0.1+/-0.02%) or PMA/I treatment (0.04+/-0.01% and 0.1+/-0.02%). However, PMA/I-stimulated CD56$^{DIM}$ NK cells from HBV patients elicited higher responses than their counterparts from control subjects (p<0.0001, Figure 4.5D).

In the absence of *ex vivo* stimulation, the mean frequencies of CD56$^{BRIGHT}$ NK cells expressing IL-10 were 0.9+/-0.3% and 2.5+/-0.5% of all CD56$^{BRIGHT}$ NK cells in controls and HBV patients, respectively. These frequencies were not increased when PBMC were stimulated with the T cell mitogen, anti-CD3 and anti-CD28 mAb (0.9+/-0.3% and 2.4+/-0.5% of CD56$^{BRIGHT}$ NK cells). However, after PMA/I stimulation, 2.4+/-0.7% of CD56$^{BRIGHT}$ NK cells from controls expressed IL-10 and this was significantly higher in HBV patients (3.8+/-0.7%; p=0.02, Figure 4.5E). When calculated as a proportion of total lymphocytes, frequencies of IL-10-expressing CD56$^{BRIGHT}$ NK cells were consistently higher in HBV patients but at a percentage of less than 0.4% of all lymphocytes. It is therefore unlikely that they significantly contributed to overall IL-10 production and modulation of inflammatory responses (Figure 4.5F).

These data indicate that both the IL-10-producing CD56$^{DIM}$ and CD56$^{BRIGHT}$ subsets of NK cells are higher in HBV patients but at such low frequencies, it is difficult to propose a possible role for such cells in immune responses against HBV.

**Figure 4.4**

**Figure 4.5**

### 4.3.3 The frequencies of circulating IL-10-producing NT cells are slightly higher in HBV patients than in healthy control subjects

In the absence of *ex vivo* stimulation, the mean frequencies of NT cells expressing IL-10 were 1.7+/-0.3% and 2.8+/-0.4% of all NT cells in controls and HBV patients, respectively. These frequencies were increased when PBMC were stimulated with anti-CD3 and anti-CD28 mAb, (2.4+/-0.4% and 3.2+/-0.5% of NT cells) or PMA/I (7.1+/-1.1% in controls and 11.9+/-2.1% of NT cells in HBV patients, Figure 4.6A). Although frequencies of IL-10-producing NT cells were slightly higher in chronic HBV patients than healthy control subjects, statistical testing revealed that such differences were insignificant. When calculated as a percentage of total lymphocytes, the frequencies of IL-10-producing NT cells were 0.1+/-0.03% and 0.1+/-0.04%; in controls and HBV patients, respectively. These percentages were increased by anti-CD3 and anti-CD28 mAb treatment (0.2+/-0.04% and 0.3+/-0.06%) and PMA/I treatment (0.2+/-0.04% and 0.7+/-0.1%, Figure 4.6B).

These data suggest that the frequencies of circulating IL-10-producing NT cells are similar in control subjects and HBV patients.

### 4.3.4 The frequencies of circulating IL-10-producing T cells are similar in HBV patients and in healthy control subjects

In the absence of *ex vivo* stimulation, the mean frequencies of T cells expressing IL-10 were 0.6+/-0.3% and 0.3+/-0.04% of all T cells in controls and HBV patients, respectively. These frequencies were increased when PBMC were stimulated with anti-CD3 and anti-CD28 mAb, (1.5+/-0.6% and 0.6+/-0.1% of T cells) or PMA/I (0.8+/-0.1% and 0.9+/-0.1%, Figure 4.6C). When calculated as a percentage of total lymphocytes, the frequencies of IL-10-producing T cells were 0.4+/-0.1% and 0.2+/-0.01% in controls and HBV patients, respectively. These percentages were increased by anti-CD3 and anti-CD28 mAb treatment (0.9+/-0.3% and 0.3+/-0.04%) and PMA/I treatment (0.4+/-0.04% and 0.4+/-0.04%, Figure 4.6D).

These data suggest that the frequencies of circulating IL-10-producing T cells are similar in control subjects and HBV patients and thus, play no significant role in immune responses against HBV.

**4.3.5 There are no significant differences in the frequencies of circulating IL-10-producing lymphocytes between HBV patients and healthy control subjects**

FITC-labelled anti-IL-10 mAb was used to identify all IL-10-producing lymphocytes. In the absence of stimulation, the frequencies of IL-10-producing lymphocytes as a percentage of total lymphocytes ranged from 0.03% to 2.7% in control subjects and from 0% to 7% in HBV patients, with means of 0.58+/-0.14% and 1.5+/-0.37%, respectively (Figure 4.6E, p=0.16). In the presence of PMA/I stimulation, the frequencies of IL-10-producing lymphocytes as a percentage of total lymphocytes ranged from 0.13% to 9% in control subjects and from 0.01% to 12.1% in HBV patients, with means of 2.3+/-0.5% and 3.1+/-0.5%, respectively (Figure 4.6E, p=0.4).

These data suggest that, despite the presence of higher frequencies of circulating IL-10-producing NK cells and slightly higher frequencies of circulating IL-10-producing NT cells, the overall number of circulating IL-10-producing lymphocytes is similar in chronic HBV patients and healthy control subjects.

Figure 4.6

**4.4 IL-13 expression by stimulated NK, NT, T and total lymphocytes from HBV patients and healthy controls**

Freshly isolated PBMC from HBV-infected or control subjects were incubated for 4 hours in medium alone or with PMA/I or plate-bound anti-CD3 mAb and anti-CD28 mAb, in the presence of brefeldin A. PE-labelled anti-IL-13 mAb was used in combination with FITC-labelled anti-CD56 mAb and PE-Cy5-labelled anti-CD3 mAb to identify IL-13-producing NK cells, NT cells, conventional T cells and total lymphocytes (Figure 4.7A, B).

**4.4.1 Frequencies of circulating IL-13-producing NK cells are low in both control subjects and HBV patients but, responses to *in vitro* stimulation are significantly reduced in HBV infection**

In the absence of *ex vivo* stimulation, the mean frequencies of NK cells expressing IL-13 were 2.7+/-0.3% and 2+/-0.2% of all NK cells in controls and HBV patients, respectively. These frequencies were not increased when PBMC were stimulated with anti-CD3 and anti-CD28 mAb (3.9+/-1% and 2.4+/-0.5% of NK cells). However, after PMA/I stimulation, 10.4+/-1.1% of NK cells from controls expressed IL-13, but this was significantly lower in PBMC from HBV patients (5+/-0.8%; p<0.05, Figure 4.8A). When calculated as a percentage of total lymphocytes, the mean frequencies of IL-13-producing NK cells were 0.2+/-0.1% and 0.2+/-0.04% in controls and HBV patients and 0.3+/-0.1% and 0.2+/-0.1% following stimulation with anti-CD3 and anti-CD28 mAb. PMA/I stimulation increased percentages of IL-13-producing NK cells to 0.9+/-0.04% in controls and 0.6+/-0.1% in HBV patients (Figure 4.8B).

These data indicate that NK cells from HBV patient are less likely to produce IL-13 than their counterparts in healthy controls.

**Figure 4.7**

**Figure 4.8**

**4.4.2 Frequencies of circulating IL-13-producing NT cells are low in both control subjects and HBV patients and, there are no significant differences between the two groups**

In the absence of *ex vivo* stimulation, the mean frequencies of NT cells expressing IL-13 were 1.8+/-0.4% and 1.8+/-0.3% of all NT cells in controls and HBV patients, respectively. These frequencies were increased when PBMC were stimulated with anti-CD3 and anti-CD28 mAb (7.9+/-1.6% and 6.6+/-0.7% of NT cells) or PMA/I (12.2+/-1.9% and 12.1+/-2.4% of NT cells, Figure 4.9A). When calculated as a percentage of total lymphocytes, the mean frequencies of IL-13-producing NT cells were 0.2+/-0.03% and 0.3+/-0.1% in controls and HBV patients and 0.3+/-0.1% and 0.4+/-0.1% following stimulation with anti-CD3 and anti-CD28 mAb. PMA/I stimulation increased percentages of IL-13-producing NT cells to 0.4+/-0.1% in controls and 0.7+/-0.2% in HBV patients (Figure 4.9B).

These data show that the numbers of circulating IL-13–producing NT cells are unchanged in HBV infection.

**4.4.3 Frequencies of circulating IL-13-producing T cells are higher in HBV infection**

In the absence of *ex vivo* stimulation, the mean frequencies of T cells expressing IL-13 were 0.5+/-0.1% and 0.8+/-0.1% of all T cells in controls and HBV patients, respectively (p<0.05). These frequencies were increased when PBMC were stimulated with anti-CD3 and anti-CD28 mAb (1.4+/-0.7% and 1.4+/-0.5% of T cells) or PMA/I (1.1+/-0.3% in controls and 3.6+/-0.9% in HBV patients, p=0.002, Figure 4.9C). When calculated as a percentage of total lymphocytes, the mean frequencies of IL-13-producing T cells were 0.4+/-0.1% and 0.8+/-0.2% in controls and HBV patients (p=0.0005), and 1.1+/-0.7% and 1.4+/-0.7% following stimulation with anti-CD3 and anti-CD28 mAb. PMA/I stimulation increased percentages of IL-13-producing T cells to 0.8+/-0.3% in controls and this increase was significantly enhanced in HBV patients (3.2+/-1.3%; p=0.0008, Figure 4.9D).

These data show that the frequencies of IL-13-producing T cells are significantly higher in HBV infection.

**4.4.4 The frequencies of circulating IL-13-producing lymphocytes are higher in HBV patients than in healthy control subjects**

In PBMC incubated in medium alone, the frequencies of IL-13-producing lymphocytes as a percentage of total lymphocytes ranged from 0.06% to 1.1% in control subjects and from 0.43% to 2.3% in HBV patients, with means of 0.41+/-0.09% and 1.3+/-0.18%, respectively (Figure 4.9E, p=0.004). After PMA/I stimulation, the frequencies of IL-13-producing lymphocytes as a percentage of total lymphocytes ranged from 0.02% to 2.4% in control subjects and from 1.2% to 7.5% in HBV patients, with means of 0.86+/-0.22% and 2.6+/-0.5%, respectively (Figure 4.9E, p=0.01).

These data suggest that higher frequencies of circulating IL-13-producing T cells contribute to an overall higher number of circulating IL-13-producing lymphocytes in chronic HBV infection.

**Figure 4.9**

**4.5 IL-17 expression by stimulated NK, NT, T and total lymphocytes from HBV patients and healthy controls**

Freshly isolated PBMC from HBV-infected or control subjects were incubated for 4 hours in medium alone or with PMA/I or plate-bound anti-CD3 mAb and anti-CD28 mAb, in the presence of brefeldin A. PE-labelled anti-IL-17 mAb was used in combination with FITC-labelled anti-CD56 mAb and PE-Cy5-labelled anti-CD3 mAb to identify IL-17-producing NK cells, NT cells, conventional T cells and total lymphocytes (Figure 4.10A, B).

**4.5.1 There are no significant differences in the frequencies of circulating IL-17-producing NK cells between HBV patients and healthy control subjects**

In the absence of *ex vivo* stimulation, the mean frequencies of NK cells expressing IL-17 were 2.6+/-1% and 1.9+/-0.4% of all NK cells in controls and HBV patients, respectively. These frequencies were not increased anti-CD3 and anti-CD28 mAb (3.4+/-1% and 2.8+/-1.1% of NK cells). However, after PMA/I stimulation, 8.8+/-2% of NK cells from controls expressed IL-17, and this was similar in PBMC from HBV patients (8.7+/-3.2%, Figure 4.11A). When calculated as a percentage of total lymphocytes, the mean frequencies of IL-17-producing NK cells were 0.3+/-0.1% and 0.2+/-0.04% in controls and HBV patients and 0.3+/-0.1% and 0.3+/-0.1% following stimulation with anti-CD3 and anti-CD28 mAb. PMA/I stimulation increased percentages of IL-17-producing NK cells to 1+/-0.2% in controls and 0.9+/-0.3% in HBV patients (Figure 4.11B).

These data show that there are no significant differences in the frequencies of circulating IL-17-producing NK cells between HBV patients and healthy control subjects.

**Figure 4.10**

**Figure 4.11**

**4.5.2 There are no significant differences in the frequencies of circulating IL-17-producing NT cells between HBV patients and healthy control subjects**

In the absence of *ex vivo* stimulation, the mean frequencies of NT cells expressing IL-17 were 2.3+/-0.5% and 1.9+/-0.4% of all NT cells in controls and HBV patients, respectively. These frequencies were increased when stimulated with anti-CD3 and anti-CD28 mAb (11.1+/-3.5% and 10.7+/-3.6%) or with PMA/I (12.4+/-1.9% and 16.1+/-6.7%, Figure 4.12A). When calculated as a percentage of total lymphocytes, the mean frequencies of IL-17-producing NT cells were 0.3+/-0.1% and 0.3+/-0.1% in controls and HBV patients and 0.4+/-0.1% and 0.5+/-0.2% following stimulation with anti-CD3 and anti-CD28 mAb. PMA/I stimulation increased percentages of IL-17-producing NT cells to 0.4+/-0.1% in controls and 0.9+/-0.4% in HBV patients (Figure 4.12B).

These data show that the frequencies of circulating IL-17-producing NT cells are similar in HBV patients and healthy control subjects.

**4.5.3 Enhanced responses of circulating IL-17-producing T cells to *in vitro* stimulation in HBV infection**

In the absence of *ex vivo* stimulation, the mean frequencies of T cells expressing IL-17 were 0.4+/-0.1% and 0.7+/-0.1% of all T cells in controls and HBV patients, respectively. These frequencies were increased when PBMC were stimulated with, anti-CD3 and anti-CD28 mAb (1.5+/-0.3% and 1.6+/-0.3% of T cells) or with PMA/I (1.5+/-0.3% and 3.5+/-0.5% of T cells; p=0.01, Figure 4.12C). When calculated as a percentage of total lymphocytes, the mean frequencies of IL-17-producing T cells were 0.4+/-0.1% and 0.7+/-0.1% in controls and HBV patients, and 0.5+/-0.2% and 1.1+/-0.3% following stimulation with anti-CD3 and anti-CD28 mAb. PMA/I stimulation increased percentages of IL-17-producing T cells to 0.6+/-0.1% in controls and 1.4+/-0.3% in HBV patients (Figure 4.12D).

These data show that there are no significant differences in the frequencies of circulating IL-17-producing T cells between HBV patients and healthy control subjects but, as a proportion of CD56⁻ T cells, IL-17-producing T cells respond superiorly in HBV infection.

### 4.5.4 The frequencies of circulating IL-17-producing lymphocytes are similar in HBV patients and healthy control subjects

In PBMC incubated in medium alone, the frequencies of IL-17-producing lymphocytes as a percentage of total lymphocytes ranged from 0.02% to 1.6% in control subjects and from 0.09% to 2.3% in HBV patients, with means of 0.48+/-0.11% and 0.7+/-0.21%, respectively (Figure 4.12E, p=0.9). After PMA/I stimulation, the frequencies of IL-17-producing lymphocytes as a percentage of total lymphocytes ranged from 0.26% to 2.5% in control subjects and from 0.26% to 5.5% in HBV patients, with means of 1.15+/-0.17% and 1.3+/-0.37%, respectively (Figure 4.12E, p=0.6).

These data suggest that the frequencies of IL-17-producing lymphocytes are not significantly different in HBV infection.

**Figure 4.12**

**4.6 Cytokine RNA levels in whole PBMC isolated from HBV patients and healthy controls**

**4.6.1 Significantly higher levels of IFN-γ RNA in PBMC isolated from HBV patients, compared with those taken from healthy control subjects**

Quantitative RT-PCR for IFN-γ was performed on cDNA from the PBMC of 10 HBV patients and 10 healthy control subjects. The duplicate Ct values for both IFN-γ and β-actin were very close (<1.5 cycles apart) which suggests that minimal pipetting error occurred. The Ct Values for β-actin ranged from 16.8 to 19.8 in control subjects and from 17.5 to 18.9 in HBV patients. These values were considered to be within or close to range, suggesting that the cDNA was of acceptable quality and quantity (Figure 4.13A). The slope of the standard curves for both β-actin and IFN-γ was within range, with a slope of -3.4 and -3.2, respectively (Figure 4.13B). This confirms that the standard curves were created successfully. Finally the standard curve axes were switched and the new equation of the line was used to calculate the copy numbers (CN) for each sample by substituting the Ct value for x (Figure 4.13C). For example, after switching the axes, the equation of the line for the IFN-γ standard curve became $y = -0.3101x + 12.606$. By substituting a Ct value of 27.08 for x and solving for y ($y = -0.3101*(27.08) + 12.606$), a copy number of 3.99 was computed. The corrected copy number (CCN) was then calculated by normalisation against the corresponding copy number for β-actin. The corrected copy numbers of IFN-γ ranged from $10^{2.3}$ to $10^{4.5}$ in healthy control subjects with a mean copy number of $10^{3.3}$ +/- $10^{0.02}$ and from $10^{3.6}$ to $10^{4.4}$ in HBV patients with a mean copy numbers of $10^{4}$ +/- $10^{0.07}$ (Figure 4.13D, p=0.008).

These data support the results from the cytokine production assay and show that in addition to higher IFN-γ protein levels, the copy number of IFN-γ RNA is significantly higher in PBMC isolated from persons with HBV infection. This also indicates that the cytokine-mediated antiviral responses to HBV infection are regulated at the genetic level and that certain HBV proteins may interact with transcription factors that regulate IFN-γ expression.

**Figure 4.13**

## 4.6.2 Similar levels of IL-10 RNA in PBMC isolated from HBV patients and healthy control subjects

QRT-PCR for IL-10 was performed on cDNA from the PBMC of 10 HBV patients and 10 healthy control subjects. The duplicate Ct values for both IL-10 and β-actin were very close which suggests that the occurrence of pipetting error was minimal. The Ct Values for β-actin ranged from 17.5 to 18.7 in control subjects and from 16.7 to 18.7 in HBV patients. These values were considered to be within or close to range, suggesting that the cDNA was of acceptable quality and quantity (Figure 4.14A). The slope of the standard curves for both β-actin and IL-10 was within range, with a slope of -3.4 and -3.2, respectively (Figure 4.14B). This confirms that the standard curves were created successfully. Finally the standard curve axes were switched and the new equation of the line was used to calculate the copy numbers for each sample by substituting the Ct value for x (Figure 4.14C). The corrected copy numbers of IL-10  ranged from $10^{1.9}$ to $10^{3.4}$ in healthy control subjects with a mean copy number of $10^{2.5}$ + / - $10^{0.21}$ and, from $10^{1.6}$ to $10^{3.2}$ in HBV patients with a mean copy numbers of $10^{2.6}$ + / - $10^{0.21}$ (Figure 4.14D, p=0.8).

These data show that the copy number of IL-10 RNA is similar in PBMC isolated from HBV patients and healthy control subjects. This is in agreement with what was observed in the total lymphocyte population. This indicates that there is not a strong anti-inflammatory response at play in such subjects and that the higher frequencies of IL-10-producing innate lymphocytes may be a control measure to prevent uncontrollable IFN-γ production and immune-mediated damage

**Figure 4.14**

## 4.6.3 Undetectable levels of IL-17A RNA in PBMC isolated from HBV patients and healthy control subjects

QRT-PCR for IL-17A was carried out on cDNA from the PBMC of 8 HBV patients and 9 healthy control subjects. The duplicate Ct values for $\beta$-actin were very close which suggests that minimal pipetting error occurred. The Ct Values for $\beta$-actin ranged from 17.7 to 18.9 in control subjects and from 16.7 to 18.7 in HBV patients. These values were considered to be within or close to range, suggesting that the cDNA was of acceptable quality and quantity (Figure 4.15A). The Ct values for IL-17A were undetermined suggesting that IL-17A copy numbers in PBMC of HBV patients and healthy control subjects are too low to be detected by qRT-PCR. The slopes of the standard curves for $\beta$-actin and IL-17A were within range, with a slope of -3.5 and -3.2, respectively (Figure 4.15B). This confirms that the standard curves were created successfully and more importantly, that IL-17A primers and probes worked. The standard curve axes were not switched because the corrected copy numbers of IL-17A could not be calculated due to undetermined Ct values. All of the experimental controls used suggest that this is due to very low levels of IL-17A RNA.

These data suggest that the copy number of IL-17A RNA is too low to be detected in PBMC isolated from HBV patients and healthy control subjects. It is possible that IL-17A RNA may only be detected in subjects where its production is significantly augmented and a pro-inflammatory response prevails.

**Figure 4.15**

**4.7 IFN-γ-producing lymphocytes in HBV patient groups**

**4.7.1 The frequencies of circulating IFN-γ-producing NK cells do not correlate with viral load, disease severity, gender or age.**

The frequencies of circulating IFN-γ-producing NK cells were calculated following incubation in medium alone or medium containing with PMA/I or anti-CD3 mAb and anti-CD28 mAb. The frequencies of IFN-γ-producing NK cells were quantified as a percentage of total NK cells (Figure 4.16A-D (left)) and as a percentage of lymphocytes (Figure 4.16A-D (right)), and compared between 19 - 25 HBV patients within the study cohort, based on clinical parameters. All data are shown in Tables 4.1 and 4.2. A Mann-Whitney test was carried out to ascertain whether there were differences in the frequencies of IFN-γ-producing NK cells between HBV-infected individuals with low viral load (less than 100,000 copies / ml) and high viral load (100,000 – $3.2x10^8$ copies / ml), and between subjects with low ALT (less than 40 IU/ml) and high ALT (greater than 40 IU/ml). A Mann-Whitney test was also used to determine any differences based on gender and age. All p-values are shown in Tables 4.1 and 4.2. No statistical differences were found. Furthermore, spearman correlation testing revealed that there were no correlations between frequencies of IFN-γ-producing NK cells and disease severity, gender or age. All r-values are shown in Tables 4.1 and 4.2.

All data shown suggest that the frequencies of circulating IFN-γ-producing NK cells do not correlate with viral load, disease severity, gender or age.

**Table 4.1**

**The frequencies of circulating IFN-γ-producing NK cells in HBV patient subsets:**
The frequencies of IFN-γ-producing NK cells (shown as a percentage of total NK cells)
within divisions of the HBV patient study cohort, based on varying ALT, viral load, age
and gender.

| | Condition | ALT <40 | ALT >40 | Low Viral Load | High Viral Load | Age <35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|---|
| **Mean%** | **Medium** | 8.4 | 4.5 | 7.3 | 6.5 | 7.2 | 6.7 | 5.6 | 8.9 |
| | **PMA/I** | 22.4 | 19.1 | 20.9 | 22.5 | 24.3 | 17.4 | 18.4 | 25.5 |
| | **anti-CD3** | 4.5 | 1.8 | 4.1 | 3.1 | 3.9 | 3.6 | 3.7 | 3.9 |
| | | | | | | | | | |
| **SEM %** | **Medium** | 1.3 | 1.2 | 1.4 | 1.1 | 1.3 | 1.4 | 1 | 1.5 |
| | **PMA/I** | 3.1 | 2.4 | 2.9 | 3 | 3 | 2.5 | 2.5 | 3.2 |
| | **anti-CD3** | 0.6 | 0.4 | 0.6 | 0.8 | 0.7 | 0.5 | 0.6 | 0.7 |
| | | | | | | | | | |
| **N*** | **Medium** | 18 | 7 | 17 | 8 | 15 | 10 | 14 | 11 |
| | **PMA/I** | 13 | 6 | 13 | 6 | 11 | 8 | 11 | 8 |
| | **anti-CD3** | 15 | 5 | 15 | 5 | 13 | 7 | 10 | 10 |
| | | | | | | | | | |
| **p*** | **Medium** | | 0.2 | | 1 | | 0.9 | | 0.2 |
| | **PMA/I** | | 0.7 | | 0.8 | | 0.1 | | 0.2 |
| | **anti-CD3** | | 0.1 | | 0.4 | | 0.9 | | 0.9 |
| | | | | | | | | | |
| **r*** | **Medium** | | -0.4 | | -0.1 | | 0.007 | | - |
| | **PMA/I** | | -0.3 | | -0.3 | | -0.4 | | - |
| | **anti-CD3** | | -0.5 | | 0.04 | | -0.1 | | - |

**\* Condition** refers to the 4 hour incubation of the cells in medium alone or, in medium
treated with PMA/I, prior to staining for flow cytometry. **Low viral load** is below
10,000 copies / ml, **high viral load** ranges from 100,000 to $3.2 \times 10^8$ copies / ml. **N*** is
the number of subjects. The p-value **p*** is a product of the Mann-Whitney U-test and,
indicates statistical significance when it falls below 0.05. The correlation coefficient **r***
is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a
linear correlation between two arrays of numbers. The columns with headers **M*** and **F***
comprise the male and female subjects, respectively.

**Table 4.2**

**The frequencies of circulating IFN-γ-producing NK cells in HBV patient subsets:**
The frequencies of IFN-γ-producing NK cells (shown as a percentage of lymphocytes) within divisions of the HBV patient study cohort, based on varying ALT, viral load, age and gender.

| | Condition | ALT <40 | ALT >40 | Low Viral Load | High Viral Load | Age <35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|---|
| **Mean%** | **Medium** | 1.1 | 0.6 | 0.9 | 1.1 | 1.1 | 0.7 | 0.9 | 0.9 |
| | **PMA** | 3.3 | 1.6 | 3 | 2.4 | 3.2 | 2.3 | 2.4 | 3.4 |
| | **anti-CD3** | 1.1 | 0.6 | 0.9 | 0.9 | 0.9 | 0.8 | 0.7 | 1.1 |
| **SEM %** | **Medium** | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.1 | 0.2 | 0.2 |
| | **PMA** | 0.5 | 0.3 | 0.5 | 0.5 | 0.5 | 0.4 | 0.5 | 0.4 |
| | **anti-CD3** | 0.3 | 0.05 | 0.2 | 0.2 | 0.3 | 0.1 | 0.1 | 0.3 |
| **N*** | **Medium** | 18 | 7 | 17 | 8 | 15 | 10 | 14 | 11 |
| | **PMA** | 13 | 6 | 13 | 6 | 11 | 8 | 11 | 8 |
| | **anti-CD3** | 15 | 5 | 15 | 5 | 13 | 7 | 10 | 10 |
| **p*** | **Medium** | | 0.1 | | 0.9 | | 0.8 | | 0.6 |
| | **PMA** | | 0.1 | | 0.5 | | 0.3 | | 0.1 |
| | **anti-CD3** | | 0.9 | | 0.5 | | 0.4 | | 1 |
| **r*** | **Medium** | | -0.3 | | 0.2 | | -0.1 | | - |
| | **PMA** | | -0.3 | | -0.1 | | -0.4 | | - |
| | **anti-CD3** | | -0.3 | | -0.04 | | 0.01 | | - |

* **Condition** refers to the 4 hour incubation of the cells in medium alone or, in medium treated with PMA/I, prior to staining for flow cytometry. **Low viral load** is below 10,000 copies / ml, **high viral load** ranges from 100,000 to $3.2 \times 10^8$ copies / ml. **N*** is the number of subjects. The p-value **p*** is a product of the Mann-Whitney U-test and, indicates statistical significance when it falls below 0.05. The correlation coefficient **r*** is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a linear correlation between two arrays of numbers. The columns with headers **M*** and **F*** comprise the male and female subjects, respectively.

**Figure 4.16**

**4.7.2 The frequencies of circulating IFN-γ-producing NT cells do not correlate with viral load, disease severity, gender or age.**

The frequencies of circulating IFN-γ-producing NT cells were calculated following incubation in medium alone or medium containing with PMA/I or anti-CD3 mAb and anti-CD28 mAb. The frequencies of IFN-γ-producing NT cells were quantified as a percentage of total NT cells (Figure 4.17A-D (left)) and as a percentage of lymphocytes (Figure 4.17A-D (right)), and compared between 17 - 21 HBV patients within the study cohort, based on clinical parameters. All data are shown in Tables 4.3 and 4.4. A Mann-Whitney test was carried out to ascertain whether there were differences in the frequencies of IFN-γ-producing NT cells between HBV-infected individuals with low viral load (less than 100,000 copies / ml) and high viral load (100,000 – $3.2\text{x}10^8$ copies / ml), and between subjects with low ALT (less than 40 IU/ml) and high ALT (greater than 40 IU/ml). A Mann-Whitney test was also used to determine differences based on gender and age. All p-values are shown in Tables 4.3 and 4.4. No statistical differences were found. Furthermore, Spearman correlation testing revealed that there were no correlations between frequencies of IFN-γ-producing NT cells and disease severity, gender or age. All r-values are shown in Tables 4.3 and 4.4.

All data shown suggest that the frequencies of circulating IFN-γ-producing NT cells do not correlate with viral load, disease severity, gender or age.

**Table 4.3**

**The frequencies of circulating IFN-γ-producing NT cells in HBV patient subsets:**
The frequencies of IFN-γ-producing NT cells (shown as a percentage of total NT cells) within divisions of the HBV patient study cohort, based on varying ALT, viral load, age and gender.

| | Condition | ALT <40 | ALT >40 | Low Viral Load | High Viral Load | Age <35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|---|
| **Mean%** | **Medium** | 2 | 1.7 | 1.9 | 1.8 | 1.6 | 2.3 | 1.7 | 2.1 |
| | **PMA** | 41.1 | 37.4 | 42.3 | 36.9 | 39.4 | 40.6 | 35.4 | 46.1 |
| | **anti-CD3** | 7.1 | 8.5 | 7.7 | 7.3 | 8.6 | 5.9 | 6.2 | 9.7 |
| | | | | | | | | | |
| **SEM %** | **Medium** | 0.4 | 0.2 | 0.4 | 0. | 0.3 | 0.5 | 0.4 | 0.3 |
| | **PMA** | 5.6 | 2.1 | 4.9 | 3.5 | 5.4 | 4 | 4.2 | 5.3 |
| | **anti-CD3** | 1.4 | 1 | 1.3 | 1.2 | 1.4 | 0.8 | 1.2 | 1.3 |
| | | | | | | | | | |
| **N*** | **Medium** | 15 | 6 | 14 | 7 | 12 | 9 | 13 | 8 |
| | **PMA** | 13 | 6 | 15 | 6 | 10 | 8 | 11 | 8 |
| | **anti-CD3** | 11 | 6 | 12 | 5 | 11 | 6 | 10 | 7 |
| | | | | | | | | | |
| **p*** | **Medium** | | 0.9 | | 0.9 | | 0.5 | | 0.4 |
| | **PMA** | | 0.7 | | 0.7 | | 0.7 | | 0.3 |
| | **anti-CD3** | | 0.5 | | 1 | | 0.3 | | 0.2 |
| | | | | | | | | | |
| **r*** | **Medium** | | -0.1 | | 0.1 | | 0.3 | | - |
| | **PMA** | | 0.0004 | | -0.01 | | 0.1 | | - |
| | **anti-CD3** | | 0.2 | | 0.2 | | -0.1 | | - |

* **Condition** refers to the 4 hour incubation of the cells in medium alone or, in medium treated with PMA/I, prior to staining for flow cytometry. **Low viral load** is below 10,000 copies / ml, **high viral load** ranges from 100,000 to $3.2 \times 10^8$ copies / ml. **N*** is the number of subjects. The p-value **p*** is a product of the Mann-Whitney U-test and, indicates statistical significance when it falls below 0.05. The correlation coefficient **r*** is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a linear correlation between two arrays of numbers. The columns with headers **M*** and **F*** comprise the male and female subjects, respectively.

**Table 4.4**

**The frequencies of circulating IFN-γ-producing NT cells in HBV patient subsets:**
The frequencies of IFN-γ-producing NT cells (shown as a percentage of lymphocytes)
within divisions of the HBV patient study cohort, based on varying ALT, viral load, age
and gender.

|  | Condition | ALT <40 | ALT >40 | Low Viral Load | High Viral Load | Age <35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|---|
| **Mean%** | **Medium** | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.08 | 0.1 |
|  | **PMA** | 2 | 1.8 | 2 | 1.8 | 2 | 1.9 | 1.8 | 2.2 |
|  | **anti-CD3** | 0.4 | 0.5 | 0.4 | 0.4 | 0.4 | 0.3 | 0.3 | 0.6 |
|  |  |  |  |  |  |  |  |  |  |
| **SEM %** | **Medium** | 0.02 | 0.01 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
|  | **PMA** | 0.3 | 0.1 | 0.3 | 0.2 | 0.2 | 0.3 | 0.2 | 0.2 |
|  | **anti-CD3** | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.04 | 0.04 | 0.1 |
|  |  |  |  |  |  |  |  |  |  |
| **N\*** | **Medium** | 15 | 6 | 14 | 7 | 12 | 9 | 13 | 8 |
|  | **PMA** | 13 | 6 | 15 | 6 | 10 | 8 | 11 | 8 |
|  | **anti-CD3** | 11 | 6 | 12 | 5 | 11 | 6 | 10 | 7 |
|  |  |  |  |  |  |  |  |  |  |
| **p\*** | **Medium** |  | 0.7 |  | 0.9 |  | 0.8 |  | 0.2 |
|  | **PMA** |  | 0.5 |  | 0.6 |  | 1 |  | 0.5 |
|  | **anti-CD3** |  | 0.3 |  | 0.9 |  | 0.5 |  | 0.07 |
|  |  |  |  |  |  |  |  |  |  |
| **r\*** | **Medium** |  | -0.1 |  | 0.1 |  | 0.2 |  | - |
|  | **PMA** |  | 0.02 |  | 0.01 |  | 0.1 |  | - |
|  | **anti-CD3** |  | 0.3 |  | 0.2 |  | -0.1 |  | - |

**\* Condition** refers to the 4 hour incubation of the cells in medium alone or, in medium
treated with PMA/I, prior to staining for flow cytometry. **Low viral load** is below
10,000 copies / ml, **high viral load** ranges from 100,000 to $3.2 \times 10^8$ copies / ml. **N\*** is
the number of subjects. The p-value **p\*** is a product of the Mann-Whitney U-test and,
indicates statistical significance when it falls below 0.05. The correlation coefficient **r\***
is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a
linear correlation between two arrays of numbers. The columns with headers **M\*** and **F\***
comprise the male and female subjects, respectively.

**Figure 4.17**

**4.7.3 The frequencies of circulating IFN-γ-producing T cells do not correlate with viral load, disease severity, gender or age.**

The frequencies of circulating IFN-γ-producing T cells were calculated following incubation in medium alone or medium containing with PMA/I or anti-CD3 mAb and anti-CD28 mAb. The frequencies of IFN-γ-producing T cells were quantified as a percentage of total T cells (Figure 4.18 A-D (left)) and as a percentage of lymphocytes (Figure 4.18 A-D (right)), and compared between 17 - 25 HBV patients within the study cohort, based on clinical parameters. All data are shown in Tables 4.5 and 4.6. A Mann-Whitney test was carried out to ascertain whether there were differences in the frequencies of IFN-γ-producing T cells between HBV-infected individuals with low viral load (less than 100,000 copies / ml) and high viral load (100,000 – 3.2x10$^8$ copies / ml), and between subjects with low ALT (less than 40 IU/ml) and high ALT (greater than 40 IU/ml). A Mann-Whitney test was also used to determine an differences based on gender and age. All p-values are shown in Tables 4.5 and 4.6. As a proportion of T cells, frequencies of IFN-γ-producing T cells isolated from HBV patients with high ALT and high viral load appear to be significantly more responsive to stimulation with anti-CD3 mAb and anti-CD28 mAb (p=0.04 and p=0.03, respectively). However, spearman correlation testing did not indicate any significant correlations between increasing viral load, disease severity and numbers of IFN-γ-producing T cells. Furthermore, as a proportion of total lymphocytes, these statistical differences were not observed. All other p and r-values are shown in Tables 4.5 and 4.6, and suggest that there are no differences in the frequencies of IFN-γ-producing T cells between HBV patient groups.

Overall, these data suggest that the frequencies of circulating IFN-γ-producing T cells do not correlate with viral load, disease severity, gender or age.

## Table 4.5

**The frequencies of circulating IFN-γ-producing T cells in HBV patient subsets:** The frequencies of IFN-γ-producing T cells (shown as a percentage of total T cells) within divisions of the HBV patient study cohort, based on varying ALT, viral load, age and gender.

| | Condition | ALT <40 | ALT >40 | Low Viral Load | High Viral Load | Age <35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|---|
| **Mean%** | **Medium** | 1.4 | 2.2 | 1.7 | 1.4 | 1.7 | 1.4 | 1.2 | 2.1 |
| | **PMA** | 9.6 | 15.8 | 11.7 | 11.2 | 10.3 | 13.2 | 13.5 | 8.8 |
| | **anti-CD3** | 1.6 | 2.9 | 1.7 | 3 | 2.1 | 2 | 2.6 | 1.5 |
| **SEM %** | **Medium** | 0.4 | 0.5 | 0.5 | 0.3 | 0.5 | 0.3 | 0.3 | 0.5 |
| | **PMA** | 0.6 | 2.1 | 1.6 | 1.1 | 1.2 | 1.7 | 1.6 | 0.7 |
| | **anti-CD3** | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 |
| **N*** | **Medium** | 17 | 8 | 17 | 8 | 15 | 10 | 14 | 11 |
| | **PMA** | 13 | 6 | 13 | 6 | 11 | 8 | 11 | 8 |
| | **anti-CD3** | 11 | 6 | 12 | 5 | 11 | 6 | 9 | 8 |
| **p*** | **Medium** | | 0.4 | | 1 | | 0.9 | | 0.4 |
| | **PMA** | | 0.2 | | 0.9 | | 0.3 | | 0.1 |
| | **anti-CD3** | | 0.04 | | 0.03 | | 0.9 | | 0.07 |
| **r*** | **Medium** | | 0.5 | | 0.01 | | 0.1 | | - |
| | **PMA** | | 0.1 | | 0.2 | | 0.1 | | - |
| | **anti-CD3** | | 0.3 | | 0.4 | | 0.03 | | - |

**\* Condition** refers to the 4 hour incubation of the cells in medium alone or, in medium treated with PMA/I, prior to staining for flow cytometry. **Low viral load** is below 10,000 copies / ml, **high viral load** ranges from 100,000 to $3.2 \times 10^8$ copies / ml. **N*** is the number of subjects. The p-value **p*** is a product of the Mann-Whitney U-test and, indicates statistical significance when it falls below 0.05. The correlation coefficient **r*** is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a linear correlation between two arrays of numbers. The columns with headers **M*** and **F*** comprise the male and female subjects, respectively.

**Table 4.6**

**The frequencies of circulating IFN-γ-producing T cells in HBV patient subsets:** The frequencies of IFN-γ-producing T cells (shown as a percentage of lymphocytes) within divisions of the HBV patient study cohort, based on varying ALT, viral load, age and gender.

| | Condition | ALT <40 | ALT >40 | Low Viral Load | High Viral Load | Age < 35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|---|
| **Mean%** | **Medium** | 0.5 | 0.6 | 0.5 | 0.6 | 0.5 | 0.6 | 0.5 | 0.5 |
| | **PMA** | 4.8 | 3.4 | 4.5 | 4.1 | 4.2 | 4.7 | 3.9 | 5 |
| | **anti-CD3** | 0.9 | 0.9 | 0.9 | 0.9 | 1 | 0.7 | 0.8 | 1.1 |
| | | | | | | | | | |
| **SEM %** | **Medium** | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 |
| | **PMA** | 0.6 | 0.3 | 0.5 | 0.5 | 0.5 | 0.6 | 0.5 | 0.5 |
| | **anti-CD3** | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.2 | 0.2 | 0.2 |
| | | | | | | | | | |
| **N*** | **Medium** | 17 | 8 | 17 | 8 | 15 | 10 | 14 | 11 |
| | **PMA** | 13 | 6 | 13 | 6 | 11 | 8 | 11 | 8 |
| | **anti-CD3** | 11 | 6 | 12 | 5 | 11 | 6 | 9 | 8 |
| | | | | | | | | | |
| **p*** | **Medium** | | 0.5 | | 0.7 | | 0.5 | | 0.4 |
| | **PMA** | | 0.5 | | 0.9 | | 0.5 | | 0.3 |
| | **anti-CD3** | | 0.8 | | 1 | | 0.7 | | 0.4 |
| | | | | | | | | | |
| **r*** | **Medium** | | -0.01 | | 0.2 | | 0.003 | | - |
| | **PMA** | | -0.1 | | 0.1 | | 0.2 | | - |
| | **anti-CD3** | | 0.2 | | 0.3 | | -0.04 | | - |

**\* Condition** refers to the 4 hour incubation of the cells in medium alone or, in medium treated with PMA/I, prior to staining for flow cytometry. **Low viral load** is below 10,000 copies / ml, **high viral load** ranges from 100,000 to $3.2 \times 10^8$ copies / ml. **N\*** is the number of subjects. The p-value **p\*** is a product of the Mann-Whitney U-test and, indicates statistical significance when it falls below 0.05. The correlation coefficient **r\*** is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a linear correlation between two arrays of numbers. The columns with headers **M\*** and **F\*** comprise the male and female subjects, respectively.

**Figure 4.18**

**4.8 Higher frequencies of IFN-γ-producing NK and NT cells following incubation with serum from HBV patients or HBV surface antigen**

Since the frequencies of IFN-γ-producing NK cells were found to be higher in HBV patients and the responses of IFN-γ-producing NT cells to *in vitro* stimulation were enhanced in such subjects, it was sought to determine whether HBV antigens could induce the production of IFN-γ by these cell subsets. PBMC from 3 healthy donors were incubated in medium supplemented with FBS, medium supplemented with serum from healthy control subjects or HBV patients or, medium containing 1μg of HBV surface antigen (HBsAg) or 3μg of HBsAg, for 24 hours. Following this incubation, cells were treated with brefeldin A and incubated for a further 4 hours. Surface and intracellular staining with PE-labelled anti-CD56, PerCP-labelled anti-CD3 and FITC-labelled IFN-γ was subsequently performed.

The mean frequencies of IFN-γ-producing NK cells, as a percentage of lymphocytes, were 1.3+/-0.4% in medium supplemented with FBS i.e. cRPMI (Figure 4.19A). These frequencies were not significantly different following incubation with serum from healthy donors (1.44+/-0.2%). However, the mean frequencies of IFN-γ-producing NK cells rose to 2.3+/-1.1% and 2.5+/-1.7% following incubation with serum from HBV patients and 3μg HBsAg, respectively. Such increases were not observed when PBMC were incubation with 1μg of HBsAg (mean; 0.7+/-0.3%).

The mean frequencies of IFN-γ-producing NT cells, as a percentage of lymphocytes, were 1.5+/-0.1% in medium supplemented with FBS (Figure 4.19B). These frequencies were not significantly different following incubation with serum from healthy donors (0.9+/-0.5%). However, the mean frequencies of IFN-γ-producing NT cells rose to 2.7+/-1.6%, 2.6+/-0.6% and 2.5+/-1.7% following incubation with serum from HBV patients, 1μg of HBsAg and 3μg of HBsAg, respectively.

As all healthy donors tested were HBV vaccinees, the frequencies of IFN-γ-producing T cells were used as a control to show that the HBsAg was functional and to determine whether the serum contained HBV antigens. The mean frequencies of IFN-γ-producing T cells were 0.1+/-0.03% under the following incubation conditions; medium supplemented with FBS, medium supplemented with serum from healthy donors and medium supplemented with serum from HBV patients (Figure 4.19C). These frequencies were increased to 0.4+/-0.1% when medium containing 1μg of HBsAg or 3μg of HBsAg was used. This shows that the commercial HBsAg was intact and raised

memory responses in these subjects but raises a question over whether the patient serum used contained sufficient quantities of HBV antigens. Paired t-tests revealed that the differences observed in the frequencies of IFN-γ-producing NK, NT and T cells following incubation with HBV serum or HBsAg were not significant ($p > 0.05$).

Overall these data suggest that HBsAg induces IFN-γ production by NK and NT cells and supports the hypothesis that such cells play a role in immune responses against HBV. Furthermore, these data, while only preliminary, suggest that HBsAg may facilitate recognition of HBV by these innate lymphocytes and is in agreement with the findings of Albarran *et al.* (2005). The results gathered here also indicate that detection of HBV antigens in patient serum should be performed before such sera is used in any immune mechanism experiments.

**Figure 4.19**

## 4.9 Bonferroni Correction method

In the flow cytometric investigation of the cytokine profiles of innate lymphocytes, 104 statistical tests were performed. The tests that yielded significant results are shown in Tables 4.7, 4.8 and 4.9. By applying the Bonferroni correction method to the p-values obtained from the Mann-Whitney U tests, the most substantial results were filtered out. Both the p-values and corrected p-values ($p_c$) for all tests are listed in Tables 4.7, 4.8 and 4.9. Following this correction, the remaining statistical differences between HBV-infected subjects and uninfected controls were observed in the frequencies of IFN-γ-producing NT and T cells, upon PMA/I stimulation, both as a proportion of each T cell population and as a proportion of total lymphocytes, with a $p_c$-value of less that 0.01 (Table 4.7). The other statistically significant finding after Bonferroni correction was the higher frequency of IL-10-producing CD56$^{DIM}$ NK cells with a $p_c$-value of less than 0.01 (Table 4.8). No differences observed in the frequencies of IL-13- and IL-17-producing cells were deemed statistically significant after application of the Bonferroni correction method.

These data suggest that the frequencies of IFN-γ-expressing NT and T cells isolated from the peripheral blood of HBV-infected individuals are significantly more responsive to *in vitro* stimulation than those isolated from uninfected controls, as are the IL-10-expressing CD56$^{DIM}$ NK cells.

**Table 4.7**

**Statistical analysis of the significant differences observed in the frequencies of IFN-γ⁺ lymphocytes in HBV infection:** The p-values and corrected p-values for 15 of 32 statistical tests performed in the study of the frequencies of IFN-γ-expressing lymphocytes in HBV infection.

| TEST | Medium | | PMA/I | | anti-CD3/28 | |
|---|---|---|---|---|---|---|
| | p value | $p_c$ value | p value | $p_c$ value | p value | $p_c$ value |
| IFN-γ⁺ NK cells (% of lymphocytes) | 0.01 | 1.04 | | | 0.03 | 3.1 |
| IFN-γ⁺ CD56$^{DIM}$ NK cells (% of NK cells) | | | 0.006 | 0.624 | | |
| IFN-γ⁺ CD56$^{DIM}$ NK cells (% of lymphocytes) | 0.01 | 1.04 | | | | |
| IFN-γ⁺ CD56$^{BRIGHT}$ NK cells (% of NK cells) | 0.08 | 8.3 | 0.02 | 2.1 | 0.01 | 1.04 |
| IFN-γ⁺ NT cells (% of NT cells) | | | <0.0001 | *<0.01* | 0.004 | 0.416 |
| IFN-γ⁺ NT cells (% of lymphocytes) | | | <0.0001 | *<0.01* | <0.0001 | *<0.01* |
| IFN-γ⁺ CD56⁻ T cells (% of CD56⁻ T cells) | | | <0.0001 | *<0.01* | | |
| IFN-γ⁺ CD56⁻ T cells (% of lymphocytes) | | | <0.0001 | *<0.01* | | |
| Total % of IFN-γ⁺ lymphocytes | 0.01 | 1.04 | 0.05 | 5.2 | | |

Significant results after Bonferroni correction ($p_c$ values) are highlighted in italics.

**Table 4.8**

**Statistical analysis of the significant differences observed in the frequencies of IL-10$^+$ lymphocytes in HBV infection:** The p-values and corrected p-values for 12 of 32 statistical tests performed in the study of the frequencies of IL-10-expressing lymphocytes in HBV infection.

| TEST | Medium | | PMA/I | | anti-CD3/28 | |
|---|---|---|---|---|---|---|
| | p value | $p_c$ value | p value | $p_c$ value | p value | $p_c$ value |
| IL-10$^+$ NK cells (% of NK cells) | | | 0.04 | 4.16 | | |
| IL-10$^+$ NK cells (% of lymphocytes) | 0.005 | 0.52 | 0.005 | 0.52 | 0.005 | 0.52 |
| IL-10$^+$ CD56$^{DIM}$ NK cells (% of NK cells) | | | 0.002 | 0.208 | | |
| IL-10$^+$ CD56$^{DIM}$ NK cells (% of lymphocytes) | 0.005 | 0.52 | <0.0001 | *<0.01* | 0.005 | 0.52 |
| IL-10$^+$ CD56$^{BRIGHT}$ NK cells (% of NK cells) | | | 0.02 | 2.08 | | |
| IL-10$^+$ NT cells (% of NT cells) | 0.004 | 0.416 | | | 0.02 | 2.08 |
| IL-10$^+$ NT cells (% of lymphocytes) | | | 0.001 | 0.1 | | |

Significant results after Bonferroni correction ($p_c$ values) are highlighted in italics.

**Table 4.9**

**Statistical analysis of the significant differences observed in the frequencies of IL-13$^+$ and IL-17$^+$ lymphocytes in HBV infection:** The p-values and corrected p-values for 7 of 20 statistical tests performed in the study of the frequencies of IL-13-expressing lymphocytes and 1 of 20 statistical tests performed in the study of the frequencies of IL-17-expressing lymphocytes in HBV infection.

| TEST | Medium | | PMA/I | | anti-CD3/28 | |
|---|---|---|---|---|---|---|
| | P value | p$_c$ value | p value | p$_c$ value | p value | p$_c$ value |
| IL-13$^+$ NK cells (% of NK cells) | | | 0.03 | 3.12 | | |
| IL-13$^+$ CD56$^-$ T cells (% of CD56$^-$ T cells) | 0.02 | 2.08 | 0.002 | 0.2 | | |
| IL-13$^+$ CD56$^-$ T cells (% of lymphocytes) | 0.0005 | 0.52 | 0.0008 | 0.08 | | |
| Total % of IL-13$^+$ lymphocytes | 0.004 | 0.416 | 0.01 | 1.04 | | |
| IL-17$^+$ CD56$^-$ T cells (% of CD56$^-$ T cells) | | | 0.01 | 1.04 | | |

**4.10 Discussion**

The findings of this part of the study suggest that HBV-infected subjects have higher frequencies of cytokine-producing lymphocytes than uninfected subjects. The IFN-γ-producers appear to be the predominant cytokine-producing lymphocyte population and this is to be expected as part of an active antiviral immune response (Guidotti & Chisari 2001; Guidotti *et al.* 1999). The higher frequencies of IL-10-producing lymphocyte subsets may be a direct immunomodulatory response to the higher IFN-γ but overall, it appears that the HBV-infected subjects studied here may be a model of an efficient antiviral response i.e. an immune response which is strong enough to prevent the occurrence of high viral titres but low enough to prevent immunopathogenesis.

Higher frequencies of cytokine-producing lymphocytes were observed when the subpopulations were quantified as a proportion of both individual populations and as a percentage of the overall lymphocyte population. This dual quantification was performed for several reasons. By examining the IFN-γ-producing NK cells, for example, as a proportion of the overall lymphocyte population, the importance of any differences could be weighted in terms of the overall immune response. However, it was important to show the data as a proportion of the total NK cell population in order to assess any specific effects HBV infection might have on NK cells. Also, there are shortfalls of gating on the total lymphocyte population; such gating gives an estimate of the overall number of cells without using any specific surface markers. This population was gated based on size and granularity of total PBMC and it could be contaminated with dead cells which can non-specifically bind antibodies, debris and small monocytes for example which would skew the overall percentage values. By gating on the $CD56^+CD3^-$ lymphocyte population in the identification of NK cells, a large degree of this non-specificity was removed and therefore, these data can be considered to be more accurate. Further surface staining with markers such as propidium iodide (PI) and anti-CD14 could have been used to exclude the dead cells and monocytes from the lymphocyte population, respectively. While PI staining was not included in our flow cytometric analyses, all samples were analysed by flow cytometry on the day of sample collection and EB/AO staining was always performed and only PBMC with a cell viability of 90% or greater were examined. The higher frequencies observed in HBV can only be considered as proportions of lymphocyte populations because absolute numbers were not measured. Absolute numbers could not be calculated when PBMC

from buffy coat packs were used as the original volume of fresh blood was unknown. Quantitative RT-PCR was used as another method of cytokine quantification and it confirmed the differences in cytokine production at the level of gene expression.

Frequencies of circulating IFN-γ- and IL-10-producing NK cells were found to be slightly but consistently higher in HBV-infected subjects and the CD56$^{DIM}$ cells were identified as the predominant cytokine producing subset of NK cells. This is contrary to other findings which indicate that the CD56$^{BRIGHT}$ cells are the main cytokine producers (Cooper *et al.* 2001). It must be noted that cytokine production was measured immediately after a 4 hour incubation in medium containing PMA/I and previous work from our lab has shown that PMA/I stimulation can reduce the fluorescence intensity of CD56 for up to 72 hours after stimulation (Kelly-Rogers *et al.* 2006).

NT cells were found to be potent IFN-γ secretors as a population, particularly those isolated from HBV-infected subjects. The cytokine-producing capacity of NT cells was most evident when PBMC from HBV-infected patients were stimulated with PMA/I.

Frequencies of IFN-γ-, IL-10- and IL-17-producing CD56$^-$ T cells were consistently higher in HBV which is an indication of an active immune response. As mentioned in the introductory paragraph, this could be expected in a virally-infected individual. It is possible that the higher levels in IL-10 are in response to higher levels of IFN-γ and, its role is antagonistic and anti-inflammatory. This supports the hypothesis of these subjects providing a model of an ideal immune response. IL-17 is a pro-inflammatory cytokine implicated in several inflammatory diseases and in immune responses against bacterial infections. It has recently been implicated in disease progression and the development of liver fibrogenesis in HBV (Zhang *et al.* 2010; Xu *et al.* 2009). However, while IFN-γ has been identified as a suppressor of Th17 cell induction, IL-17 has also been implicated as a negative regulator of IFN-γ (Mills 2008). Therefore, higher frequencies of IL-17-producing cells in HBV may be another mechanism by which the immune response in these subjects is regulated.

The findings reported in section 4.6 show that there are no correlations between the frequencies of cytokine-producing lymphocytes and viral load, disease severity, age or gender. One major factor would be that most subjects have a relatively low viral load and normal ALT level. These low values indicate that the majority if not all patients are free of liver diseases (if all patients have no liver disease then, the minority with high

ALT may be experiencing hepatic flares which are common in HBV infection). The low viral load of the majority of subjects removed the need for therapy and suggests that the immune response is working strongly enough to keep viral titres at a low level. The low ALT levels in these subjects suggest that the immune response is well-regulated and is not causing any liver damage.

Following the Bonferroni correction method, the frequencies of IFN-γ-producing NT and T cells were shown to be significantly higher in HBV following *in vitro* stimulation, with almost 100% of NT cells from HBV patients producing IFN-γ following stimulation, in some cases. This implicates these cells and IFN-γ as predominant players in the immune responses against HBV. The results of qRT-PCR support the hypothesis of a central role for IFN-γ in immune control of HBV with significantly higher IFN-γ RNA expression observed in patients compared to controls.

The Bonferroni correction method also revealed significantly higher frequencies of IL-10-producing CD56$^{DIM}$ NK cells, as a proportion of total lymphocytes, in HBV patients, following *in vitro* stimulation. Although, these cells only constituted less that 1% of lymphocytes, they may still represent a regulatory mechanism by which the antiviral responses are controlled. However, the shortfalls of measuring percentages as a proportion of lymphocytes must be noted along with the general finding of low frequencies of circulating IL-10-producing lymphocytes. At such low frequencies, if the CD56$^{DIM}$ NK cells are the only true population of IL-10-producing lymphocytes that are higher in these HBV patients, then, this may explain why the higher level was not detected using qRT-PCR. One hypothesis is that IL-10 may act as a safety net allowing the antiviral response to prevail but if IFN-γ production surpasses a certain threshold, IL-10 gene expression may be induced and a Treg response might be stimulated to prevent immune-mediated damage. This is a more favourable cytokine milieu for viral clearance rather than that observed in chronic viremic HCV patients where NK cells appeared to preferentially produce IL-10 rather than IFN-γ (De Maria *et al.* 2007). Indeed, the innate lymphocytes in the HBV-infected subjects examined in this study appear to have significantly higher IFN-γ and IL-10 production capacities compared to that of uninfected subjects. However, IL-10 production appears to be sustained at relatively low levels compared to IFN-γ so that the antiviral response is allowed to control viral replication but is regulated so that it may not become too excessive and cause immune mediated damage.

In addition, it was found that IL-17-producing T cells display enhanced responses to PMA/I stimulation compared to those from uninfected controls. However, it was also found that IL-17A RNA was undetectable in PBMC from patients and controls. Since IL-17 has been implicated in disease progression, decreases in Th1 cell frequencies and the development of liver fibrosis in HBV, these data suggest that its transcription might be under strict control in this group of asymptomatic HBV patients so as not to cause liver damage or inhibit IFN-γ expression (Xu *et al.* 2009; Zhang *et al.* 2010, Ge *et al.* 2010). However, sufficient stimulation can trigger IL-17 production suggesting that this pro-inflammatory cytokine plays a supporting role in the control of HBV infection.

Our observations indicate that NT cells are key players in the control of HBV infection. This supports the findings of Northfield *et al.* (2008) who have found higher frequencies of tissue-infiltrating $CD161^+CD8^+$ T cells in HBV infection. Billerbeck *et al.* (2010) have recently shown that these $NKR^+CD8^+$ T cells exhibit a strong Th17 bias. Since CD161 (NKRP1A) is an NK cell cytotoxicity coreceptor and since IL-17 has been implicated in HBV-associated liver injury, Billerbeck *et al.* (2010) investigated association of such cells with liver disease in HCV infection and found that the repertoires of $CD161^+CD8^+$ cells co-producing IL-17 and IFN-γ were most abundant in the livers of HCV patients with mild liver disease. The authors proposed that such cells mediated immune control and that their functions might be depleted in HCV patients with severe liver disease. These findings along with our own identification of expansions of circulating $CD56^+$ T cells in HBV, suggest that such $NKR^+CD8^+$ T cells or $NKR^+$ T cells which may have lost CD8 expression play a crucial role in the immune control of HBV infection. So, while HBV-specific $CD8^+$ T cell numbers are significantly lower in those individuals who develop chronic HBV infection (Maini *et al.* 2000; Sobao *et al.* 2002, Yang *et al.* 2009) and large numbers of non-antigen specific $CD8^+$ T cells are found in the livers of patients with uncontrolled HBV infections (Bertoletti & Maini 2000; Reignat *et al.* 2002), the control of HBV may be effectively mediated by a group of $CD8^+$ T cells that upregulate NKRs (CD161 or CD56) in an effort to control HBV infection in a manner which minimises liver damage. Viral clearance via non-cytolytic mechanisms has already been shown for HBV (Guidotti et al. 1999) and in our group of asymptomatic carriers, it appears that control but not clearance of HBV is sufficient since a persistent infection with no disease is a safer

option for the host.

The role of NK and NT cells in immunity against HBV has previously been highlighted in responders to vaccination with HBsAg, where these cells exhibited higher IFN-γ and IL-2 expression compared to non-responders (Albarran *et al.* 2005). PBMC from healthy donors were incubated with serum from HBV patients or with HBsAg and then examined IFN-γ production by the NK, NT and T cell populations. From this, it was have shown similar results to Albarran *et al.* (2005) and have supported our hypothesis that elevated IFN-γ production by innate lymphocytes is a direct response to HBV infection. These results were obtained using whole PBMC from HBsAg vaccinated individuals and therefore, the interaction between NK and NT cells with memory T cells may contribute to the enhanced IFN-γ production in response to HBsAg. From these results, it is proposed that NK and NT cells are involved in conferring protective immunity against HBV, along with HBV-specific T cells. Furthermore, HBsAg, unlike the envelope protein E2 of HCV, does not appear to modulate NK cell functions (Tseng and Klimpel 2002).

The HBV-infected subjects studied here may represent a preliminary model of the evolutionary changes that immune cells must undergo to control viral replication without causing liver disease. It is proposed that certain T cell subsets undergo phenotypic changes, minimise their cytolytic activity and maximise their antiviral cytokine production to promote control of HBV infection. Lessons learned here could be used to develop novel immunotherapies to treat HBV patients with higher viral loads but first, a similar study of such patients should be performed.

# Chapter 5

# Phenotypic and functional analysis of γδ T cells in HBV infection

## 5.1 Introduction

γδ T cells are a group of T lymphocytes that elicit potent cytokine production and cytotoxic activity usually in a much faster manner than their $αβ^+$ counterparts but they account for only 1-5% of circulating lymphocytes (Hayday 2000). All γδ T cells express TCRs consisting of a γ- and a δ-chain, as opposed to the α- and β-chain of the conventional T cell (Hayday 2000). Some γδ T cells exhibit TCR-mediated recognition of glycolipids (Spada *et al.* 2000; Russano *et al.* 2007) and phosphoantigens which can lead to significant expansion and activation of these cells (Constant *et al.* 1994; Tanaka *et al.* 1995; Morita *et al.* 1995; Jomaa *et al.* 1999). However, they can also recognise danger molecules in a TCR-independent manner via the expression of NKRs such as NKG2D and NKG2A (Battistini *et al.* 1997; von Lilienfeld-Toal *et al.* 2006; Toutirais *et al.* 2009). The multi-faceted nature of γδ T cell activation and their powerful cytokine production and cytotoxicity capabilities make them ideal targets for cell-based immunotherapies.

The importance of γδ T cells in antiviral immune responses has been reported in EBV, HIV and HSV infections (De Paolo *et al.*1990; Poccia *et al.*1999; Maccario *et al.*1995). γδ T cells have also been implicated in immune responses to HCV and are believed to play a role in the liver injury associated with the virus (Tseng *et al.*2001; Nikolopoulou *et al.*1995). Several studies have investigated the role of γδ T cells in HBV infection; Sing *et al.* (1998) found an association between expansions of γδ T cells and seroconversion while Chen *et al.* (2008) showed that their frequencies are depleted in the peripheral blood of persistent HBV infection.

So far, a significant expansion of circulating γδ T cells in HBV-infected persons has been found compared to uninfected control subjects. Although all patients had persistent HBV infection (HBsAg-positive), the vast majority of them had a low viral burden (<20,000 copies/ml) and were free of liver disease (ALT below 40). Therefore, our patient cohort can be considered as representative of a controlled immune response to HBV, which is under sufficient regulatory control so that does not cause significant pathology but it fails to completely eliminate the virus. Since γδ T cells appear to play a role in this immune response, the subsets which are numerically changed in the peripheral blood have been investigated here. Their phenotypes, differentiation status expression of stimulatory receptors and their capacity to express IFN-γ and the cytotoxicity-associated receptor NKG2D have also been evaluated.

There are 3 main groups of γδ T cells in humans that are divided based on their δ chain usage i.e. Vδ1, Vδ2 and Vδ3 T cells. Vδ2 T cells are the most abundant γδ T cell in circulation while Vδ1 subsets are most prevalent in the tissues and mucosal surfaces and Vδ3 T cells have only been described in blood and liver (Dechanet *et al.* 1999; Kenna *et al.* 2004).

Vδ2 T cells are the predominant γδ T cell subset in circulation and their TCR most often consists of a Vδ2 chains paired with a Vγ9 chain (Eberl *et al.* 2009). They exhibit potent cytotoxic activity and predominantly produce Th1 cytokines such as IFN-γ and TNF-α (Eberl *et al.* 2009; Conti *et al.* 2005; Angelini *et al.* 2004; Dudal *et al.* 2006; ; Unpublished work from our lab). Vγ9Vδ2 T cells play central roles in innate and adaptive immune responses through their ability to recruit neutrophils, induce maturation of DC into APC and provide help to B cells promoting their maturation into antibody-producing plasma cells (Conti *et al.* 2005; Ismaili *et al.* 2002; Agrati *et al.* 2009; Caccamo *et al.* 2006). Vγ9Vδ2 T cells have also been shown to have antigen presentation properties and upregulate HLA-DR, and the lymph node homing receptor CCR7, when activated (Brandes 2005). Resting Vγ9Vδ2 T cells lack these properties therefore indicating that an antigen presentation role is assumed in an inflammatory environment. Vγ9Vδ2 T cells have also been implicated in wound repair via the production of tissue growth factors (Workalemahu *et al.* 2004). Their multi-functional capacity makes Vγ9Vδ2 T cells ideal candidates for immunotherapy and they are already the centre of several clinical trials as immunotherapeutics in cancer (Dieli *et al.* 2007; Bennouna *et al.* 2008). The role of Vδ2 T cells in immunity against HBV is uncertain but their frequencies in the peripheral blood of chronic HBV patients with varying degrees of liver disease were shown to be decreased in a study by Chen *et al.* (2008). Here, circulating Vδ2 T cells were quantified in order to confirm if they are the γδ T cell population that is expanded in our patient cohort.

Vδ1 T cells are mostly found in the tissues and mucosal surfaces and constitute 70-90% of γδ T cells in the intestine where they have been shown to exhibit immunoregulatory functions (Groh *et al.* 1998; Bhagat *et al.* 2008). However, IFN-γ- and TNF-α-expressing Vδ1 T cells have also been isolated from tissue suggesting that they play a role in antiviral immunity (Spada *et al.* 2000). Vδ1 T cells are expanded in tumor-bearing livers and in HIV and HSV infection (Fenoglio *et al.* 2009; Kenna *et al.*

2004). Furthermore, research by Rossol *et al.* (1998) yielded data which suggest that circulating Vδ1 T cell frequencies are unchanged in HSV-1, HSV-2, HCV and HBV infection and have proposed that their expansion is unique to HIV infection. Interestingly, Agrati *et al.* (2001) have reported that Vδ1 T cells are the main γδ T cell subset infiltrating HCV-infected liver and have suggested that their Th1 cytokine profile makes them possible contributors to liver inflammation. Here, the frequencies of circulating Vδ1 T cells have been quantified to determine if they contribute to the expansions of γδ T cells previously observed in our patient cohort. The frequencies of CD56-expressing subsets of these cells have also been investigated to determine if expanded NT and γδ T cell populations observed in Chapter 3 are overlapping shared populations.

Phenotypic analysis has revealed that HBV-specific CD8$^+$ T cells in PBMC from patients with acute HBV infection predominantly express a memory T cell phenotype suggesting that they are actively involved in immunity against HBV (Sobao *et al.* 2002; Urbani *et al.* 2002). Since γδ T cells appear to play a role in immune control of HBV in this patient cohort, the differentiation status of circulating γδ T cell subsets were examined using a method described by Dieli et al (2003). According to this scheme, γδ T cells can be divided into memory subsets using the markers CD27 and CD45RA. Naïve γδ T cells express both CD27 and CD45RA while central memory γδ T cells lose CD45RA but retain CD27 expression (Dieli *et al.* 2003). Both naïve and central memory γδ T cells tend to home to the secondary lymphoid organs and usually lack immediate effector functions i.e. cytotoxicity and rapid cytokine production (Eberl *et al.* 2002). Effector memory (T$_{EM}$) γδ T cells lack both CD45RA and CD27 surface expression but CD45RA re-appears on terminally differentiated effector memory γδ T cells (Dieli *et al.* 2003). Effector memory γδ T cells home to the sites of infection where they exhibit immediate effector functions (Eberl *et al.* 2002). Dieli and colleagues have shown that the majority of γδ T cells express a central memory or T$_{CM}$ phenotypes (Dieli *et al.* 2003).

Here, the frequencies of naïve, T$_{CM}$, T$_{EM}$ and terminally differentiated effector memory (T$_{EMRA}$) Vδ2 and Vδ1 T cells were compared between HBV-infected subjects and uninfected control subjects by quantifying the frequencies of circulating

CD45RA$^+$CD27$^+$, CD45RA$^-$CD27$^+$, CD45RA$^-$ CD27$^-$ and CD45RA$^+$CD27$^-$ V$\delta$2 and V$\delta$1 T cells.

Since the frequencies of $\gamma\delta$ T cells were found to be higher in HBV infection, the effector functions of such cells were examined by quantifying their IFN-$\gamma$ production. The V$\delta$2 T cells were investigated in this part of the study because they are the predominant $\gamma\delta$ T cell subset present in the peripheral blood and their expansion in HBV infection was more pronounced than that of total $\gamma\delta$ T cells. IFN-$\gamma$ was the cytokine of choice because several studies, including studies from our own lab, have revealed that cytokine production by V$\delta$2 T cells is limited to the Th1 cytokines IFN-$\gamma$ and TNF-$\alpha$ (Eberl *et al.* 2009; Conti *et al.* 2005; Angelini *et al.* 2004; Unpublished work from our lab). Furthermore, IFN-$\gamma$ is the classical antiviral cytokine and can elicit a variety of functions that promote viral clearance (Guidotti & Chisari 2001). In addition, a study in HBV-infected chimpanzees revealed that the clearance of HBV DNA from liver occurred at the same time as the appearance of IFN-$\gamma$ thus implicating a central role for the cytokine in the control and resolution of HBV infection (Guidotti *et al.* 1999). Chen *et al.* (2008) have also found that $\gamma\delta$ T cell-derived IFN-$\gamma$ is significantly decreased in chronic HBV infection thus suggesting that impairment of $\gamma\delta$ T cell function is associated with the failure to eliminate the virus.

NKG2D is an activating receptor originally identified on NK cells but, it can also mediate cytotoxicity by some T cells. It is an important component of the antiviral response because it facilitates the recognition of virus-infected cells in the absence of classical MHC-restricted antigen presentation. The activating receptor has been manipulated by several viruses in order to evade immune recognition. For instance, CMV and HIV have evolved mechanisms by which the expression of NKG2D ligands is inhibited (Gonzalez *et al.* 2008). Furthermore, the frequencies of circulating NKG2D-expressing NK cells are significantly lower in HBV infection and in even more so in HBV patients with liver cirrhosis and HCC. These decreases correlated with the cytotoxic activity of the NK cells suggesting that NKG2D downregulation may be a means through which HBV evades NK cell cytotoxicity (Zeng *et al.* 2009). Recent work by Chen *et al.* (2008) has revealed that $\gamma\delta$ T cell cytotoxicity is impaired in persistent HBV infection but they have not shown the mechanism through which this putative dysfunction is mediated. In this study, the expression of NKG2D on V$\delta$2 T cells was investigated as a measure of their cytotoxic potential in controlled HBV infection. If our

data support that of Chen *et al.* (2008) then, this work may have identified a mechanism through which inhibition of γδ T cell cytotoxicity is mediated.

Overall, this study should provide a good insight into the γδ T cell repertoires needed to provide immune control of HBV infection without causing liver injury.

**5.2 γδ T cell subpopulations in HBV infection**

**5.2.1 The frequencies of circulating Vγ9⁺Vδ2⁺ T cells are higher in HBV patients than in control subjects**

PBMC were prepared from the fresh blood of 33 HBV patients and from the buffy coat packs of 55 control subjects. Surface staining with FITC-labelled anti-Vγ9 mAb, PE-labelled anti-Vδ2 mAb and PE-Cy5-labelled anti-CD3 mAb was performed to identify Vγ9⁺Vδ2⁺ T cells in the peripheral blood (Figure 5.1A and 5.1B). It was found that most Vγ9⁺ T cells express Vδ2 (mean; 81.2 +/- 3.4%, Figure 5.1A) and most Vδ2⁺ T cells express Vγ9 (mean; 90+/- 3.9%) and therefore, it was only necessary to use one marker to identify the Vγ9⁺Vδ2⁺ T cell population. PE-labelled anti-Vδ2 mAb was the marker of choice and the Vγ9⁺Vδ2⁺ T cell population is herein referred to as the Vδ2 T cells. Another interesting observation made when using FITC-labelled anti-Vγ9 mAb and PE-labelled anti-Vδ2 mAb together was that the occurrence of 2 different Vγ9⁺Vδ2⁺ T cell populations with differing intensities of Vγ9 or Vδ2 (Figure 5.1A). The frequencies of circulating Vδ2 T cells, as a percentage of total T cells, ranged from 0.3% to 11.7% in controls, and from 1.8% to 21.2% in HBV. The mean frequencies were 3.6+/-0.38% in controls and 7.8+/-0.95% in HBV. This difference is significant ($p < 0.0001$, Figure 5.1C).

**Figure 5.1**

**5.2.2 The frequencies of circulating Vδ1 T cells are higher in HBV infection**

The frequencies of T cells with the Vδ1 chain were also quantified to determine which γδ T cell subset was expanded in HBV infection. PBMC from 23 HBV-infected subjects and from the buffy coat packs of 21 control subjects were indirectly surface stained with unconjugated anti-Vδ1 mAb (murine IgG), followed by PE-labelled anti-mouse IgG, in combination with direct surface staining with PE-Cy5-labelled anti-CD3 mAb. Vδ1 T cells were identified as those T cells which stained positive for CD3 (Figure 5.1A) and Vδ1 (Figure 5.2A). The frequencies of circulating Vδ1 T cells were then quantified, as a percentage of total T cells. Frequencies of circulating Vδ1 T cells ranged from 0.1% to 3.8% in controls, and from 0.1% to 2.9% in HBV, with mean frequencies of 0.77+/-0.19% and 1.2+/-0.16%, respectively (p = 0.02, Figure 5.2B).

These data show that there are statistically significantly higher frequencies of circulating Vδ1 and Vδ2 T cells in HBV infection.

**Figure 5.2**

**5.2.3 The frequencies of circulating CD56$^+$ Vδ2 T cells are similar in HBV-infected and control subjects**

The findings documented in Chapter 3 indicated that the frequencies of both circulating CD56$^+$ T cells and γδ T cells are higher in HBV-infected persons. It was next sought to determine if frequencies of CD56$^+$ γδ T cell subsets were higher in HBV infection. Firstly, the frequencies of CD56$^+$ Vδ2 T cells were quantified. PBMC from 18 HBV-infected subjects and from the buffy coat packs of 30 control subjects were surface stained with FITC-labelled anti-CD56 mAb, PE-labelled anti-Vδ2 mAb and PE-Cy5-labelled anti-CD3 mAb to identify CD56$^+$ Vδ2 T cells in the peripheral blood (Figure 5.3A). When the proportion of CD56$^+$ T cells were quantified as a percentage of Vδ2 T cells, the mean frequencies of CD56$^+$ Vδ2 T cells were 23.8+/-3.1% in controls and 18.6+/-3.2% in HBV-infected individuals (p = 0.4, Figure 5.3B). These differences were not significant. The frequencies of circulating CD56$^+$Vδ2 T cells, as a percentage of total T cells ranged from 0.07% to 2.7% in controls, and from 0.2% to 6.4% in HBV, with means of 0.92+/-.14% and 1.53+/-0.40%, respectively (p = 0.4, Figure 5.3C).

These data show that the higher frequencies of CD56$^+$ T cells in HBV infection is not due to higher frequencies of CD56$^+$ Vδ2 T cells.

**Figure 5.3**

**5.2.4 The frequencies of circulating CD56$^+$ Vδ1 T cells are significantly higher in HBV infection as a proportion of total T cells, but not as a proportion of Vδ1 T cells**

The frequencies of CD56$^+$ Vδ1 T cells were also investigated to determine if such cells were responsible for the higher frequencies of CD56$^+$ T cells in HBV infection. PBMC from 17 HBV patients and from the buffy coat packs of 15 control subjects were directly surface stained with FITC-labelled anti-CD56 mAb and PE-Cy5-labelled anti-CD3 mAb and indirectly stained with unconjugated anti-Vδ1 mAb (murine IgG), followed by PE-labelled anti-mouse IgG to identify CD56$^+$ Vδ1 T cells in the peripheral blood (Figure 5.4A). When quantified as a percentage of Vδ1 T cells, the mean frequencies of CD56$^+$ Vδ1 T cells were 30.6+/-7.9% in controls and 37.7+/-3.8% in HBV-infected individuals and this difference was not significant (p = 0.4, Figure 5.4B). The frequencies of circulating CD56$^+$ Vδ1 T cells, as a percentage of total T cells, ranged from 0% to 1.3% in controls, and from 0.08% to 1.6% in HBV, with means of 0.31+/-0.11% and 0.61+/-0.11%, respectively. The numbers of CD56$^+$ Vδ1 T cells in the peripheral blood were slightly but significantly higher in HBV-infected subjects than in control subjects, when quantified as a proportion of the total CD3$^+$ lymphocyte population (p = 0.01, Figure 5.4C).

These data show that a significant proportion of Vδ1 T cells in patients and controls express CD56 and that Vδ1 T cells make a significant contribution to the higher frequencies of CD56$^+$ T cells observed in HBV infection in Chapter 3.

**Figure 5.4**

**5.3 Memory phenotypes of γδ T cell subpopulations in HBV infection**

**5.3.1 Proportions of naïve and memory Vδ2 T cells are altered in HBV infection**

PBMC from 27 HBV-infected subjects and from the buffy coat packs of 40 control subjects were surface stained with PE-labelled anti-Vδ2 mAb, PerCP-labelled anti-CD3 mAb, FITC-labelled anti-CD45RA mAb and APC-labelled anti-CD27 mAb to identify the naïve, $T_{CM}$, $T_{EM}$ and $T_{EMRA}$ Vδ2 T cells (Figure 5.5A).

The frequencies of naive Vδ2 T cells, as a proportion of the total Vδ2 T cell population, ranged from 1.2% to 61.1% in controls and from 1% to 38.4% in HBV, with means of 23.75+/-2.4% and 14.1+/-2.8%, respectively, (Figure 5.5B, p=0.006). The frequencies of $T_{CM}$ Vδ2 T cells ranged from 1.8% to 89.3% in controls and from 0% to 55.1% in HBV, with means of 33.7+/-3.3% and 22.2+/-3.7%, respectively. These lower frequencies of $T_{CM}$ Vδ2 T cells in HBV were significant (Figure 5.5C, p=0.03). The frequencies of $T_{EM}$ Vδ2 T cells ranged from 0% to 57.1% in controls and from 2.3% to 72.3% in HBV with means of 25.3+/-2.8% and 33.8+/-5.3%, respectively. This difference was not statistically significant (Figure 5.5D, p=0.1). The frequencies of $T_{EMRA}$ Vδ2 T cells ranged from 0% to 71% in controls and from 0.9% to 64.1% in HBV, with means of 17.1+/-2.6% and 26.8+/-4.3%, respectively (Figure 5.5E, p=0.02).

These data show that the frequencies of $T_{CM}$ and naive Vδ2 T cells are significantly lower in HBV infection, while the frequencies of $T_{EMRA}$ Vδ2 T cells are higher, suggesting that the effector Vδ2 T cells are expanded in order to facilitate the control of HBV infection.

**Figure 5.5**

**5.3.2 Proportions of naïve and memory Vδ1 T cells are altered in HBV infection**

Further phenotypic studies were also performed to ascertain whether the frequencies of $T_{EM}$, $T_{CM}$, naïve and $T_{EMRA}$ Vδ1 T cells were altered in HBV infection. PBMC from 17 HBV-infected subjects and from the buffy coat packs of 21 control subjects were indirectly surface stained with unconjugated anti-Vδ1 mAb (murine IgG), followed by PE-labelled anti-mouse IgG and directly surface stained with PerCP-labelled anti-CD3 mAb, FITC-labelled anti-CD45RA mAb and APC-labelled anti-CD27 mAb. The frequencies of naive Vδ1 T cells, as a proportion of the total Vδ1 T cell population, ranged from 17.5% to 100% in controls and from 4.6% to 50% in HBV, with means of 61+/-5.2% and 28.2+/-3.6%, respectively (Figure 5.6A, p=0.0002). The frequencies of $T_{CM}$ Vδ1 T cells ranged from 0% to 33.9% in controls and from 0% to 36.4% in HBV, with means of 7+/-2.1% and 14.3+/-2.5%, respectively (Figure 5.6B, p=0.02). The frequencies of $T_{EM}$ Vδ1 T cells ranged from 0% to 16.7% in controls and from 0% to 48.2% in HBV with means of 3+/-1.2% and 12.9+/-3.2%, respectively. This difference was significant (Figure 5.6C, p=0.004). The frequencies of $T_{EMRA}$ Vδ1 T cells ranged from 0% to 65.6% in controls and from 6.4% to 77.3% in HBV, with means of 26.1+/-4.2% and 44.6+/-5.4%, respectively (Figure 5.6D, p=0.02).

These data show that the frequencies of all memory subsets of Vδ1 T cells are expanded in HBV infection while, naïve Vδ1 T cells are significantly lower, suggesting that Vδ1 T cells are actively involved in the successful immune control of HBV.

**Figure 5.6**

**5.4 γδ T cell subpopulations in HBV patient groups**

**5.4.1 The frequencies of circulating Vδ2 T cells do not correlate with viral load, disease severity and age in HBV but are slightly higher in female subjects**

The Vδ2 T cell frequencies of the HBV patients within the study cohort were compared based on clinical parameters. Figure 5.7A shows the frequencies of circulating Vδ2 T cells as a percentage of total T cells for 23 HBV patients with a low viral load i.e. below 100,000 copies/ml and 5 HBV patients with a high viral load i.e. between 100,000 and $5 \times 10^8$ copies/ml. The frequencies of circulating Vδ2 T cells in patients with low viral load ranged from 3.1% to 10.8% (mean 6.6+/-0.4%) and in patients with high viral load, ranged from 3.6% to 12.2% (mean 8+/-0.7%). There was no significant difference between these numbers (p=0.6). Furthermore, a Spearman correlation test yielded a correlation coefficient of r=-0.1, suggesting that there is no linear correlation between the frequencies of Vδ2 T cells and viral load. Figure 5.7B shows the frequencies of Vδ2 T cells for 19 HBV patients with a low ALT and 7 HBV patients with a high ALT. For those patients with low ALT, the frequencies of Vδ2 T cells ranged from 1.8% to 12.2% (mean 7.9+/-0.5%) and for those with a high ALT, the frequencies ranged from 3.1 % to 21.2% (mean 9.2+/-1%). There were no significant differences (p=0.2) and moreover, there was no linear correlation between the numbers of Vδ2 T cells and ALT levels (r=0.03). Figure 5.7C shows the frequencies of circulating Vδ2 T cells in 12 males and 17 females. The frequencies ranged from 3.1% to 14.9% (mean 6.8+/-0.7%) and from 1.8% to 21.2% (mean 10.2+/-0.8%), respectively. There was slightly but significantly higher frequencies of Vδ2 T cells in HBV-infected females than HBV-infected males (p=0.02). Figure 5.7D shows the frequencies of circulating Vδ2 T cells in 17 HBV-infected subjects aged between 19 and 35 years of age and 12 HBV-infected subjects aged between 35 and 55 years of age. The frequencies of Vδ2 T cells ranged from 1.8% to 21.2% in the younger group of patients (mean 9+/-0.6%) and ranged from 7.6% to 14.9% in the older group of patients (mean 7.8+/-0.8%). There was no significant difference (p=0.9) and there was no linear correlation between the frequencies of circulating Vδ2 T cells and the age of the HBV patients (r=0.05).

These data suggest that the frequencies of circulating Vδ2 T cells are slightly higher in female HBV-infected subjects than their male counterparts but the numbers do not correlate with viral load, disease severity or age.

**Figure 5.7**

**5.4.2 Frequencies of circulating Vδ1 T cells are not significantly different between HBV patient groups**

The frequencies of Vδ1 T cells in HBV patients within the study cohort were compared based on clinical parameters. Figure 5.8A shows the frequencies of circulating Vδ1 T cells as a percentage of total T cells for 19 HBV patients with a low viral load i.e. below 100,000 copies/ml and 3 HBV patients with a high viral load i.e. between 100,000 and $5 \times 10^8$ copies/ml. The frequencies of circulating Vδ1 T cells in patients with low viral load ranged from 0.1% to 2.9% (mean 1.5+/-0.1%) and in patients with high viral load, ranged from 0.6% to 2.3% (mean 1.3+/-0.5%). There was no significant difference between these numbers (p=0.9) and there was no linear correlation between the frequencies of Vδ1 T cells and viral load (r=-0.01). Figure 5.8B shows the frequencies of Vδ1 T cells for 13 HBV patients with a low ALT and 7 HBV patients with a high ALT. For those patients with low ALT, the frequencies of Vδ1 T cells ranged from 0.1% to 2.9% (mean 1.1+/-0.2%) and for those with a high ALT, the frequencies ranged from 0.4% to 1.9% (mean 1.1+/-0.1%). There were no significant differences (p=0.8) and moreover, there was no linear correlation between the numbers of Vδ1 T cells and ALT levels (r=0.003). Figure 5.8C shows the frequencies of circulating Vδ1 T cells in 11 males and 12 females. The frequencies ranged from 0.2% to 2.9% (mean 1.2+/-0.1%) and from 0.1% to 2.7% (mean 1.6+/-0.1%), respectively. There was no significant difference between the frequencies of Vδ1 T cells in HBV-infected females and HBV-infected males (p=0.5). Figure 5.8D shows the frequencies of circulating Vδ1 T cells in 10 HBV-infected subjects aged between 19 and 35 years of age and 12 HBV-infected subjects aged between 35 and 55 years of age. The frequency of circulating Vδ1 T cells ranged from 0.1% to 2.3% in the younger group of patients (mean 1+/-0.1%) and ranged from 0.2% to 2.9% in the older group of patients (mean 1.3+/-0.2%). There was no significant difference (p=0.9) and there was no linear correlation between the frequencies of circulating Vδ1 T cells and the age of the HBV patients (r=0.05).

These data suggest that the frequencies of circulating Vδ1 T cells do not correlate with viral load, disease severity, gender or age.

**Figure 5.8**

### 5.4.3 Frequencies of circulating CD56^{+} Vδ2 T cells are not significantly different between HBV patient groups

The frequencies of CD56^{+} Vδ2 T cells in HBV patients within the study cohort were compared based on clinical parameters. Figure 5.9A shows the frequencies of circulating CD56^{+} Vδ2 T cells as a percentage of total T cells for 13 HBV patients with a low viral load i.e. below 100,000 copies/ml and 4 HBV patients with a high viral load i.e. between 100,000 and 5 x $10^8$ copies/ml. The mean frequencies of circulating Vδ2 T cells in patients with low and high viral load were 1.4+/-0.4% and 2.2+/-0.8%, respectively. There was no significant difference between these numbers (p=1) and there was no linear correlation between the frequencies of CD56^{+} Vδ2 T cells and viral load (r=-0.3). Figure 5.9B shows the frequencies of Vδ2 T cells for 9 HBV patients with a low ALT and 9 HBV patients with a high ALT. The mean frequencies of CD56^{+} Vδ2 T cells in patients with low and high ALT were 1.8+/-0.5% and 1.3+/-0.7%, respectively (p=0.2, r=-0.2). Figure 5.9C shows the frequencies of circulating CD56^{+} Vδ2 T cells in 8 males and 10 females. The mean frequencies for males and females were 1.1+/-0.3% and 1.9+/-0.7%), respectively (p=0.7). Figure 5.9D shows the frequencies of circulating CD56^{+} Vδ2 T cells in 10 HBV-infected subjects aged between 19 and 35 years of age and 8 HBV-infected subjects aged between 35 and 55 years of age. The mean frequencies of CD56^{+} Vδ2 T cells in the younger and older groups of patients were 1.7+/-0.7% and 1.3+/-0.4%, respectively (p=0.9, r=0.08).

These data suggest that the frequencies of circulating CD56^{+} Vδ2 T cells do not correlate with viral load, disease severity, gender or age.

**Figure 5.9**

**5.4.4 Frequencies of circulating CD56$^+$ Vδ1 T cells are not significantly different between HBV patient groups**

The frequencies of CD56$^+$ Vδ1 T cells in HBV patients within the study cohort were compared based on clinical parameters. Figure 5.10A shows the frequencies of circulating CD56$^+$ Vδ1 T cells as a percentage of total T cells for 13 HBV patients with a low viral load i.e. below 100,000 copies/ml and 3 HBV patients with a high viral load i.e. between 100,000 and 5 x 10$^8$ copies/ml. The mean frequencies of circulating CD56$^+$ Vδ1 T cells in patients with low and high viral load were 0.6+/-0.1% and 0.6+/-0.3%, respectively. There was no significant difference between these numbers (p=0.9) and there was no linear correlation between the frequencies of CD56$^+$ Vδ1 T cells and viral load (r=0.1). Figure 5.10B shows the frequencies of CD56$^+$ Vδ1 T cells for 9 HBV patients with a low ALT and 8 HBV patients with a high ALT. The mean frequencies of CD56$^+$ Vδ1 T cells for HBV patients with low and high ALT were 0.6+/-0.1% and 0.7+/-0.2%, respectively (p=0.8 , r=0.2). Figure 5.10C shows the frequencies of circulating CD56$^+$ Vδ1 T cells in 7 males and 10 females. The mean frequencies for males and females were 0.6 +/-0.2% and 0.6+/-0.1%, respectively (p=0.8). Figure 5.10D shows the frequencies of circulating CD56$^+$ Vδ1 T cells in 9 HBV-infected subjects aged between 19 and 35 years of age and 8 HBV-infected subjects aged between 35 and 55 years of age. The mean frequencies of circulating CD56$^+$ Vδ1 T cells in the younger and older patient groups were 0.6+/-0.2% and 0.6+/-0.2%, respectively (p=1, r=-0.1).

These data suggest that the frequencies of circulating CD56$^+$ Vδ1 T cells do not correlate with viral load, disease severity, gender or age.

**Figure 5.10**

**5.4.5 Frequencies of naïve, $T_{CM}$, $T_{EM}$ and $T_{EMRA}$ V$\delta$2 T cells do not correlate with viral load, disease severity, gender or age in HBV infection**

The frequencies of V$\delta$2 T cells of different memory phenotypes were quantified as a percentage of total V$\delta$2 T cells and compared between HBV patients within the study cohort, based on clinical parameters. The memory phenotypes were the same as those investigated in section 5.2 i.e. naïve, $T_{CM}$, $T_{EM}$, and $T_{EMRA}$ phenotypes. The minimum, maximum and mean frequencies for circulating naïve, $T_{CM}$, $T_{EM}$, and $T_{EMRA}$ V$\delta$2 T cells are shown in Table 5.1. The frequencies of naïve, $T_{CM}$, $T_{EM}$, and $T_{EMRA}$ V$\delta$2 T cells divided based viral load, ALT, gender and age are shown in Figures 5.11A, B, C and D, respectively.

All data shown suggest that there were no statistically significant differences in the frequencies of circulating naïve, $T_{CM}$, $T_{EM}$ or $T_{EMRA}$ V$\delta$2 T cells between HBV patient groups.

**5.4.6 Frequencies of naïve, $T_{CM}$, $T_{EM}$ and $T_{EMRA}$ V$\delta$1 T cells do not correlate with viral load, disease severity, gender or age in HBV infection**

The frequencies of V$\delta$1 T cells of different memory phenotypes were quantified as a percentage of total V$\delta$1$^+$T cells and compared between HBV patients within the study cohort, based on clinical parameters. The minimum, maximum and mean frequencies for circulating naïve, $T_{CM}$, $T_{EM}$ and $T_{EMRA}$ V$\delta$1 T cells are shown in Table 5.2. The frequencies of naïve, $T_{CM}$, $T_{EM}$ and $T_{EMRA}$ V$\delta$1 T cells divided based viral load, ALT, gender and age are shown in Figures 5.12A, B, C and D, respectively.

All data shown suggest that there were no statistically significant differences in the frequencies of circulating naïve, $T_{CM}$, $T_{EM}$ or $T_{EMRA}$ V$\delta$1 T cells between HBV patient groups.

Altogether, there are higher frequencies of effector-memory V$\delta$1$^+$ and V$\delta$2 T cells in HBV infection. However, these higher frequencies do not correlate with increases in viral load and disease severity, suggesting that such cells are involved in immune responses against HBV but not pathogenesis.

**Figure 5.11**

**Table 5.1**

**Differentiation status of circulating Vδ2 T cells in subgroups of HBV patients:** The frequencies of Vδ2 T cells in patient subsets, based on varying ALT, viral load, age and gender.

| | Memory Phenotype | ALT <40 | ALT >40 | Low Viral Load | High Viral Load | Age < 35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|---|
| **Min %** | **Naïve** | 3.9 | 3.2 | 3.8 | 3.2 | 3.2 | 3.9 | 6.1 | 3.2 |
| | $T_{CM}$ | 15 | 6.7 | 14.9 | 6.7 | 6.7 | 9.9 | 15 | 6.7 |
| | $T_{EM}$ | 9.4 | 6 | 7.7 | 6 | 6 | 7.8 | 7.8 | 6 |
| | $T_{EMRA}$ | 3.4 | 0.9 | 0.9 | 4.9 | 0.9 | 3.4 | 7.8 | 0.9 |
| **Max %** | **Naïve** | 38.4 | 29.3 | 38.4 | 22 | 34.4 | 38.4 | 38.4 | 29.2 |
| | $T_{CM}$ | 49.5 | 55.1 | 55.1 | 25.2 | 47.9 | 55.1 | 55.1 | 49.5 |
| | $T_{EM}$ | 50.5 | 66.9 | 50.5 | 66.9 | 66.9 | 50.5 | 66.9 | 50.5 |
| | $T_{EMRA}$ | 39.3 | 59.1 | 36.4 | 59.1 | 59.1 | 54.3 | 36.3 | 59.1 |
| **Mean%** | **Naïve** | 21.8 | 13.3 | 20.7 | 10.7 | 13.2 | 23 | 23.2 | 14 |
| | $T_{CM}$ | 34 | 25.9 | 36 | 16.2 | 26 | 35 | 34.2 | 27.4 |
| | $T_{EM}$ | 24.8 | 29 | 26.6 | 27.1 | 30.8 | 22.2 | 26.7 | 26.8 |
| | $T_{EMRA}$ | 19.5 | 23.7 | 16.5 | 33.5 | 22.9 | 19.8 | 15.7 | 25.5 |
| **SEM %** | **Naïve** | 4.3 | 3.1 | 3.3 | 3.6 | 3.5 | 4.1 | 5.1 | 2.9 |
| | $T_{CM}$ | 4 | 6.3 | 3.7 | 3.7 | 4.7 | 5.6 | 5.7 | 4.9 |
| | $T_{EM}$ | 4.8 | 7.5 | 4.3 | 10.7 | 6.3 | 5.6 | 8.4 | 4.7 |
| | $T_{EMRA}$ | 4.3 | 8.1 | 3.3 | 11.2 | 6.4 | 6.1 | 3.9 | 6.7 |
| **N*** | **Naïve** | 9 | 8 | 12 | 5 | 10 | 8 | 7 | 10 |
| | $T_{CM}$ | 9 | 8 | 12 | 5 | 10 | 8 | 7 | 10 |
| | $T_{EM}$ | 9 | 8 | 12 | 5 | 10 | 8 | 7 | 10 |
| | $T_{EMRA}$ | 9 | 8 | 12 | 5 | 10 | 8 | 7 | 10 |
| **P*** | **Naïve** | 0.2 | | 0.1 | | 0.1 | | 0.1 | |
| | $T_{CM}$ | 0.3 | | 0.02 | | 0.7 | | 0.5 | |
| | $T_{EM}$ | 0.9 | | 0.8 | | 0.4 | | 0.8 | |
| | $T_{EMRA}$ | 1 | | 0.2 | | 0.7 | | 0.5 | |
| **r*** | **Naïve** | -0.3 | | -0.5 | | 0.4 | | - | |
| | $T_{CM}$ | -0.3 | | -0.3 | | 0.1 | | - | |
| | $T_{EM}$ | 0 | | 0.07 | | -0.4 | | - | |
| | $T_{EMRA}$ | 0.01 | | -0.01 | | 0.02 | | - | |

**\* Low viral load** lies below 100,000 copies/ml, **high viral load** ranges from 100,000 to $1x10^8$ copies/ml. **N\*** is the number of subjects. The p-value **p\*** is a product of the Mann-Whitney U-test and indicates statistical significance when it falls below 0.05. The correlation coefficient **r\*** is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a linear correlation between two arrays of numbers. The columns with headers **M\*** and **F\*** comprise the male and female subjects.

**Figure 5.12**

# Table 5.2

**Differentiation status of circulating Vδ1 T cells in subgroups of HBV patients:** The frequencies of Vδ1 T cells in patient subsets, based on varying ALT, viral load, age and gender.

| | Memory Phenotype | ALT <40 | ALT >40 | Low Viral Load | High Viral Load | Age < 35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|---|
| **Min %** | **Naïve** | 16.5 | 4.6 | 4.6 | 11.8 | 7.2 | 4.6 | 4.6 | 11.8 |
| | **$T_{CM}$** | 2 | 0 | 2 | 0 | 0 | 4.6 | 0 | 2 |
| | **$T_{EM}$** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | **$T_{EMRA}$** | 11.1 | 6.4 | 6.4 | 24.1 | 6.4 | 12.2 | 11.1 | 6.4 |
| **Max %** | **Naïve** | 48 | 50 | 48 | 50 | 50 | 48 | 50 | 48 |
| | **$T_{CM}$** | 36.4 | 22.3 | 36.4 | 15.9 | 33.3 | 36.4 | 33.3 | 36.4 |
| | **$T_{EM}$** | 27.3 | 48.2 | 31.7 | 48.2 | 48.2 | 27.3 | 31.7 | 48.2 |
| | **$T_{EMRA}$** | 70 | 77.3 | 77.3 | 72.6 | 72.6 | 77.3 | 77.3 | 72.6 |
| **Mean%** | **Naïve** | 4.2 | 23.7 | 27.9 | 29 | 29.1 | 27.3 | 28.3 | 28.2 |
| | **$T_{CM}$** | 16 | 12.4 | 16.9 | 7.9 | 12.7 | 16 | 14.2 | 14.3 |
| | **$T_{EM}$** | 8.6 | 17.8 | 12.4 | 14.3 | 16.1 | 9.3 | 10.7 | 14.5 |
| | **$T_{EMRA}$** | 7.5 | 46.1 | 42.8 | 48.8 | 42.1 | 47.4 | 46.8 | 43 |
| **SEM %** | **Naïve** | 4.2 | 6 | 4.2 | 7.3 | 5.5 | 5.1 | 7.2 | 4 |
| | **$T_{CM}$** | 4.1 | 2.9 | 3 | 2.9 | 3.7 | 3.4 | 4.3 | 3.2 |
| | **$T_{EM}$** | 2.7 | 5.8 | 2.9 | 8.7 | 5.3 | 3.1 | 4 | 4.7 |
| | **$T_{EMRA}$** | 7.5 | 8.4 | 6.8 | 8.1 | 8.2 | 7.4 | 8.4 | 7.4 |
| **N*** | **Naïve** | 9 | 8 | 12 | 5 | 10 | 8 | 7 | 10 |
| | **$T_{CM}$** | 9 | 8 | 12 | 5 | 10 | 8 | 7 | 10 |
| | **$T_{EM}$** | 9 | 8 | 12 | 5 | 10 | 8 | 7 | 10 |
| | **$T_{EMRA}$** | 9 | 8 | 12 | 5 | 10 | 8 | 7 | 10 |
| **p*** | **Naïve** | 0.4 | | 1 | | 1 | | 1 | |
| | **$T_{CM}$** | 0.6 | | 0.06 | | 0.5 | | 1 | |
| | **$T_{EM}$** | 0.2 | | 0.7 | | 0.5 | | 0.5 | |
| | **$T_{EMRA}$** | 0.7 | | 0.6 | | 0.7 | | 0.9 | |
| **r*** | **Naïve** | -0.2 | | -0.2 | | 0.07 | | - | |
| | **$T_{CM}$** | -0.1 | | -0.4 | | 0.02 | | - | |
| | **$T_{EM}$** | 0.2 | | 0.06 | | 0.03 | | - | |
| | **$T_{EMRA}$** | 0.1 | | 0.2 | | 0.2 | | - | |

**\* Low viral load** lies below 100,000 copies/ml, **high viral load** ranges from 100,000 to $1 \times 10^8$ copies/ml. **N\*** is the number of subjects. The p-value **p\*** is a product of the Mann-Whitney U-test and indicates statistical significance when it falls below 0.05. The correlation coefficient **r\*** is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a linear correlation between two arrays of numbers. The columns with headers **M\*** and **F\*** comprise the male and female subjects.

**5.5 Vδ2 and Vδ1 T cell frequencies in demographically-matched healthy controls**

Our patient cohort consisted of a group of individuals of different race as discussed in Chapter 2 and 3 and our knowledge of the healthy control subjects was also limited in this part of the study. Therefore, blood samples were obtained from healthy controls that were demographically-matched to our patient cohort in an effort to minimise the risk of false-positive results based on race and not HBV infection. The demographically-matched control blood samples were taken from 13 African, 14 Caucasian and 9 Asian healthy donors. PBMC preparation and surface staining was performed in the same manner as for all other subjects and the frequencies Vδ2 and Vδ1 T cells were quantified.

The mean frequencies of circulating Vδ2 T cells were 3+/-0.5%, 2.8+/-0.4% and 3.3+/-0.3% in African, Caucasian and Asian control subjects (Figure 5.13A). The mean frequencies of circulating Vδ1 T cells were 0.9+/-0.2%, 0.6+/-0.1% and 1.6+/-0.4% in African, Caucasian and Asian control subjects (Figure 5.13B). Performance of the Mann Whitney U test revealed that neither the frequencies of Vδ2 or Vδ1 T cells were significantly different between African, Caucasian or Asian control subjects, with all p values falling outside our confidence interval of 0.05.

These data suggest that the frequencies of circulating Vδ2 and Vδ1 T cells do not significantly differ between persons from the 3 ethnic groups investigated in this study and therefore, the differences observed here between HBV patients and control subjects are not biased by the demographics of the patient group.

**Figure 5.13**

**5.6 IFN-γ production by Vδ2 T cells in HBV infection**

**5.6.1 The frequencies of circulating IFN-γ-producing Vδ2 T cells are higher in HBV patients than in healthy control subjects**

PBMC from 10 HBV-infected subjects and 18 control subjects were incubated for 4 hours in medium alone or with PMA/I, in the presence of brefeldin A. This was followed by surface staining with PE-labelled anti-Vδ2 mAb and PE-Cy5-labelled anti-CD3 mAb and intracellular staining with FITC-labelled anti-IFN-γ to identify IFN-γ-producing Vδ2 T cells (Figure 5.14A). The frequency of IFN-γ-producing Vδ2 T cells was calculated as a percentage of the Vδ2 T cells and as a percentage of the total T cells. In the absence of stimulation, the mean frequencies of IFN-γ-producing Vδ2 T cells as a percentage of total Vδ2 T cells were 2.7+/-0.7% in controls and 6.7+/-1.4% in HBV patients (Figure 5.14B, p=0.01). After PMA/I stimulation, the mean frequencies of IFN-γ-producing Vδ2 T cells were 48.7+/-7.9% and 35.6+/-5.2%, respectively (Figure 5.14C, p=0.6).

In the absence of stimulation, the mean frequencies of IFN-γ-producing Vδ2 T cells, as a percentage of T cells, were 0.06+/-0.02% in controls and 0.4+/-0.2% in HBV (Figure 5.13D, p=0.005). In the presence of stimulation, the mean frequencies of IFN-γ-producing Vδ2 T cells as a percentage of T cells were 1+/-0.2% in controls and 1.6+/-0.4% in HBV (Figure 5.14E, p=0.2).

These data show that the frequencies of IFN-γ-expressing resting Vδ2 T cells isolated from HBV-infected individuals are significantly higher than those isolated from uninfected controls. However, Vδ2 T cells isolated from control subjects have similar responses to *in vitro* stimulation, as those cells from HBV-infected subjects.

**Figure 5.14**

**5.6.2 The frequencies of circulating IFN-γ-producing Vδ2 T cells in HBV patient groups**

The frequencies of IFN-γ-producing Vδ2 T cells were quantified as a percentage of total Vδ2 T cells and compared between 10 HBV patients within the study cohort, based on clinical parameters. The minimum, maximum and mean frequencies of circulating IFN-γ-producing Vδ2 T cells following incubation in medium alone or medium conditioned with PMA/I are shown in Table 5.3. The p and r values are also shown in Table 5.3. All patients investigated in this part of the study had low viral load (i.e. below 100,000 copies/ml) and therefore, a Mann-Whitney test was carried out to ascertain whether there were differences in the frequencies of IFN-γ-producing Vδ2 T cells between HBV-infected individuals with very low viral load (10 – 1000 copies/ml) and a low to medium viral load (10 – 10,000 copies/ml). Furthermore, only 2 HBV-infected individuals in this part of the study had ALT greater than 40 IU/ml and thus, Mann-Whitney tests could not be performed to ascertain differences between patients with low and high ALT because the test requires at least 3 data points in each group. Instead, the test was performed to compare frequencies in patients with ALT less than or greater than 35 IU/ml. All data are shown in Table 5.3. The frequencies of IFN-γ-producing Vδ2 T cells divided based on viral load, ALT, gender and age are shown in Figures 5.15A, B, C and D, respectively.

All data shown suggest that the frequencies of circulating IFN-γ-producing Vδ2 T cells do not correlate with viral load, disease severity, gender or age in this patient cohort.

**Figure 5.15**

**Table 5.3**

**The frequencies of circulating IFN-γ-producing Vδ2 T cells in HBV patient subsets:**

The frequencies of IFN-γ-producing Vδ2 T cells within divisions of the HBV patient study cohort, based on varying ALT, viral load, age and gender.

| | Condition* | ALT <35 | ALT >35 | Low Viral Load | Low to Medium Viral Load | Age < 35 | Age 35-50 | M* | F* |
|---|---|---|---|---|---|---|---|---|---|
| Min % | Medium | 1.6 | 2.2 | 1.6 | 2.2 | 1.6 | 2.4 | 2.6 | 1.6 |
| | PMA/I | 22.8 | 22.9 | 22.8 | 22.9 | 22.8 | 23.6 | 22.9 | 22.8 |
| Max % | Medium | 15.2 | 10.7 | 9.5 | 15.2 | 15.2 | 6.4 | 15.2 | 9.5 |
| | PMA/I | 44.3 | 75.6 | 75.6 | 44.3 | 75.6 | 44.6 | 75.6 | 44.6 |
| Mean % | Medium | 7 | 6.2 | 6 | 7.6 | 7.9 | 3.8 | 9.5 | 4.8 |
| | PMA/I | 36.8 | 37.1 | 40.2 | 33 | 36.7 | 37.5 | 40.1 | 34.5 |
| SEM % | Medium | 2 | 2.2 | 1.4 | 3.2 | 2 | 1.1 | 2.6 | 1.3 |
| | PMA/I | 3.7 | 12.8 | 8.9 | 5 | 8.2 | 6 | 12.4 | 4.1 |
| N* | Medium | 6 | 4 | 6 | 4 | 7 | 3 | 4 | 6 |
| | PMA/I | 5 | 4 | 5 | 4 | 6 | 3 | 5 | 4 |
| p* | Medium | 1 | | 0.8 | | 0.4 | | 0.1 | |
| | PMA/I | 0.7 | | 0.9 | | 0.5 | | 1 | |
| r* | Medium | 0.02 | | 0.3 | | 0.01 | | - | |
| | PMA/I | -0.2 | | -0.3 | | 0.5 | | - | |

\* **Condition** refers to the 4 hour incubation of the cells in medium alone or, in medium treated with PMA/I, prior to staining for flow cytometry. **Low viral load** is below 1,000 copies/ml, **low to medium viral load** ranges from 1,000 to 10,000 copies/ml. **N\*** is the number of subjects. The p-value **p\*** is a product of the Mann-Whitney U-test and indicates statistical significance when it falls below 0.05. The correlation coefficient **r\*** is a product of the Spearman Correlation test and a value close to 1 or -1 indicates a linear correlation between two arrays of numbers. The columns with headers **M\*** and **F\*** comprise the male and female subjects, respectively.

**5.6.3 The frequencies of circulating IFN-γ-producing Vδ2 T cells were lower following incubation with HBsAg**

Since frequencies of IFN-γ-expressing resting Vδ2 T cells are higher in HBV, a preliminary experiment to ascertain whether HBV can induce IFN-γ production by Vδ2 T cells was performed. PBMC from 3 healthy donors were incubated in medium supplemented with FBS, medium supplemented with sera from healthy control subjects or from HBV patients with a viral load above $10^6$ copies/ml or, medium containing 1 μg of HBV surface antigen (HBsAg) or 3 μg of HBsAg, for 24 hours. Following this incubation, cells were treated with brefeldin A and incubated for a further 4 hours. Surface and intracellular staining with PE-labelled anti-Vδ2, PerCP-labelled anti-CD3 and FITC-labelled IFN-γ was subsequently performed.

The mean frequencies of IFN-γ-expressing Vδ2 T cells, as a percentage of total Vδ2 T cells, were 14.5+/-3.6% following incubation in medium supplemented with FBS. Contrary to our hypothesis, the frequencies of IFN-γ-expressing Vδ2 T cells were lower following 24 hour incubation with medium supplemented with sera from healthy control subjects or HBV patients or, medium containing 1μg of HBV surface antigen (HBsAg) or 3μg of HBsAg, with mean frequencies of 10.3+/-2.2%, 9.5+/-2.9%, 6.6+/-1.7% and 6.3+/-1.6%, respectively (Figure 5.16A). Paired t-tests revealed that only the differences observed in the presence of HBV serum were statistically significant (p=0.04)

As shown in Chapter 4, the serum from HBV patients is not a definite source of HBV antigens but the commercial HBsAg used in these experiments is intact. Therefore, these data suggest that Vδ2 T cells do not produce IFN-γ as a direct response to HBsAg, and perhaps, another viral epitope or cell of the host immune system triggers the enhanced numbers of IFN-γ-expressing Vδ2 T cells observed in HBV infection. Furthermore, these data suggest that HBsAg causes a decrease of more than 50% in the frequencies of IFN-γ-expressing Vδ2 T cells. It appears that HBsAg may have inhibited IFN-γ expression by Vδ2 T cells under the conditions provided here but in the HBsAg$^+$ carriers studied in sections 5.5.1 and 5.5.2, another arm of the immune system may counteract this inhibition and lead to overall higher frequencies of IFN-γ-expressing Vδ2 T cell and immune control. This experiment is only a preliminary investigation of the immune mechanisms in HBV and further work must be carried out before any conclusions can be made.

**5.7 NKG2D expression by Vδ2 T cells in HBV**

Surface staining with PE-labelled anti-Vδ2 mAb, PerCP-labelled anti-CD3 mAb and APC-labelled anti-NKG2D was performed to identify NKG2D-expressing Vδ2 T cells in the peripheral blood. Frequencies of NKG2D$^+$ Vδ2 T cells were similar in HBV-infected subjects and controls with mean frequencies of 43% and 46.7%, respectively (p=0.6, Figure 5.17A). However, the mean fluorescence intensity of the receptor on the surface of Vδ2 T cells in HBV infection (mean MFI: 27.2+/-3.2) was lower than that observed on the surface of such cells in uninfected controls (mean MFI: 83+/-42.6, p=0.1, Figure 5.17B).

These data suggest that the frequencies of NKG2D-expressing Vδ2 T cells are intact in HBV but, the level of NKG2D surface expression by such cells may be impaired.

**Figure 5.16**

**Figure 5.17**

## 5.8 Bonferroni correction method

Since the present study involved the analysis of the frequencies of multiple $\gamma\delta$ T cell subpopulations in patient and control subject groups, it is possible that statistically-significant differences in the two subject groups will be identified by chance alone.

For the phenotypic study of $\gamma\delta$ T cells, 14 tests were performed as shown in Table 3.5. By applying the Bonferroni correction method to the p-values obtained from the Mann-Whitney U tests, the most substantial results could be filtered out. Both the p and $p_c$ values for all tests are listed in Table 5.4. Following this correction, the differences in the frequencies of total V$\delta$2 T cells and naïve V$\delta$1 cells, only, were shown to be statistically significant with $p_c$-values of 0.01 and 0.003, respectively (Table 5.4).

These data provide strong evidence that the frequencies of circulating V$\delta$2 T cells are higher in HBV infection while, the frequencies of naïve V$\delta$1 T cells appear to be depleted.

**Table 5.4**

**Statistical analysis of the phenotypical differences observed in γδ T cell subpopulations in HBV infection:** The p-values and corrected p-values for all 14 statistical tests performed in the phenotypical study of γδ T cell subpopulations in HBV infection.

| TEST | *Change in HBV | p value | $p_c$ value |
|---|---|---|---|
| Vδ2 T cells (as % of T cells) | Higher | <0.0001 | *0.014* |
| Vδ1 T cells (as % of T cells) | Higher | 0.02 | 0.28 |
| CD56+Vδ1 T cells (as % of T cells) | Higher | 0.01 | 0.14 |
| Naïve Vδ2 T cells (as % of Vδ2 T cells) | Lower | 0.006 | 0.084 |
| $T_{CM}$ Vδ2 T cells (as % of Vδ2 T cells) | Lower | 0.03 | 0.42 |
| $T_{EMRA}$ Vδ2 T cells (as % of Vδ2 T cells) | Higher | 0.02 | 0.28 |
| Naïve Vδ1 T cells (as % of Vδ1 T cells) | Lower | 0.0002 | *0.0028* |
| $T_{CM}$ Vδ1 T cells (as % of Vδ1 T cells) | Higher | 0.02 | 0.28 |
| $T_{EM}$ Vδ1 T cells (as % of Vδ1 T cells) | Higher | 0.004 | 0.056 |
| $T_{EMRA}$ Vδ1 T cells (as % of Vδ1 T cells) | Higher | 0.02 | 0.28 |

**\***This column indicates whether the frequencies of cells are higher or lower in HBV-infected subjects, compared to control subjects.
Significant results after Bonferroni correction ($p_c$ values) are highlighted in italics.

## 5.9 Discussion

This study investigated the frequencies of circulating γδ T cell subpopulations in patients with asymptomatic HBV infection to identify which subsets of γδ T cells are expanded in HBV. Since the majority of the patients had a viral load below 20,000 and an ALT below 40, the group of patients were considered to have minimal or no liver disease and controlled viral replication. Therefore, although all patients are HBsAg carriers, they represent a model of immune control in the absence of severe immune pathology.

An association between Higher frequencies of γδ T cells and seroconversion has previously been identified in HBV thus implicating these cells in the resolution of HBV infection while, the inability to clear the virus has been linked to deficiencies in circulating Vδ2 T cells (Sing *et al.* 1998; Chen *et al.* 2008). Furthermore, impaired IFN-γ production and cytotoxic activity by Vδ2 T cells was observed in chronic HBV with the most significant deficiencies observed in those with the most severe liver disease (Chen *et al.* 2008).

Expansions of circulating Vδ2 T cells and to a lesser extent, Vδ1 T cells, have been found in our patient cohort, compared to a group of uninfected controls. It has also been found that effector memory Vδ2 and Vδ1 T cells were more abundant in the peripheral blood of HBV-infected patients than in that of uninfected control subjects while naïve subsets were substantially lower. This suggests that the expanded populations of Vδ2 and Vδ1 T cells are actively involved in the immune control of HBV in these patients. The quantification of IFN-γ- and NKG2D-expressing Vδ2 T cells in HBV-infected patients and uninfected controls revealed that the frequencies of IFN-γ-expressing Vδ2 T cells are higher in HBV but the frequencies of NKG2D-expressing Vδ2 T cells are not. While the frequencies of NKG2D$^+$ Vδ2 T cells are unchanged in the HBV patients, the surface expression of NKG2D (MFI) by Vδ2 T cells is lower. Together with previous work by Chen *et al.* (2008) which has revealed that depletions of Vδ2 T cells correlate with an increase in liver inflammation, our data suggest that higher frequencies of Vδ2 T cells are required to control HBV infection and prevent immune-mediated damage. The mechanism by which this occurs may involve the IFN-γ-mediated downregulation of NKG2D. IFN-γ has been shown to inhibit NKG2D ligand expression in melanoma and could employ a similar mechanism in HBV so that IFN-γ-mediated viral clearance can prevail and liver damage from

cytolytic activity is limited (Schwinn *et al.* 2009). As discussed in Chapter 4, non-cytolytic control of virus infection is preferable when large numbers of cells in large vital organs are infected (Guidotti & Chisari 2001). γδ T cells have previously been implicated in the liver injury associated with HCV with Th1-biased Vδ1 T cells identified as the predominant γδ T cell subset to infiltrate HCV-infected livers and therefore, need to be controlled in order to facilitate viral clearance without causing liver damage (Tseng *et al.* 2001; Nikolopoulou *et al.*1995). Furthermore, NKG2D has been implicated in NK cell-mediated hepatocyte injury in virus-induced hepatitis and liver failure in mice thus showing why its regulation is critical in the avoidance of severe liver disease (Zou *et al.* 2010). Therefore, it appears that the expansions of IFN-γ-expressing Vδ2 T cells with lower NKG2D expression may represent a repertoire of Vδ2 T cells that can control HBV replication without causing significant liver injury. Furthermore, the application of the Bonferroni correction method to our data have informed us that the expansions of Vδ2 T cells are highly significant.

The elevated frequencies of circulating Vδ1 T cells, although not as significant as those observed for Vδ2 T cells, suggest that these cells also play a role in the antiviral immune response against HBV, possibly via the production of IFN-γ and TNF-α (Spada *et al.* 2000). Their active role in immunity in these patients is evident in the expansions of their effector memory repertoires. The production of IFN-γ by these cells might also contribute to the inhibition of NKG2D-mediated cytotoxicity. Furthermore, the expansion of the CD56^{+} repertoire of Vδ1 T cells in HBV suggests that they are part of the IFN-γ-producing NT cell population which was already found to be expanded in HBV. Since Vδ1 T cells have previously been implicated in the pathogenesis of HCV infection and arthritis, they might require strict regulation in asymptomatic HBV infection (Tseng *et al.* 2001; Bank *et al.* 2002). However, they have also previously been shown to regulate inflammatory responses of CD8^{+} T cells in the small intestine via the suppression of IFN-γ, granzyme-B and NKG2D expression (Bhagat *et al.* 2008). Therefore, further work is required to determine whether this γδ T cell subset plays a predominant antiviral or regulatory role in the control of HBV infection. Future work might also examine the role of Vδ3 T cells in HBV-infected liver. This γδ T cell subpopulation is most prevalent in liver and therefore, may be of particular importance in controlling HBV infection but its investigation was not feasible for this study due to limited access to liver samples.

Since higher frequencies of circulating IFN-γ-producing Vδ2 T cells have been found in HBV patients in the absence of stimulation, the potential of these increases were mimicked *in vitro* using HBV patient serum or HBsAg. However, it was found that the frequencies of IFN-γ-producing Vδ2 T cells were lower following incubation with HBV patient serum or HBsAg, suggesting that HBV proteins may have an immunomodulatory effect on Vδ2 T cells. Chen *et al.* (2008) previously found depletions of Vδ2 T cells and reduced IFN-γ expression in chronic HBV. They found the greatest depletions in patients with the highest viral load suggesting that HBV proteins may have detrimental effects on Vδ2 T cells. Since, higher frequencies of Vδ2 T cells have been found in a group of HBV patients with low viral load, this study may have identified an environment in which HBV proteins cannot reach sufficient levels to affect Vδ2 T cell frequencies or functions. Therefore, it is proposed that the expansions of Vδ2 T cells observed in our study group may be an indirect consequence of HBV infection and a more direct effect of a highly activated cell population, most likely NT cells.

The data obtained in this study suggest that γδ T cells contribute to the control of HBV replication in these HBV patients, most likely via the production of IFN-γ. It is proposed that the expansion of such γδ T cells in HBV infection may be in response to other innate lymphocytes, rather than the virus. Since no significant differences were observed in CD1 expression in Chapter 3, it is also proposed that the expanded Vδ2 and Vδ1 T cells are not CD1-restricted populations of γδ T cells. Furthermore, the frequencies of Vδ2 and Vδ1 T cells were similar between control subjects of different race suggesting that our findings are not significantly influenced by the demographics of our patient group.

From these findings, it is proposed that innate T cells, most notably CD56$^+$ T cells and Vδ2 T cells, are crucial to the control of HBV replication and such cells may be used as the basis for future immunotherapies.

# Chapter 6

# Reciprocal interactions between Vδ2 T cells and epithelial cells

## 6.1 Introduction

HCC is a major global health problem and a potential endpoint of HBV infection (Bruix & Llovet 2003; Stefaniuk *et al.* 2010; Paraskevi *et al.* 2006; Fattovich *et al.* 2004). There are limited curative options for HCC and since surgical resection is only possible for small HCC malignancies, liver transplantation is often the best option (Jinushi *et al.* 2005; Stefaniuk *et al.* 2010; De Villa & Lo 2007). Treatment of HBV can prevent the development of liver disease and HBV-associated HCC but HBV treatments are not always effective, durable or tolerated as discussed in Chapter 1. Therefore, new immunotherapies are required for the successful treatment of HBV-associated liver cancer.

Vγ9Vδ2 T cells constitute the majority of γδ T cells in the peripheral blood and have a predominant Th1 cytokine profile (produce IFN-γ, IL-2 and TNF-α), elicit cytotoxic responses and have antigen presentation capabilities (Brandes 2005; Dudal *et al.* 2006; Eberl *et al.* 2009). While Vγ9Vδ2 T cells can directly modulate adaptive immune responses via cytokine production, they can also indirectly activate αβ T cell responses via activation of DC maturation. Blocking experiments have revealed that the Vγ9Vδ2 T cell induction of DC maturation is mediated, at least in part, by IFN-γ and TNF-α (Ismaili *et al.* 2002; Conti *et al.* 2005; Dunne *et al.* 2010). The functional plasticity of Vγ9Vδ2 T cells and the relative ease at which they can be cultured and expanded *in vitro* positions them as ideal candidates for immunotherapy. Since Vδ2 T cells usually co-express the Vγ9 chain and vice versa, the identification of Vγ9Vδ2 T cells can be achieved using an antibody against just one of the chains (Eberl *et al.* 2009; Chapter 5 of this thesis). The mAb against Vδ2 was predominantly used in this study and therefore, these cells are referred to as Vδ2 T cells throughout this work.

Since expansions of Vδ2 T cells were identified in HBV patients with controlled infection and little or no liver disease, it was proposed that these innate T cells are crucial to the control of HBV infection and in limiting progression to liver cirrhosis and HCC. Here, it was hypothesised that such cells may have potential for the treatment of HBV-associated carcinoma.

Although Vδ2 T cells are the main γδ T cell subset in peripheral blood, they still only constitute 0.5-4.5% of circulating lymphocytes and must be expanded *in vitro* in order to gain sufficient numbers to induce a therapeutic immune response. This can be achieved by stimulating whole PBMC with phosphoantigens such as HMBPP or BrHPP

or with aminobiphosphonates such as zoledronate, while using IL-2 as a growth factor. This induces significant expansion of Vδ2 T cells (Dieli *et al.* 2007; Bennouna *et al.* 2008; Wilhelm *et al.* 2003; Eberl *et al.* 2002; Abe *et al.* 2009). Various pyrophosphates or aminobisphosphonates have been used to expand and activate Vδ2 T cells in cell-based therapies that are being tested in several advanced stage malignancies (Eberl *et al.* 2009; Dieli *et al.* 2007; Bennouna *et al.* 2008; Wilhelm *et al.* 2003). Recognition of phosphoantigens is believed to be mediated through the Vδ2-TCR and these non-peptide antigens induce significant activation and expansion of the cells (Eberl *et al.* 2002). NKG2D expression is also believed to be crucial in the lysis of tumour cells by BRHPP+IL-2 or Zoledronate+IL-2-expanded Vγ9Vδ2 T cells (Bouet *et al.* 2008; Wrobel *et al.* 2007; Das *et al.* 2001).

The importance of γδ T cells in anti-tumour immune responses is evident in their isolation from the tumour infiltrates in breast, bladder, kidney, lung and ovarian cancer (Malkovsky 2003). Previous studies in our lab have shown that Vδ2 T cell frequencies are depleted in tumour-bearing livers (Kenna *et al.* 2004). They kill primary cultures of tumour cells including renal, hepatocellular and colorectal carcinoma cells (Viey *et al.* 2005; Mattarollo *et al.* 2007) and a variety of tumor cell lines (Fisch *et al.* 1990; Wrobel *et al.* 2007; Vantourout *et al.* 2009). This study aimed to investigate if Vδ2 T cells recognise and respond to HBV-associated HCC-derived cells i.e. Hep3B cells, and to determine whether such cells can inhibit or suppress Vδ2 T cell functions.

The pyrophosphate HMBPP together with IL-2 was used to propagate Vδ2 T cells *in vitro* and subsequently investigated the effects of a HBV-associated HCC cell line, Hep3B, on the phenotype and cytokine secretion profiles of the expanded Vδ2 T cells. The expression of HBsAg by this cell line makes it an ideal model for studying the possible therapeutic effects of Vδ2 T cells in HBV-associated HCC *in vitro* (Knowles *et al.* 1980).

Epithelial cells can potentially stimulate Vδ2 T cells via the expression of MICA/B or HLA-E which bind to NKG2D or NKG2A/C, respectively, on the surface of the Vδ2 T cells. In addition to ligation of the Vγ9Vδ2 TCR by phosphoantigen, ligation of NKG2D or NKG2C (dimerized with CD94) can induce the activation of Vδ2 T cells. On the other hand, ligation of NKG2A (dimerized with CD94) would inhibit their activation. The effects of co-incubation of HMBPP/IL-2-expanded Vγ9Vδ2 T cells and Hep3B cells on the expression of receptor-ligand pairs involved in T cell activation

and inhibition was evaluated to determine the immunotherapeutic potential of these cells in HCC. The effects of Hep3B cells on the expression of the antigen presentation molecule HLA-DR by Vδ2 T cells was also assessed to determine if HCC can modulate or suppress the capabilities Vδ2 T cells have in antigen presentation to αβ T cells. The expression of the adhesion molecule CD54 (ICAM-1) by Hep3B cells was also investigated following co-culture. In addition, the expression levels of a variety of inflammatory and immunomodulatory cytokines was investigated to analyse the reciprocal effects of Vδ2 T cell and Hep3B cells in co-culture. The colorectal carcinoma-derived cell line HT29 and the melanoma-derived cell line GRM were used as additional target cell populations and served as control epithelial cell lines. Fresh PBMC and PHA/IL-2-expanded αβ T cells were used as controls for expanded Vδ2 cells. PHA/IL-2-expanded αβ T cells served as an expanded population of non-Vδ2 T cells and also served as a control for any αβ T cell contamination of enriched Vδ2 cell populations. PBMC served as a control for the stimulated / expanded cells by providing resting Vδ2 T cell and αβ T cell populations which could be individually identified by flow cytometry.

Overall, the aim of this preliminary study was to gain knowledge of the immunotherapeutic potential of HMBPP/IL-2-expanded Vδ2 T cells in HBV-associated HCC and to identify any possible mechanisms by which HCC may subvert their effector functions. However, it was kept in mind that a HCC-derived cell line may not be representative of HCC and that this study should purely serve as a platform for further studies where such cellular interactions may be investigated in HCC patients.

**6.2 The effects of epithelial cells on Vδ2 T cell surface marker expression**

Vδ2 T cells were expanded from the PBMC of 4-5 donors, using the pyrophosphate antigen HMBPP in combination with IL-2 as described in Chapter 2. The Hep3B, HT29 and GRM cell lines were maintained in culture as described in Chapter 2. Each epithelial cell line was subsequently cultured in the absence or presence of 14-day old expanded Vδ2 T cells at 1:1 ratios. After 24 hours of co-culture, culture plates were centrifuged and supernatants were frozen for subsequent cytokine analysis. The pelleted cells were re-suspended in PBA and surface stained to examine NKG2A, HLA-DR and NKG2D surface expression by Vδ2 T cells (Table 2.19). The use of mAbs specific for BerEP4, Vδ2 and CD3 facilitated the exclusion of epithelial cells and ensured that surface marker expression was only examined on the Vδ2 T cell population (Figures 6.1A–E).

**6.2.1 The levels of NKG2A expression by HMBPP-expanded Vδ2 T cells are unchanged after co-culture with epithelial cells**

Surface staining with FITC-labelled anti-BerEP4, PE-labelled anti-Vδ2, PerCP-labelled anti-CD3 and APC-labelled NKG2A was performed to identify epithelial cells and Vδ2 T cells and to ascertain whether the level of NKG2A expression by the Vδ2 T cells had changed after co-culture with Hep3B, HT29 or GRM cells (Figure 6.2A).

The frequencies of 14-day old expanded Vδ2 T cells expressing NKG2A ranged from 21.6% to 99.1% (mean 56+/-13%) of total Vδ2 T cells. This did not change significantly after co-culture with Hep3B cells with frequencies ranging from 21.45% to 100% (mean 62+/-17%). Similar frequencies were also observed following co-culture with HT29 cells (range; 23.5-99.4%, mean; 71.8+/-15.5%) or with GRM cells (range; 50.8-100%, mean; 87.2+/-9.4).

The mean fluorescence intensity (MFI) of NKG2A staining by Vδ2 T cells ranged from 32.4 to 138.4 (mean 61+/-18) and from 14.7 to 178.1 (mean 64+/-27) when measured after culture in the absence and presence of Hep3B cells, respectively (Figure 6.2B). When measured after culture in the presence of HT29 cells, NKG2A expression ranged from 33 to 620.1 (mean; 169.8+/-113.4, Figure 6.2C) and, ranged from 27.8 to 82.4 (mean; 47.4+/-10) following co-culture with GRM cells (Figure 6.2D). A paired t-test revealed that no statistically significant changes in the levels of NKG2A expression

by Vδ2 T cells occurred following co-culture with Hep3B, HT29 or GRM cells (p=0.4, 0.2 and 0.2).

These data show that expression of the inhibitory receptor NKG2A by Vδ2 T cells is not altered by co-culture with Hep3B, HT29 or GRM cells.

## 6.2.2 The levels of HLA-DR expression by HMBPP-expanded Vδ2 T cells are unchanged after co-culture with epithelial cells

Surface staining with FITC-labelled anti-BerEP4, PE-labelled anti-Vδ2 and PerCP-labelled anti-CD3 was performed to identify epithelial cells and Vδ2 T cells and further surface staining with FITC-labelled anti-HLA-DR in combination with PE-labelled anti-Vδ2 was performed to ascertain whether the level of HLA-DR expression by the Vδ2 T cells had changed after co-culture with Hep3B cells (Figure 6.3A).

The frequencies of 14-day old Vδ2 T cells expressing HLA-DR ranged from 59.4% to 97.5% (mean 79+/-6%) of total Vδ2 T cells. This did not change significantly after co-culture with Hep3B cells with frequencies ranging from 88.2% to 97.2% (mean 88+/-4%, Figure 6.3A). Similar frequencies were also observed following co-culture with HT29 cells (range; 82.05-99.5%, mean; 73.8+/-18%) or with GRM cells (range; 92.4-99.2%, mean; 96+/-1.2%).

The MFI of HLA-DR expression by Vδ2 T cells also did not change and ranged from 26.9 to 190.8 (mean 107+/-33) and from 28.1 to 197 (mean 112+/-38, Figure 6.3B) when measured after culture in the absence and presence of Hep3B cells, respectively (Figure 6.3C, J, p=0.3). Furthermore, co-culture with HT29 or GRM cells did not alter HLA-DR expression levels by Vδ2 T cells with MFI values ranging from 4.8 to 98.77 (mean; 51.9+/-17.2, p=0.3) and from 38.1 to 193.7 (mean; 90.3+/-31.8, p=0.4), respectively.

These data show that expression of the antigen presentation marker HLA-DR by Vδ2 T cells is not altered by co-culture with Hep3B, HT29 or GRM cells.

## 6.2.3 The levels of NKG2D expression by HMBPP-expanded Vδ2 T cells are lower after co-culture with epithelial cells

Surface staining with FITC-labelled anti-BerEP4, PE-labelled anti-Vδ2, PerCP-labelled anti-CD3 and APC-labelled anti-NKG2D was performed to examine expression levels of NKG2D by Vδ2 T cells, in the absence and presence of epithelial cells (Figure 6.4A).

The frequencies of 14-day old Vδ2 T cells expressing NKG2D ranged from 75.7% to 100% of total Vδ2 T cells (mean 89.1+/-4.9%). This did not change significantly after co-culture with Hep3B cells with frequencies ranging from 79.2% to 100% (mean 73.8+/- 16%). Similar frequencies of NKG2D-expressing Vδ2 T cells were also observed following co-culture with HT29 cells (range; 77.2-99.6%, mean; 89+/-3.9%) or GRM cells (range; 98.2-100%, mean; 99.6+/-0.4%).

The MFI of NKG2D expression by Vδ2 T cells ranged from 109.2 to 390.4 (mean 216.3+/-50) but ranged from 55.2 to 164.9 (mean 83.2+/-22.2) when measured after culture in the presence of Hep3B cells (Figure 6.4B). A paired t-test revealed that these changes were not statistically significant (p=0.07). However, the lower NKG2D expression by Vδ2 T cells, observed following co-cultured with HT29 cells was statistically significant (range; 35.2-84.3, mean; 64+/-8.9, p<0.05, Figure 6.4C). Significantly lower levels of NKG2D expression by Vδ2 T cells were also recorded following co-culture with GRM cells (range; 45.7-163.6, mean; 86.3+/-23.5, p<0.05, Figure 6.4D).

These data show that the levels of NKG2A and HLA-DR expression by HMBPP expanded Vδ2 T cells are unchanged after co-culture with epithelial cells, while NKG2D expression is consistently lower, suggesting a possible downregulation of this activating receptor by several different carcinoma-derived cell lines.

## 6.2.4 The levels of NKG2D expression by PHA and IL-2-expanded T cells are lower after co-culture with epithelial cells

To determine if the reduction in NKG2D expression was restricted to HMBPP-enriched Vδ2 T cells, T cells were also expanded using PHA and IL-2 from PBMC as described in Chapter 2. The majority of such cells were αβ T cells with a minimal enrichment of Vδ2 T cells as shown in Figure 6.1F. Fourteen-day old PHA/IL-2-expanded T cells from 2 healthy donors were then cultured alone or in the presence of equal numbers of Hep3B, HT29 or GRM cells for 24 hours. Hep3B cells were only co-cultured with PHA/IL-2-expanded T cells from 1 donor due to limited cell numbers. Surface staining with FITC-labelled anti-BerEP4, PerCP-labelled anti-CD3 and APC-labelled anti-NKG2D was performed to identify epithelial cells and T cells and, to ascertain whether the level of NKG2D expression by the PHA/IL-2-expanded T cells had changed after co-culture with epithelial cells.

The mean MFI of NKG2D expression by PHA and IL-2-expanded T cells was 112.5+/-22.5. This was not altered by co-culture with Hep3B cells (140, Figure 6.4E). However the NKG2D expression was reduced following co-culture with HT29 (mean MFI: 60+/-9.8) or GRM cells (mean MFI: 49.9+/-4.9).

These data are only preliminary but, they suggest that the putative NKG2D downregulation observed above may not be restricted to HMBPP-expanded V$\delta$2 T cells and, that these cancers may inhibit NKG2D on all T cells expressing it.

## 6.2.5 The levels of NKG2D expression by fresh T cells are lower after co-culture with epithelial cells

To determine if the reduction in NKG2D expression was restricted to expanded V$\delta$2 T cells, fresh PBMC were prepared from the buffy coat packs of 2 donors and cultured alone or in the presence of HT29 or GRM cells for 24 hours. Only results shown to be statistically significant were investigated further and therefore, the Hep3B cells were not used for this part of the study. Surface staining with FITC-labelled anti-BerEP4, PE-labelled anti-V$\delta$2, PerCP-labelled anti-CD3 and APC-labelled anti-NKG2D was performed to identify epithelial cells and V$\delta$2 T cells and, to ascertain whether the level of NKG2D expression by the fresh V$\delta$2 T cells had changed after co-culture with HT29 cells.

The MFI values of NKG2D expression by fresh V$\delta$2 T cells were 81.6 and 73.4 in those PBMC incubated alone, 35.9 and 37.7 in those PBMC incubated with HT29 cells, and 31.6 and 30.2 in those PBMC cultured with GRM cells (Figure 6.4F). This shows that co-culture with epithelial cells causes lower levels of NKG2D expression by both fresh and expanded V$\delta$2 T cells.

NKG2D expression by the $\alpha\beta^+$ T cells in the PBMC was also quantified. MFI values of 47.4 and 42.8 on $\alpha\beta^+$ T cells incubated alone, 32.6 and 25 on $\alpha\beta^+$ T cells co-cultured with HT29 cells and 22.4 and 21 on $\alpha\beta^+$ T cells co-cultured with GRM cells, indicated NKG2D is also lower on fresh $\alpha\beta^+$ T cells after incubation with HT29 cells (Figure 6.4F).

These data show that the reduction in NKG2D expression is not specific to expanded T cells and that fresh V$\delta$2$^+$ and $\alpha\beta^+$ T cells may be subjected to the same mechanism of immune subversion by carcinoma-derived cells. Overall, it appears that

colorectal carcinoma and melanoma-derived cells may downregulate NKG2D-mediated cytotoxicity as a means of evading host immune responses.

**Figure 6.1**

**Figure 6.2**

**Figure 6.3**

**Figure 6.4**

**Figure 6.4**

**6.3 The effects of Vδ2 T cells on epithelial cell surface marker expression**

The Hep3B, HT29 and GRM cell lines were maintained in culture as described in Chapter 2. Vδ2 T cells were expanded from the PBMC of 3-5 donors, using the pyrophosphate antigen HMBPP in combination with IL-2 as described in Chapter 2. Each type of epithelial cell was subsequently cultured in the absence or presence of 14-day old expanded Vδ2 T cells at 1:1 ratios. After 24 hours of co-culture, culture plates were centrifuged and supernatants were frozen for subsequent cytokine analysis. The pelleted cells were re-suspended in PBA and surface stained to examine CD54, HLA-E and MICA/B surface expression by epithelial cells. The use of mAbs specific for BerEP4 and CD3 facilitated the exclusion of PBMC and, ensured that surface marker expression was only examined on the carcinoma-derived epithelial cells (Figures 6.1A-D).

**6.3.1 The levels of CD54 expression by epithelial cells are higher following co-culture with HMBPP-expanded Vδ2 T cells**

Surface staining with FITC-labelled anti-BerEP4, PerCP-labelled anti-CD3 and APC-labelled anti-CD54 was performed to identify epithelial cells and T cells and, to ascertain whether the level of CD54 expression by epithelial cells had changed after co-culture with Vδ2 T cells (Figure 6.5A).

The MFI of CD54 expression by Hep3B cells ranged from 19.8 to 318.6 with a mean MFI of 80.6+/-59.5. Following co-culture in the presence of HMBPP-expanded Vδ2 T cells, the MFI ranged from 21.9 to 250.3 (mean; 90.8+/-45.3, Figure 6.5B). Higher CD54 expression by Hep3B cells was observed after co-culture with Vδ2 T cells from 4/5 donors and lower after co-culture with Vδ2 T cells from 1/5 donors. A paired t-test revealed that these changes were not statistically significant (p=0.4).

The MFI of CD54 expression by HT29 cells ranged from 26.9 to 50.4 with a mean MFI of 35.9+/-5.5. Following co-culture in the presence of HMBPP-expanded Vδ2 T cells, the MFI ranged from 129.3 to 1122.6 (mean; 547+/-166.6, Figure 6.5C). Higher CD54 expression by HT29 cells was observed following co-culture with Vδ2 T cells from 5/5 donors (p=0.02).

The MFI of CD54 expression by GRM cells ranged from 18.7 to 507.2 with a mean MFI of 121.7+/-96.5. Following co-culture in the presence of HMBPP-expanded Vδ2 T cells, the MFI ranged from and from 340.2 to 913.6 (mean; 558.9+/-101, Figure

6.5D). Higher levels of CD54 expression by GRM cells were observed following co-culture with Vδ2 T cells from 5/5 donors (p=0.02).

These data show that co-culture with HMBPP-expanded Vδ2 T cells leads to higher levels of CD54 expression by carcinoma-derived cells, particularly those derived from colorectal carcinoma and melanoma. These findings suggest that HMBPP-expanded Vδ2 T cells may increase the visibility of certain tumours and enhance anti-tumour immune responses, therefore, making these cells potential candidates for immunotherapies against these cancers.

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### 6.3.2 The levels of CD54 expression by epithelial cells are higher following co-culture with PHA and IL-2-expanded T cells

To determine if the enhanced CD54 expression on epithelial cells was restricted to co-culture with HMBPP-enriched Vδ2 T cells, T cells were also expanded using PHA and IL-2 from PBMC as described in Chapter 2. The majority of such cells were αβ T cells with a mean 12.8% of Vδ2 T cells as shown in Figure 6.1F. HT29 or GRM cells were cultured alone or in the presence of equal numbers of 14-day old PHA and IL-2-expanded T cells from each of 2 donors for 24 hours. Only PHA and IL-2-expanded T cells from 1 donor were co-cultured with equal numbers of Hep3B cells due to limited cell numbers. Surface staining with FITC-labelled anti-BerEP4, PerCP-labelled anti-CD3 and APC-labelled anti-CD54 was performed to identify epithelial cells, exclude all T cells and any PBMC from analysis and, to ascertain whether the level of CD54 expression by epithelial cells had changed after co-culture with PHA and IL-2-expanded T cells

CD54 expression by HT29 cells rose from 26.89 to a mean MFI of 425.91+/-35.72 following co-culture with PHA and IL-2-expanded T cells (Figure 6.5E). CD54 expression by GRM cells rose from 18.72  to a mean MFI of 102.46+/-46.6 following co-culture and the MFI of Hep3B cells (318.56) almost doubled following a single co-culture experiment with PHA and IL-2-expanded T cells (611.91, Figure 6.5E). Since these experiments were only performed using PHA and IL-2-expanded T cells from 1–2 donors, paired T test could not be performed because this significance test requires a minimum of 3 pairs of data.

These data suggest that PHA and IL-2 expanded T cells can enhance CD54 expression by epithelial cells to a level comparable with that following co-culture with

HMBPP and IL-2-expanded T cells. However, it must be noted that these data are preliminary and must be repeated with PHA and IL-2 expanded T cells from more donors.

### 6.3.3 The levels of CD54 expression by HT29 and GRM cells are higher following co-culture with freshly isolated PBMC

To determine if the enhanced CD54 expression by epithelial cells was specific to co-culture with expanded T cells, fresh PBMC from 2 donors were incubated at 1:1 ratios with HT29 or GRM cells for 24 hours. Only results shown to be statistically significant using expanded T cells above were investigated further and therefore, the Hep3B cells were not used for this part of the study. Surface staining with FITC-labelled anti-BerEP4, PerCP-labelled anti-CD3 and APC-labelled anti-CD54 was performed to identify epithelial cells, exclude PBMC from analysis and to ascertain whether the level of CD54 expression by epithelial cells was altered following co-culture with fresh PBMC.

The MFI of CD54 expression by the HT29 cells rose from 50.4 to 84.2 and 75.1, after a 24 hour incubation with PBMC from each of 2 healthy donors, thus showing that a much smaller increase in CD54 expression by HT29 cells occurred when fresh PBMC were used instead of HMBPP- or PHA-expanded T cells (Figure 6.5F, $p = 0.05$).

The MFI of CD54 expression by the GRM cells rose from 45.04 to 71.2 and 65.1, after a 24 hour incubation with PBMC from each of 2 healthy donors. These data show that a much smaller increase in CD54 expression by GRM cells occurred when fresh PBMC were used instead of HMBPP-expanded V$\delta$2 T cells (Figure 6.5F, $p = 0.04$).

These data show that CD54 expression is enhanced by co-culture with fresh PBMC but, the magnitude of enhancement is lower than that observed with HMBPP-expanded V$\delta$2 T cells.

### 6.3.4 The levels of HLA-E expression by HT29 cells, but not Hep3B or GRM cells, are slightly but significantly altered following co-culture with HMBPP-expanded V$\delta$2 T cells

Surface staining with FITC-labelled anti-BerEP4, PE-labelled anti-HLA-E and PerCP-labelled anti-CD3 was performed to ascertain the levels of HLA-E expression by

epithelial cells, following culture in the presence and absence of HMBPP-expanded V$\delta$2 T cells (Figure 6.6A).

The MFI of HLA-E expression by Hep3B cells ranged from 2 to 10 with mean MFI of 7.3+/-2.6. The MFI of HLA-E expression by Hep3B cells ranged from 2.7 to 16 when measured after co-culture with HMBPP-expanded V$\delta$2 T cells (mean; 6.6+/-2.8, Figure 6.6B). Higher HLA-E expression was observed following co-culture with HMBPP-expanded V$\delta$2 T cells from 3/4 donors and lower following co-culture with HMBPP-expanded V$\delta$2 T cells from 1/4 donors. A paired t-test revealed that these changes were not statistically significant (p=0.4).

The MFI of HLA-E expression by HT29 cells was 1.83 but rose slightly to 2.6, 2.9 and 4 when measured after co-culture with HMBPP-expanded V$\delta$2 T cells from each of 3 healthy donors (Figure 6.6C, p=0.04).

The MFI of HLA-E expression by GRM cells ranged from 1.98 to 199.4 with mean MFI of 51.3+/-44.1. The MFI of HLA-E expression by GRM cells ranged from 2.9 to 50 when measured after co-culture with HMBPP-expanded V$\delta$2 T cells (mean; 14+/-10.5, Figure 6.6D). Higher HLA-E expression was observed after co-culture with HMBPP-expanded V$\delta$2 T cells from 3/4 donors and lower after co-culture with HMBPP-expanded V$\delta$2 T cells from 1 donor (p=0.2).

These data show that slightly but statistically significantly higher levels of HLA-E expression by HT29 cells occur after co-culture with HMBPP-expanded PBMC, but its expression by the other carcinoma-derived cells remains unchanged under the same conditions.


**6.3.5 PHA and IL-2-expanded T cells have comparable effects to HMBPP-expanded V$\delta$2 T cells on HLA-E expression by HT29 cells, but not Hep3B or GRM cells**

HT29 or GRM cells were cultured alone or in the presence of equal numbers of 14-day old PHA and IL-2-expanded T cells from each of 2 donors for 24 hours. Only PHA and IL-2-expanded T cells from 1 donor were co-cultured with equal numbers of Hep3B cells due to limited cell numbers. Surface staining with FITC-labelled anti-BerEP4, PE-labelled HLA-E and PerCP-labelled anti-CD3 was performed to identify epithelial cells, exclude all T cells and any PBMC from analysis and, to ascertain whether the level of

HLA-E expression by epithelial cells was altered following co-culture with PHA and IL-2-expanded T cells.

HLA-E expression by HT29 cells rose slightly from 1.83 to a mean MFI of 3.1+/-0.1 following co-culture with PHA and IL-2-expanded T cells (Figure 6.6E). Co-culture did not alter HLA-E expression by GRM cells with MFI of 2 when cultured alone and mean MFI of 2.1+/-0.2 following the co-culture. The MFI of Hep3B cells (2) also rose slightly following a single co-culture experiment with PHA and IL-2-expanded T cells (3.4, Figure 6.6E).

These data show that PHA and IL-2-expanded T cells do not greatly induce HLA-E expression on epithelial cells and only have subtle effects like those observed with HMBPP-expanded Vδ2 T cells.

## 6.3.6 The levels of HLA-E expression by HT29 cells are not significantly altered following co-culture with fresh PBMC

To determine if the slightly enhanced HLA-E expression by HT29 cells was specific to co-culture with HMBPP-expanded Vδ2 T cells, fresh PBMC from 2 donors were incubated at 1:1 ratios with the colorectal carcinoma-derived cells. The MFI of HLA-E expression by the HT29 cells was 1.83 following culture alone and 1.6 and 1.5 following culture with fresh PBMC, thus showing that the same slight increases in HLA-E expression by HT29 cells did not occur following co-culture with fresh PBMC (Figure 6.6F).

## 6.3.7 The levels of MICA/B expression by epithelial cells are reduced following co-culture with HMBPP-expanded Vδ2 T cells

Surface staining with FITC-labelled anti-BerEP4, PerCP-labelled anti-CD3 and APC-labelled anti-MICA/B was performed to determine if co-culture with HMBPP-expanded Vδ2 T cells altered MICA/B expression by epithelial cells (Figure 6.7A).

The MFI of MICA/B expression by Hep3B cells ranged from 19 to 29.3 with a mean MFI of 24.3+/-3.2. Following co-culture in the presence of HMBPP-expanded Vδ2 T cells, the MFI ranged from 14.3 to 19.4 (mean; 16.8+/-1.6, Figure 6.7B). The expression of MICA/B by Hep3B cells, although lower, was not statistically significantly altered according to a paired t-test (p=0.3).

The MFI of MICA/B by HT29 cells ranged from 32.1 to 147.3 (mean; 60.9+/-25.8) and from 11.2 to 124.6 when measured after co-culture with HMBPP-expanded Vδ2 T cells (mean; 42.1+/-24.6, Figure 6.7C). The expression of MICA/B by HT29 cells was significantly lower following co-culture with HMBPP-expanded Vδ2 T cells (p=0.002).

The MFI of MICA/B by GRM cells ranged from 13.2 to 40 (mean; 33.3+/-6) and from 10.9 to 17.1 when measured after co-culture with HMBPP-expanded Vδ2 T cells (mean 13.4+/-1.2, Figure 6.7D). The expression of MICA/B by GRM cells was reduced following co-culture with HMBPP-expanded Vδ2 T cells from each of 4 donors (p=0.02).

These data show that MICA/B expression by HT29 and GRM cells is significantly reduced following co-culture with HMBPP-expanded Vδ2 T cells, suggesting that MICA/B may mediate the NKG2D downregulation observed on the Vδ2 T cells after the same co-culture experiments.

### 6.3.8 The levels of MICA/B expression by epithelial cells are reduced following co-culture with PHA and IL-2-expanded T cells

To determine if the reduced MICA/B expression on epithelial cells was restricted to co-culture with HMBPP-enriched Vδ2 T cells, conventional T cells were also expanded using PHA and IL-2 from PBMC of 2 donors. HT29 or GRM cells were cultured alone or in the presence of equal numbers of 14-day old PHA and IL-2-expanded PBMC from each of 2 donors for 24 hours. Only PHA and IL-2-expanded PBMC from 1 donor were co-cultured with equal numbers of Hep3B cells due to limited cell numbers. Surface staining with FITC-labelled anti-BerEP4, PerCP-labelled anti-CD3 and APC-labelled anti-MICA/B was performed to identify epithelial cells, exclude T cells and any PBMC from analysis and, to ascertain whether the level of MICA/B expression by epithelial cells had changed after co-culture with PHA and IL-2-expanded T cells

MICA/B expression by HT29 cells fell from 32.1 to a mean MFI of 15.5+/-1.7 following co-culture with PHA and IL-2-expanded PBMC (Figure 6.7E). MICA/B expression by GRM cells fell from 40 to a mean MFI of 12.3+/-1.3 following the co-culture and, the MFI of Hep3B cells (29.3) was reduced to 14.6 following a single co-culture experiment with PHA and IL-2-expanded PBMC (Figure 6.7E).

These data show that MICA/B expression by epithelial cells is reduced following co-culture with PHA and IL-2-expanded T cells, suggesting that MICA/B may mediate the NKG2D downregulation observed on such T cells after the same co-culture experiments. Again, it must be noted that these data are preliminary and must be repeated and expanded to determine the role, if any, of MICA/B in the putative downregulation of NKG2D on T cells.

**6.3.9 The levels of MICA/B expression by epithelial cells are unchanged following co-culture with fresh PBMC**

HT29 or GRM cells were incubated alone or in the presence of equal numbers of freshly prepared PBMC from each of 2 donors for 24 hours to ascertain if the reductions in MICA/B expression were specific to co-culture with expanded T cells. Surface staining was performed as before and analysis revealed that, after a 24 incubation with PBMC from each of 2 healthy donors, the MFI of MICA/B by the HT29 cells remained unchanged from 62.1 to 62.2 and 58.1, respectively, thus showing that co-culture with fresh PBMC has very little effect on MICA/B expression by HT29 cells (Figure 6.7F, p=0.3). The MFI of MICA/B by the GRM cells slightly changed from 122.1 to 124 and 113, respectively, thus showing that co-culture with fresh PBMC has very little effect on MICA/B expression by GRM cells (Figure 6.7F, p=0.3).

These data show that MICA/B expression by HT29 or GRM cells is not altered by co-culture with fresh PBMC.

Overall, these data suggest that HMBPP-expanded V$\delta$2 T cells may increase tumour visibility by inducing CD54 upregulation. Furthermore, these data also indicate that such tumours may employ immune evasion strategies such as the downregulation of NKG2D on V$\delta$2 T cells. The lower surface expression of MICA/B by Hep3B cells which is concurrent with the reduced NKG2D expression by V$\delta$2 T cells suggests that these decreases are caused by ligand-receptor binding. It must be noted that tumour cells can shed MICA/B to evade immune responses and that Hep3B might have employed this strategy to mediate NKG2D downregulation in these experiments. However, further experiments to quantify the amount of soluble MICA/B would be required before such conclusions can be drawn.

Higher levels of MICA/B expression by epithelial cells were observed when fresh PBMC were used but these higher levels cannot be attributed to V$\delta$2 T cells alone

as they are a much smaller proportion of fresh PBMC. Interestingly, reduced NKG2D expression by both, $\alpha\beta^+$ and $V\delta2^+$ T cells in fresh PBMC was observed, suggesting that this a general immune evasion strategy used by the tumours and, it is not specific to HMBPP-expanded $V\delta2$ T cells. The simultaneous reduction in MICA/B expression by the epithelial cells was observed to a much lower extent when fresh PBMC were used, suggesting that another factor is involved in the putative NKG2D downregulation.

**Figure 6.5**

**Figure 6.5**

**Figure 6.6**

**Figure 6.6**

**Figure 6.7**

**Figure 6.7**

**6.4 The effects of epithelial cell / Vδ2 T cell co-incubation on cytokine expression**

The BD™ Cytometric Bead Array (CBA) was used to quantify cytokine expression by Vδ2 T cells and epithelial cells, following 24 hours of culture alone and culture together. The culture plates were then centrifuged and the supernatants removed and stored at -20ºC, The supernatants were later thawed and then prepared for multiplex analysis of cytokine levels as described in Chapter 2, along with the standards for IL-4, IL-6, IL-10, IL-12, IL-13, IFNγ and TGF-β1. Analysis was performed as described in Chapter 2 such that the MFI of each cytokine standard corresponded to the concentration in pg/ml. The concentration of each cytokine could then be compared between supernatants from cultures of Vδ2 T cells from 3 healthy donors, from Hep3B cells, HT29 cells or GRM cells alone and from cultures containing both Vδ2 T cells from 5 donors and Hep3B, HT29 or GRM cells.

**6.4.1 Co-incubation of epithelial cells with Vδ2 T cells had no significant effects on IL-4 expression by either cell type**

Using an appropriate gating technique, the concentrations of IL-4 were determined in each sample of interest (Figure 6.8A-D). The mean concentration of IL-4 in the supernatants from Vδ2 T cell cultures was 6.4+/-1.9 pg/ml (Figure 6.8E). The concentration of IL-4 in the supernatants from Hep3B, HT29 and GRM cells was 1.6, 5.7 and 1.5 pg/ml, respectively (Figure 6.8E). The supernatants from Hep3B cell / Vδ2 T cell co-cultures had a mean concentration of 4.9+/-2.1 pg/ml of IL-4, while mean concentrations from HT29 cell / Vδ2 T cell and GRM cell / Vδ2 T cell co-cultures were 4.4+/-0.9 and 5.9+/-1.9 pg/ml, respectively (Figure 6.8E). A paired t-test revealed that there were no significant differences between IL-4 levels in the cultures containing Vδ2 T cells, Hep3B cells, HT29 cells or GRM cells alone and in the cultures containing both, Vδ2 T cells and Hep3B, HT29 or GRM cells.

These data show that co-culture has no significant effects on IL-4 secretion by Vδ2 T cells, Hep3B, HT29 or GRM cells.

**6.4.2 There were no significant differences in IL-6 protein levels between the supernatants of epithelial cell or Vδ2 T cell cultures and the supernatants of co-cultures containing both**

The concentrations of IL-6 were also determined in each sample of interest (Figure 6.9A-D). The mean concentration of IL-6 in the supernatants from Vδ2 T cell cultures was 79.1+/-22 pg/ml (Figure 6.9E). The concentration of IL-6 in the supernatants from Hep3B, HT29 and GRM cells was 2.1, 3.1 and 42.6 pg/ml, respectively (Figure 6.9E). The supernatants from Hep3B cell / Vδ2 T cell co-cultures had a mean concentration of 171.26+/-102.9 pg/ml of IL-6, while mean concentrations from HT29 cell / Vδ2 T cell and GRM cell / Vδ2 T cell co-cultures were 124.02+/-86.1 and 1921.32+/-1380.749 pg/ml, respectively (Figure 6.9E). A paired t-test revealed that there were no significant differences between IL-6 levels in the cultures containing Vδ2 T cells, Hep3B cells, HT29 cells or GRM cells alone and in the cultures containing Vδ2 T cells and any of the epithelial cells.

These data show that co-culture has no significant effects on IL-6 secretion by Vδ2 T cells, Hep3B, HT29 or GRM cells.

**6.4.3 IL-10 expression is significantly higher in Hep3B cell / Vδ2 T cell co-cultures, compared to Vδ2 T cell cultures**

The concentrations of IL-10 were determined in each sample of interest (Figure 6.10A-D). The mean concentration of IL-10 in the supernatants from Vδ2 T cell cultures was 8.3+/-0.8 pg/ml (Figure 6.10E). The concentration of IL-10 in the supernatants from Hep3B, HT29 and GRM cells was 42.7, 9.7 and 1.5 pg/ml, respectively (Figure 6.10E). The supernatants from Hep3B cell / Vδ2 T cell co-cultures had a mean concentration of 55.1+/-14.3 pg/ml of IL-10 and a paired t-test revealed that this was significantly higher, compared to the concentration of IL-10 in the supernatants from Vδ2 T cell cultures (p=0.03). The mean IL-10 concentrations from HT29 cell / Vδ2 T cell and GRM cell / Vδ2 T cell co-cultures were 20.3+/-6.1 and 31.4+/-15.3 pg/ml, respectively (Figure 6.10E).

These data show that IL-10 expression is significantly higher when Hep3B cells and Vδ2 T cells are co-cultured, compared to when Vδ2 T cells are cultured alone. However, the levels of IL-10 are similar between Hep3B cell cultures and Hep3B/ Vδ2

T cell co-cultures. Therefore, this suggests that Hep3B cells are the predominant producers of IL-10 in these co-culture experiments.

**6.4.4 Co-incubation of Vδ2 T cells with epithelial cells had no significant effects on IL-13 expression**

Gating on IL-13-conjugated beads only was performed to determine the IL-13 protein level in each sample of interest (Figure 6.11A-D). The mean concentration of IL-13 in the supernatants from Vδ2 T cell cultures was 236.5+/-100.5 pg/ml (Figure 6.11E). The concentration of IL-13 in the supernatants from Hep3B, HT29 and GRM cells was 10.5, 62 and 16.5 pg/ml, respectively (Figure 6.11E). The supernatants from Hep3B cell / Vδ2 T cell co-cultures had a mean concentration of 1176.6+/-849.7 pg/ml of IL-13, while mean concentrations from HT29 cell / Vδ2 T cell and GRM cell / Vδ2 T cell co-cultures were 873.2+/-422.8 and 1467.2+/-915.6 pg/ml, respectively (Figure 6.11E). A paired t-test revealed that there were no significant differences between IL-13 levels in the cultures containing Vδ2 T cells, Hep3B cells, HT29 cells or GRM cells alone and in the cultures containing both, Vδ2 T cells and Hep3B, HT29 or GRM cells.

These data show that co-culture had no significant effects on IL-13 expression by Vδ2 T cells, Hep3B, HT29 or GRM cells

**6.4.5 Co-incubation of epithelial cells and Vδ2 T cells had no significant effect on IFN-γ expression**

The concentrations of IFN-γ were determined in each sample of interest (Figure 6.12A-D). The mean concentration of IFN-γ in the supernatants from Vδ2 T cell cultures was 1619+/-837.1 pg/ml (Figure 6.12E). The concentration of IFN-γ in the supernatants from Hep3B, HT29 and GRM cells was 36, 1248.6 and 43.8 pg/ml, respectively (Figure 6.12E). The supernatants from Hep3B cell / Vδ2 T cell co-cultures had a mean concentration of 281.8+/-91.1 pg/ml of IFN-γ, which is a marked reduction from that observed in cultures of Vδ2 T cells alone but not statistically significant (p=0.4). The mean concentrations from HT29 cell / Vδ2 T cell and GRM cell / Vδ2 T cell co-cultures were 1186.4+/-892.9 and 536.7+/-453.2 pg/ml, respectively (Figure 6.12E).

These data show that IFN-γ release is almost always higher when epithelial cells are co-cultured with Vδ2 T cells compared to when epithelial cells are cultured alone,

most probably because the HMBPP-expanded Vδ2 T cells are potent producers of this cytokine. More importantly, although no changes in IFN-γ secretion were deemed statistically significant, its reduction in the supernatants of Vδ2 T cell co-cultured with Hep3B cells compared to when Vδ2 T cells were cultured alone suggests that these HCC-derived cells might suppress the cytokine-mediated effector functions of HMBPP/IL-2-expanded Vδ2 T cells. One might speculate that this putative inhibition of IFN-γ release is mediated by Hep3B cell-derived IL-10.

### 6.4.6 The levels of TGF-β1 protein are depleted from supernatant of the co-cultures, compared to when the epithelial cells are cultured alone

Using an appropriate gating technique, the concentrations of TGF-β1 were determined in each sample of interest (Figure 6.13A-D). The mean concentration of TGF-β1 in the supernatants from Vδ2 T cell cultures was 106.3+/-79.6 pg/ml (Figure 6.13E). The concentration of TGF-β1 in the supernatants from Hep3B, HT29 and GRM cells was 168.1, 228.1 and 89.5 pg/ml, respectively (Figure 6.13E). The supernatants from Hep3B cell / Vδ2 T cell co-cultures had a mean concentration of 90.8+/-14.3 pg/ml of TGF-β1 which is a marked reduction from that observed in cultures of Hep3B cells alone (Figure 6.13E, p=0.005). The mean concentrations from HT29 cell / Vδ2 T cell was also significantly lower than that observed in culture of HT29 cells alone (mean; 107.2+/-29.8 pg/ml, p=0.02, Figure 6.13E). The mean concentration of TGF-β1 in the supernatants from GRM cell / Vδ2 T cell co-cultures was 86+/-16.7 pg/ml which is similar to that observed in cultures of GRM cells alone and slightly lower than the levels observed in the supernatants of Vδ2 T cell cultures (Figure 6.13E).

These data suggest that the expression of TGF-β1 by Hep3B and HT29 cells is reduced in the presence of Vδ2 T cells. Further experiments might show that this reduction could be due to the engagement of TGF-β1 with the Vδ2 T cells as part of its role in the downregulation of NKG2D. However, such conclusions could only be drawn after the effects of exogenous TGF-β1 or neutralizing antibodies against TGF-β1 on NKG2D expression are evaluated.

### 6.4.7 Undetectable levels of IL-12 in supernatants from epithelial cell and Vδ2 T cell cultures

Using an appropriate gating technique, the concentrations of IL-12 in each standard tube could be determined (Figure 6.14A, B, C). However, IL-12 protein was undetectable in the supernatants collected from Hep3B, HT29, GRM and Vδ2 T cell cultures and co-cultures. As the IL-12 standards worked as expected these data suggest that HMBPP-expanded Vδ2 T cells do not produce IL-12 under the culture conditions provided.

**Figure 6.8**

**Figure 6.9**

**Figure 6.10**

**Figure 6.11**

**Figure 6.12**

**Figure 6.13**

**Figure 6.14**

## 6.5 Discussion

In the phenotypic analysis of innate lymphocyte populations in HBV infection (Chapter 3), the frequencies of circulating Vδ2 T cells were found to be significantly expanded in patients with HBV infection who had low viral loads and were either asymptomatic or had mild liver disease. From this it was proposed that these cells played an active role in the immune control of HBV. Since HCC is a potential endpoint of chronic HBV infection with a 100-fold higher risk in chronically infected persons compared to age-matched uninfected controls, the anti-tumour potential of Vδ2 T cells in this malignancy was evaluated (Beasley 1988; Beasley *et al.* 1981). Only 0.2% of asymptomatic carriers are diagnosed with HCC compared to approximately 5% of chronically infected patients with liver cirrhosis (Beasley 1988; Beasley *et al.* 1981; Rehermann & Nascimbeni 2006). The expansions of Vδ2 T cells observed in asymptomatic carriers may be responsible for the low prevalence of HCC in similar HBV-infected subjects. If this is the case, Vδ2 T cells expanded *in vitro* could be administered as an immunotherapy to those chronically infected patients who are at a higher risk of developing the malignancy.

To evaluate the immunotherapeutic potential of Vδ2 T cells in HBV-associated HCC, the HCC-derived Hep3B cells were co-cultured with HMBPP-expanded Vδ2 T cells and then investigated the expression of several surface markers and cytokines by both cell types. The cytotoxicity of Hep3B cells by Vδ2 T cells was also investigated by analysis of cell surface CD107 expression, but these experiments proved to be unsuccessful.

It was found that NKG2D surface expression by HMBPP-expanded Vδ2 T cells is lower following co-culture with Hep3B cells. It was also found that MICA/B surface expression by Hep3B cells is lower following co-culture with Vδ2 T cells. Interestingly, altered cytokine expression levels were also observed; the levels of IL-10 were higher in the supernatants of Vδ2 T cell/Hep3B cell co-cultures compared to the supernatants of Vδ2 T cells or Hep3B cells alone while the IFN-γ levels were lower. Furthermore, the levels of the immunomodulatory growth factor TGF-β1 are significantly lower in the supernatants from the Vδ2 T cell/Hep3B cell co-cultures compared to the supernatants of Hep3B cells alone. These alterations indicate that NKG2D and IFN-γ expression by Vδ2 T cells is reduced following co-culture with Hep3B cells and it is proposed that the Hep3B cells are mediating this reduction via the shedding of MICA and MICB.

Previous studies have revealed that NKG2D is a target for immune evasion and that the frequencies of circulating NKG2D-expressing NK cells are depleted in HCC, HBV and cervical cancer (Zeng *et al.* 2009; Arreygue-Garcia *et al.* 2008). MICA and MICB are natural ligands of NKG2D and their expression by tumour and virus-infected cells is crucial to NKG2D-mediated recognition (Wrobel *et al.* 2007; Bouet *et al.* 2008; Corvaiser *et al.* 2005). The engagement of NKG2D with MICA/B results in internalisation of the receptor and the bound ligand and, phosphorylation of DAP10 which triggers a cascade of events that ultimately result in calcium flux and cytotoxicity (Mistry & O'Callaghan 2007). In the immune subversion of NKG2D-mediated effector functions, MICA and MICB can be shed from tumour cells in a soluble form that impairs immune responses against the tumours by reducing NKG2D-ligand densities on malignant cells (Salih *et al.* 2006). This can lead to endocytosis and degradation of NKG2D by the cells on which it is expressed thus reducing the capacity of those cells to recognise tumour or virus-infected cells (Gonzalez *et al.* 2008). TGF-β can also suppress NKG2D-mediated recognition of tumours, by reducing NKG2D ligand expression (Eisele *et al.* 2006). TGF-β-mediated downregulation of NKG2D has been reported on both CD8$^+$ T cells and NK cells and epithelial cells and Treg cells are a common source of such TGF-β (Ghiringhelli *et al.* 2006; Clayton *et al.* 2008). Inhibition of NKG2D most probably exploits the mechanism by which this receptor is downregulated in the absence of malignancies or viral infections. The involvement of NKG2D in autoimmune diseases such as rheumatoid arthritis, autoimmune diabetes and coeliac disease shows why such regulation is required (Obeidy & Sharland 2008).

From these studies and our current findings, it is proposed that the reduction of MICA and MICB expression by Hep3B cells is due to shedding of the molecules in a soluble form. The soluble MICA and MICB then bind to NKG2D on the surface of Vδ2 T cells and cause its internalisation and degradation, resulting in reduced NKG2D expression and impaired NKG2D-mediated effector functions. Since, the expression of NKG2D on BRHPP+IL-2 or zoledronate+IL-2-expanded Vδ2 T cells was found to be important in the lysis of HCC primary cells and HCC-derived cell lines, it might be hypothesised that this immune subversion may be indicative of HCC *in vivo* (Bouet *et al.* 2008). It is possible that the reduced detection of NKG2D on Vδ2 T cells and MICA/B on Hep3B cells in these experiments may be due to NKG2D-MICA/B engagement and therefore, further experiments would be required to confirm MICA/B

shedding and/or NKG2D downregulation. Such experiments would include the addition of exogenous soluble MICA/B to Vδ2 T cell cultures or the performance of ELISAs to compare the amount of soluble MICA/B in the supernatants of Hep3B cell cultures to those in which Hep3B cells and Vδ2 T cells are cultured together.

Depleted levels of TGF-β1 were also found in the Vγ9Vδ2 T cell/Hep3B cell supernatants compared to the supernatants of Hep3B cells alone. This is possibly due to Vδ2 T cell-mediated inhibition of TGF-β1 expression. The suppression of TGF-β1 might help to facilitate an effective Vδ2 T cell-mediated anti-tumour immune response since the growth factor can serve as both an immunomodulator and a tumour promoter.

Reduced IFN-γ and augmented IL-10 production were also apparent in the supernatants of Vδ2 T cell/Hep3B cell co-cultures compared to supernatants taken from Vδ2 T cells cultured alone suggesting that epithelial cell-derived IL-10 may serve to modulate the inflammatory responses of the Vδ2 T cells.

It must be noted that the cellular source of the cytokines in the co-culture supernatants are unknown but the source can be gauged by comparing levels in supernatants of Vδ2 T cell or Hep3B cell single cultures to those of the co-cultures. However this is only an estimation and therefore, future experiments using intracellular cytokine staining and flow cytometry would be desirable to confirm the source of IFN-γ, IL-10 and TGF-β1. However, the cytometric bead assay was considered as the best option for this preliminary study because it allowed the detection of 7 cytokines and facilitated the identification of the cytokines which are involved in Vδ2 T cell-Hep3B cell interactions.

CD54 (ICAM-1) expression by HT29 and GRM, but not Hep3B cells, was significantly higher following co-culture with Vδ2 T cells. CD54 is expressed by APCs, memory and activated T cells and tumour cells (Roebuch & Finnegan 1999; Sun *et al.* 1999; Alexiou *et al.* 2001) and its primary function as a cell adhesion molecule enables intercellular communication and can facilitate MHC-restricted antigen presentation to T cells (Roy *et al.* 2001). CD54 expression can be induced by IFN-γ, IL-1, IL-2, IL-6, TNF-α and LPS and can be inhibited by IL-4 and IL-10 (Roy *et al.* 2001; Dymicka-Pierkarska & Kemona 2009). Therefore, the absence of CD54 upregulation by Hep3B cells following co-culture with Vδ2 T cells may be due to the presence of IL-10 and the apparent inhibition of IFN-γ and might represent an immune evasion strategy of the HCC-derived cells. Furthermore, its enhanced expression by HT29 and GRM cells

following co-culture with HMBPP/IL-2-expanded Vδ2 T cells, fresh PBMC or PHA/IL-2-expanded αβ T cell may increase recognition of these epithelial cells and facilitate their immune-mediated destruction. Shedding of CD54 is an immune evasion strategy used by tumours to facilitate metastasis (Sun *et al.* 1998; Dymicka-Pierkarska & Kemona 2009). In this study, the lack of CD54 surface expression upregulation by Hep3B cells may indicate that the CD54 has been shed as an immune evasion strategy of the HCC-derived cells. Higher serum levels of CD54 have been found in HCC and soluble CD54 is believed to play a role in the resistance of such tumours to immunotherapy. It can achieve this by blocking attachment of CTLs and NK cells to tumour cells and therefore, increases the potential for tumour development and metastasis (Sun *et al.* 1999). Future experiments may confirm if Hep3B cells shed CD54 by comparing the ratios of soluble and surface expressed CD54 in Hep3B cell cultures to Hep3B cell/ Vδ2 T cell co-cultures.

The use of PHA/IL-2 expanded αβ T cells instead of HMBPP/IL-2-expanded Vδ2 T cells in one co-culture experiment with Hep3B cells appeared to have similar effects on MICA/B expression by Hep3B cells but not on NKG2D expression by the αβ T cells suggesting that these cells are not susceptible to the same suppression mechanisms as their γδ T cells. However, no real conclusions can be drawn from a single result. Furthermore, the proportion of PHA/IL-2-expanded αβ T cells expressing NKG2D (mean; 54.2%) was significantly lower than the proportion of HMBPP/IL-2-expanded Vδ2 T cells expressing the activating receptor (mean 89.1%), suggesting that it does not play as large a role in the effector functions of conventional T cells.

All data shown here have been obtained using HMBPP-expanded Vγ9Vδ2 T cells from a maximum of 5 healthy donors and are therefore, preliminary. Despite this, the findings raise some important issues for the use of these cells in therapeutics against HCC. At present, measures are being taken to curb similar tumour cell-mediated inhibition such as the use of metalloproteinase inhibitors to inhibit MICA shedding or *in vitro* testing of MICA conjugated mAbs specific for tumour-associated antigens to sensitize NKG2D-mediated lysis (Gonzalez *et al.* 2008). Furthermore, Wrobel *et al.* (2007) suggest that NKG2D suppression could be overcome by 'forced' TCR-dependent tumour cell recognition which might be achieved by using aminobiphosphonates and BrHPP in combination. They also suggest that tumours whose recognition by Vγ9Vδ2 T cells is solely mediated by NKG2D would not be

suitable candidates for a Vγ9Vδ2 T cell-based immunotherapy. TCR-dependent recognition of F1-ATPase is another means whereby Vγ9Vδ2 T cells can recognise hepatocytes and it may be a preferable option for the treatment of HCC (Wrobel *et al.* 2007).

The investigation of Vγ9Vδ2 T cell/HT29 cell and Vγ9Vδ2 T cell/GRM cell co-culture experiments have yielded equally interesting results with significantly lower levels of NKG2D expression by Vδ2 T cells and MICA/B expression by HT29 and GRM cells therefore, suggesting that the same effects may take place in the use of cell-based immunotherapies for the treatment of CRC and melanoma. In fact, many signs of immune subversion appear to be more striking for these cancers suggesting that they might be more difficult to treat with such immunotherapies than HCC.

From these findings, it is hypothesised that cell-based immunotherapies may be the way forward for the treatment of HCC but the effector cell type and the mechanisms by which they are activated *in vitro* and through which they recognise tumour cells *in vivo* must be carefully considered.

# Chapter 7

# Final Discussion

## 7.1 Immune control in HBV infection

Despite the availability of a vaccine since the 1970s, HBV is one of the major causes of liver disease worldwide and is responsible for over 1 million deaths each year (Millman & Blumberg 1978; Pisani *et al.* 1990; Evans & Landon 1998; Christina & Page 2001; Lok & McMahon 2001; Lauer & Walker 2001). While, the majority of adult HBV infections are resolved, an alarming 95% of neonates develop persistent infection (Stevens *et al.* 1975; Rehermann & Nascimbeni 2006; Chisari & Ferrari 1975). A serious endpoint of chronic HBV infection is liver cirrhosis which occurs in 2-5% of HBV patients and can lead to the development of hepatocellular carcinoma (HCC) and, often liver failure. Approximately 5% of chronically infected patients who develop liver cirrhosis are diagnosed with HCC (Beasley *et al.* 1981; 1988; Rehermann & Nascimbeni 2006). The majority of chronically-infected patients however, do not develop liver disease and are said to be asymptomatic carriers. They are also referred to as HBsAg-positive and a cohort of such subjects have been investigated in this study. Most of the HBV-infected persons in our study cohort had a viral load below 20,000 copies/ml and an ALT below 40 which indicates that the virus is maintained at a low replicative phase and that no or little liver damage has arisen from the antiviral immune response. Therefore, despite the failure to completely eliminate the virus, these subjects exhibit a good antiviral immune response that keeps viral load at a low level without causing immune-mediated damage. However, it should be noted that definitive classification of asymptomatic disease would ideally require liver biopsy and/or fibroscan testing which was not possible in the present study.

Resolution of HBV infection is associated with strong, polyclonal and multi-specific CTL responses directed against epitopes within the polymerase, envelope and core and pre-core proteins of HBV while, persistent HBV infection is characterised by lower numbers of HBV-specific $CD8^+$ T cells (Penna *et al.* 1991; Missale et al; 1992; Bertoletti *et al.* 1991; Nayersina *et al.* 1993; Rehermann *et al.* 1995; Maini *et al.* 1999; Maini *et al.* 2000; Sobao *et al.* 2002, Thimme *et al.* 2003; Yang *et al.* 2009). Furthermore, the presence of large numbers of non-antigen specific $CD8^+$ T cells in the livers of patients with uncontrolled HBV infections and the ability of CTLs cells to produce IFN-γ and TNF-α in chronic HBV may contribute to liver inflammation clearing the virus (Bertoletti & Maini 2000; Reignat *et al.* 2002; Das *et al.* 2008). Inefficient T cell priming by DC and immunomodulation by Treg cells have been

implicated as factors in the inadequate T cell responses that are seen in persistent HBV infection (Duan *et al.* 2006; Fu *et al.* 2007). Moreover, HBV variants carrying mutations in cytotoxic T cell epitopes have been detected in chronic hepatitis B suggesting that viral immune escape mechanisms have a part to play in the establishment of persistent infection (Bertoletti *et al.* 1994; Rehermann *et al.* 1995). Since HBV is a non-cytopathic virus, it is quite possible that non-specific CD8$^+$ T cells that were once specific but are now redundant due to antigenic drift play a role in the liver pathology associated with HBV. CD8$^+$ T cells have also been shown to upregulate NK receptors in murine influenza A suggesting that the expression of such receptors increases the chances of antigen recognition and viral clearance (Kambayashi *et al.* 2000).

Since innate lymphocytes can modulate DC maturation and adaptive immune responses, it was proposed that deficiencies in their numbers or functions might result in impaired adaptive immune responses and a failure to resolve HBV infection (Kakimi *et al.* 2000; Liu *et al.* 2000; Nishimura *et al.* 2000; Cooper *et al.* 2001; Guidotti & Chisari 2001; Vincent *et al.* 2002; Ismaili *et al.* 2002; Martino *et al.* 2002; Dieli *et al.* 2004; Devilder *et al.* 2006; Ing & Stevenson 2009; Dunne *et al.* 2010). In this study the role of innate lymphocytes in immune responses against HBV has been investigated by quantifying their frequencies in the peripheral blood and examining their cytokine profiles in HBV-infected subjects and uninfected controls. Since the HBV patient cohort primarily consists of asymptomatic HBV carriers, it was expected that the findings would reflect the requirements of an effective immune response.

Treatment of HBV infection is burdened with several challenges including poor tolerance of immunomodulatory therapy and resistance to antiviral treatment (van Zonneveld *et al.* 2005; Janssen *et al.* 1990; Buster & Janssen 2006). Therefore, novel effective and well-tolerated immunotherapies are very necessary for the treatment of HBV. Several clinical trials have evaluated the potential of innate lymphocytes in immunotherapeutics and many have proven to be effective and well-tolerated (Schmidt-Wolf *et al.* 1999; Shi *et al.* 2004; Leemhuis *et al.* 2005; Chang *et al.* 2005; Bennouna *et al.* 2008; Motohashi *et al.* 2009; Dieli *et al.* 2009). It was proposed that the identification of innate lymphocyte populations that mediate effective immune control without causing liver disease may pave the way for the development of new immunotherapies in HBV.

Expansions of circulating innate lymphocytes have been identified in our patient cohort which might represent an active antiviral response. NK, NT and γδ T cells, but not iNKT cells, were more abundant in the peripheral blood of HBV-infected patients than in that of uninfected control subjects. This suggests that these cells are important players in the control of HBV replication. After the application of the Bonferonni correction method to eliminate significant differences that may arise solely as a result of multiple testing, higher frequencies of circulating NT cells and γδ T cells in HBV infection remained statistically significant. Further phenotypical studies revealed that the Vδ2 T cell subset of γδ T cells were most significantly expanded in the peripheral blood of these patients, while Vδ1 T cells were expanded to a lesser extent. Interestingly, in this study it has been found that effector memory Vδ2 and Vδ1 T cells were more abundant in the peripheral blood of HBV-infected patients than in that of uninfected control subjects while the frequencies of naïve subsets were substantially higher. This suggests that the expanded populations of effector Vδ2 and Vδ1 T cells are actively involved in the immune control of HBV in these patients.

The identification of an active antiviral immune response is further evident in the expansions of circulating IFN-γ-producing NT and T cells in HBV, particularly following *in vitro* stimulation. This implicates these cells and IFN-γ as predominant players in the immune responses against HBV. The results of quantitative RT-PCR support the hypothesis of a central role for IFN-γ in immune control of HBV as do the higher frequencies of IFN-γ-expressing NK cells, γδ T cells and total lymphocytes. However, upon application of Bonferroni correction, it was the expansions of IFN-γ-producing NT and T cells which proved to be most significant. These findings are in agreement with the findings of studies which have indicated that viral clearance is mediated non-cytolytically by IFN-γ and that NT cell-derived IFN-γ plays a role in immune responses against HBV (Guidotti *et al.* 1999; Albarran *et al.* 2005). Furthermore, IFN-γ-deficient mice exhibit impaired control of HBV replication and delayed clearance of HBV protein suggesting that the cytokine plays an important role in such mice but that another mechanism of viral clearance is employed in its absence (Yang *et al.* 2009).

Higher frequencies of IL-10-producing NK and NT cells were also observed in HBV patients. However, after Bonferroni correction, only the higher frequencies of IL-10-producing CD56$^{DIM}$ NK cells in HBV patients were found to be significant thus

identifying a putative immunomodulatory role for NK cells in controlled HBV infection and a possible measure of protection against liver disease. Since these cells constitute less that 1% of lymphocytes and are the only IL-10-producing lymphocytes whose expansions are highly significant in these HBV patients, it may explain why the higher levels of IL-10 were not detected using PCR. From these results, one may speculate that an abundance of IFN-γ-expressing cells and low numbers of IL-10-expressing cells may be the required composition of an immune response that controls viral replication in HBV without causing liver damage. Since IL-10-producing CD56$^{DIM}$ NK cells are higher, particularly in response to *in vitro* stimulation, it is proposed that they may proliferate rapidly in response to excessive IFN-γ production *in vivo* and quickly regulate the inflammatory response before it causes immune-mediated damage. However, further experimentation is required before such putative immune mechanisms can be proven because it may just be a balancing act between the pro-inflammatory and anti-inflammatory cytokines and the source of IL-10 and IFN-γ may not be important once the ratio of the two cytokines is optimal for viral clearance.

IL-17 has been implicated in disease progression, the development of liver fibrosis and the inhibition of IFN-γ-production in HBV infection (Xu *et al.* 2009; Zhang *et al.* 2010; Ge *et al.* 2010). These findings suggest that IL-17 promotes liver disease but not necessarily viral clearance in HBV infection. However, more recently Billerbeck *et al.* (2010) have identified IL-17-producing CD161$^{+}$CD8$^{+}$ T cells whose abundance in HCV-infected liver correlates with less severe liver disease. Enhanced responses of IL-17-producing T cells to *in vitro* stimulation have been identified in our HBV patient cohort. IL-17 production is not significantly enhanced among unstimulated PBMC from HBV patients and this finding has been confirmed by qRT-PCR. Since IL-17 has been identified as a negative regulator of IFN-γ (Mills 2008), this suggests that such enhanced responses in HBV may be another mechanism by which the immune response in these subjects is regulated. It is proposed that an IL-17-producing subset has been identified that does not elicit highly inflammatory responses when resting but upon sufficient stimulation, exhibits superior antiviral and/or immunoregulatory functions.

Since it is possible that the NT cells can be generated from CD8$^{+}$ T cells (Kelly-Rogers *et al.* 2006), it is possible that these expanded IFN-γ-producing cells may have previously been the virus-specific CD8$^{+}$ T cells that became redundant due to weakened recognition capacity caused by antigenic drift. HBV variants carrying mutations in CTL

epitopes have been detected in chronic HBV and this evolutionary escape mechanism of the virus might be challenged by an evolutionary mechanism of the host (Rehermann *et al.* 1995; Bertoletti *et al.* 1994). Therefore, the host immune system may have evolved from an inefficient specific response to an efficient non-specific response to control HBV replication. The expression of NK receptors by CD8^{+} T cells and the acquisition of NK-like, MHC-unrestricted cytotoxic activity has also been reported in murine influenza A infection suggesting that such changes from a specific adaptive immune response to a more innate-like response may occur in viral infections where the virus exhibits a lot of genetic variation (Kambayashi *et al.* 2000). Furthermore, CD161^{+}CD8^{+} T cells have been found in HBV and HCV and their abundance in HCV-infected liver has been associated with milder liver disease (Northfield *et al.* 2008; Billerbeck *et al.* 2010). This may represent a model in which CD8^{+} T cells upregulate NK receptors in an effort to increase their recognition capacity and increase their ability to control but not clear certain genetically heterogeneous viruses and ultimately, to elicit effector functions that are most beneficial to the host.

While NT cells represent one population of expanded IFN-γ-expressing cells in our patients, Vδ2 T cells are a second. The quantification of the frequencies of IFN-γ- and NKG2D-expressing Vδ2 T cells in HBV-infected patients and uninfected controls revealed that the frequencies of IFN-γ-expressing Vδ2 T cells are higher in HBV but the frequencies of Vδ2 T cells expressing the cytotoxicity-associated receptor NKG2D are not. However, the surface density of NKG2D is reduced on Vδ2 T cells in HBV indicating that the expanded populations of Vδ2 T cells may be mediating immune control via non-cytolytic mechanisms rather than cell-killing. This might involve the IFN-γ-mediated downregulation of NKG2D. NKG2D has been implicated in NK cell-mediated hepatocyte injury and liver failure in murine virus-induced hepatitis (Zou *et al.* 2010) and since IFN-γ has been shown to inhibit NKG2D ligand expression in melanoma, the cytokine might employ a similar mechanism in HBV so that liver injury from cytolytic activity is limited (Schwinn *et al.* 2009). As discussed in Chapter 4, non-cytolytic control of virus infection is preferable when large numbers of cells in large vital organs are infected and the dominance of IFN-γ in our patient cohort may represent such control (Guidotti & Chisari 2001). These expansions may represent an active but regulated hepatic immune response that maintains a low viral load without causing liver disease.

Previous work by Chen *et al.* (2008) has revealed depletions of circulating Vδ2 T cells in chronic HBV infection with the most significant decreases observed in the 57 patients with mild to severe liver disease. However, contrary to our findings, Chen *et al.* (2008) also found that frequencies of Vδ2 T cells were slightly but significantly lower in 46 asymptomatic patients and 38 patients with low grade liver disease, compared to healthy donors. They have used the same PE-conjugated anti-Vδ2 (BD Bioscience) that was used in this study but the demographics of their patient cohort is different to ours. Our cohort is racially diverse comprising African, Caucasian and Asian patients while theirs is entirely Asian and endogenous to the city of Chongqing. The endogeneity of their population may also explain why slightly higher frequencies of Vδ2 T cells in Asians were found here compared to the Africans and Caucasians since the Asians investigated in this study were from different regions. The discrepancy between our results may also be due to another factor such as genotype of the virus but since this is unknown for our study, this can only be proposed as a determining factor. Overall, the significantly lower frequencies of circulating Vδ2 T cells in HBV patients with higher severity of liver disease and ALT 3 times the upper limit of normal reported by Chen *et al.* (2008) supports our proposal that Vδ2 T cells are important in the control of HBV replication and in limiting progression to liver disease. Since, HBV patient serum and HBsAg had negative effects on IFN-γ production by Vδ2 T cells from healthy donors, it is proposed that HBV proteins have immunomodulatory effects on this γδ T cell subset and that other cells may be required to keep viral load at a minimum so that the frequencies of Vδ2 T cells can reach a sufficient level to take part in an antiviral response. Since IFN-γ production by NT cells is higher in asymptomatic HBV patients and its production by NT cells from healthy donors is enhanced in response to HBV patient serum or HBsAg, it is proposed that these cells are the key players in the control of HBV replication and can reduce HBV replication to levels at which HBV proteins cannot significantly immunomodulate Vδ2 T cell responses. Once HBV has been maintained at a low replicative phase, the Vδ2 T cells may help sustain the antiviral response. Further immune mechanism experiments using HBV proteins should be performed to confirm their immunomodulatory effect on Vδ2 T cells.

Although they are much less prevalent in peripheral blood than Vδ2 T cells, the frequencies of Vδ1 T cells have also been found to be higher in HBV patients in this study but their cytokine production capabilities have not been elucidated. However, the

effector memory repertoires of Vδ1 T cells are higher while the frequencies of naïve subsets are lower, suggesting that they play an active role in the antiviral response in these patients. These γδ T cells have been found to be producers of antiviral cytokines (Spada *et al.* 2000) and have also been implicated in the pathogenesis of HCV and arthritis (Tseng *et al.* 2001; Bank *et al.* 2002). However, they have also been shown to regulate inflammatory responses in the small intestine via the suppression of IFN-γ, granzyme-B and NKG2D expression by CD8^{+} T cells and perhaps, they prevent excessive IFN-γ production and NKG2D-associated cytotoxicity in our patient cohort too (Bhagat *et al.* 2008). While IFN-γ production by Vδ2 T cells is enhanced in the absence of *in vitro* stimulation in HBV, its production in response to PMA/I-stimulation is lower. This indicates that while IFN-γ appears to play a predominant role in the control of HBV replication, its expression is under strict control and can be inhibited if it rises above a certain threshold. Vδ1 T cells may be involved in the regulation of IFN-γ or alternatively, they may contribute to the IFN-γ production. The higher proportion of CD56^{+} Vδ1 T cells in HBV indicates that this γδ T cell subset may form part of the expanded IFN-γ-producing NT population. However, further experiments to assess the cytokine producing capabilities and effector functions of Vδ1 T cells in controlled HBV infection are needed to elucidate whether their role is predominantly inflammatory or anti-inflammatory.

Chromium release assays were performed using unstimulated, IL-2-stimulated or IFN-α-stimulated PBMC as effector cells and K562 cells as target cells. Cytotoxicity was slightly but consistently enhanced in our HBV patient cohort compared to the controls and since K562 are widely used as NK cell targets, this indicates that NK cytolytic activity is not impaired. IL-2 induced cytotoxicity was found to be significantly higher in HBV and since this cytokine is a strong activator of T cell proliferation and NT cell-mediated cytotoxicity (Jin *et al.* 1998; Zoll *et al.* 1998; Kelly Rogers *et al.* 2006; Dieli *et al.* 2007; Bennouna *et al.* 2008) it is proposed that the IL-2 induced cytotoxicity may be mediated by the expanded NT cell population in our patient cohort (Arreygue-Garcia *et al.* 2008; Zeng *et al.* 2009). This proposal is supported by our observations of lower NKG2D expression by Vδ2 T cells and the findings of other groups which revealed that numbers of NKG2D-expressing NK cells are depleted in chronic HBV infection and in HBV-associated HCC. Further work using enriched populations must be performed to confirm this proposal. Furthermore, since

cytotoxicity was consistently but only slightly enhanced under most conditions provided, it might only play a small role in the antiviral response in these asymptomatic HBV patients.

Overall, this study yields strong evidence that NT and γδ T cells are expanded in controlled HBV infection and appear to play a crucial role in the immune responses in this study cohort via predominantly non-cytolytic mechanisms. Furthermore, the frequencies of these innate T cells do not strongly correlate with differences in gender, age, viral load or disease severity but higher frequencies of both NT and total γδ T cells are observed in patients with elevated ALT levels, although these differences are not statistically significant. This suggests that if not regulated, the expansions of these cells and inflammatory cytokine producers may lead to liver damage. However, it must be noted that the highest ALT level among our patient cohort was 100 with most ALT values falling around the upper normal limit of 40. Other studies have used 3 times the upper limit of normal as a marker of liver disease and to distinguish between asymptomatic/low grade HBV patients and patients with mild/severe liver disease (Chen *et al.* 2008). However, none of our patients had such elevated ALT levels and so, the evaluation of the association between cell frequencies and high ALT was very limited.

The HBV-infected subjects studied here may represent a preliminary model of the immune responses required to maintain low viral load without causing liver disease in HBV and lessons learned here could be used to predict outcome of infection and to develop novel immunotherapies for patients with higher viral loads. However, a similar phenotypical study of HBV patients with mild to severe liver disease should be performed before this can achieved. Such a study would reveal which immune responses are prevalent and deficient in HBV-associated liver disease.

Questions may be raised concerning the usefulness of a therapy that can boost immune responses to a level that will control HBV infection but not clear it since such therapies would not reduce the prevalence of HBV. However it may reduce the viral burden and also prevent progression to liver cirrhosis and the development of HCC thus removing the need for liver transplantation. The persistence of HBV DNA may be due to the cccDNA pool in the nucleus of the hepatocytes. This transcriptional template is accumulated in the nucleus when there is a disruptive excess of proteins in the endoplasmic reticulum. The cccDNA is believed to serve as a reservoir for viral

replication and spread of infection and such reservoirs are important in the maintenance of chronic HBV infection (Zoulim 2004; Rehermann & Nascimbeni 2006). Therefore, while IFN-γ might target HBV via post transcriptional degradation of RNA and post translational inhibition of HBV capsid formation, its antiviral activity may not affect the persistence of cccDNA in the nucleus once it has been established (Guidotti & Chisari 2001). Therefore, perhaps cytolytic mechanisms are required to destroy the hepatocytes that harbour the nuclear cccDNA. However, the cytolytic mechanisms carry a risk of liver disease. Therefore, for a HBV-infected patient in which liver inflammation is evident, there is a high chance of progression to liver cirrhosis and in which resistance to antiviral treatment has emerged, a cell-based immunotherapy which boosts the immune response and lowers viral burden, may be preferred.

## 7.2 Immunomodulation by HBV-associated HCC

While only 0.2% of asymptomatic HBsAg carriers are diagnosed with HCC, 5% of chronically infected patients with liver cirrhosis develop HCC (Beasley 1988; Beasley *et al.* 1981; Rehermann & Nascimbeni 2006). Therapeutic options are limited for HCC and surgical resection is only suitable for small malignancies (Jinushi *et al.* 2005; Stefaniuk *et al.* 2010). Therefore, new therapies are required for the treatment of this cancer and since immunotherapies using innate lymphocytes have proven to be effective and well-tolerated in other cancers, they are an obvious choice for the treatment of liver cancer (Chang *et al.* 2005; Bennouna *et al.* 2008; Motohashi *et al.* 2009; Dieli *et al.* 2009). When developing a cell-based immunotherapy, it is important to assess the effects of the target tumour cells on the effector cells in order to identify any suppressive effects that may hinder immunotherapeutic potential. In this study, the reciprocal effects of Vδ2 T cells and the HCC-derived Hep3B cells on stimulatory and inhibitory receptor/ligand expression and cytokine expression by both cell types were investigated. Expansions of circulating Vδ2 T cells have already been found in controlled HBV infection in this work and it can be hypothesised that they are key players in a well-balanced immune response against the virus and that they might be responsible for the low prevalence of HCC in similar patients with low grade HBV. The anti-tumour potential of phosphoantigen-activated Vδ2 T cells is well-documented but here, the immunomodulatory effects of HCC-derived cells on their effector functions was determined (Dieli *et al.* 2009; Bennouna *et al.* 2008). Furthermore, Hep3B cells

were derived from the tumour of a HBV-infected person with HCC and even express HBsAg thus making them an ideal model of HBV-associated HCC (Knowles *et al.* 1980).

Lower NKG2D expression by Vδ2 T cells and lower MICA/B and higher levels of CD54 expression by the epithelial cells following co-culture was found here.

The lower NKG2D surface expression by HMBPP-expanded Vδ2 T cells following co-culture with Hep3B cells is not surprising as similar decreases have been reported for NK cells in HBV and HCC (Zeng *et al.* 2009). Furthermore, since NKG2D expression by BRHPP+IL-2 or zoledronate+IL-2-expanded Vδ2 T cells was found to be important in the lysis of HCC primary cells and HCC-derived cell lines, its downregulation would be an ideal immune evasion mechanism (Bouet *et al.* 2008; Arreygue-Garcia *et al.* 2008). Since MICA/B surface expression by Hep3B cells is reduced following co-culture, it can be proposed that the Hep3B cells cause downregulation of NKG2D in an effort to suppress the cytolytic functions of Vδ2 T cells. Hep3B cells might achieve this by shedding MICA and MICB which are the natural ligands of NKG2D. The shedding of these molecules from tumour cells in a soluble form impairs immune responses against the tumours by reducing NKG2D-ligand densities on malignant cells (Salih *et al.,* 2006). This can lead to internalisation and degradation of NKG2D by the cells on which it is expressed thus reducing the capacity of those cells to recognise tumour or virus-infected cells (Gonzalez *et al.,* 2008). Further experiments are required to confirm the speculative shedding of MICA and MICB by Hep3B cells since the reduced detection of NKG2D and MICA/B expression may only be due to their engagement. Perhaps, ELISAs could be performed to quantify soluble MICA/B in the supernatants and flow cytometry could be used to measure surface bound MICA/B in an effort to compare the ratios of surface bound and soluble MICA/B in Hep3B cultures and Hep3B cell/Vδ2 T cell co-cultures. Furthermore, the addition of exogenous MICA/B to Vδ2 T cell cultures might reveal if the ligand in soluble form can cause downregulation of NKG2D expression by Vδ2 T cells. Since similar and greater decreases in NKG2D expression by Vδ2 T cells and MICA/B expression by HT29 cells and GRM cells were observed after their co-culture, it can be suggested that these cell lines may employ the MICA/B-shedding strategy of immune subversion, also. Again, further experimentation is needed to confirm these suggestions,

followed by studies in HCC, CRC and melanoma patients to confirm that the findings of cell-line *in vitro* studies are representative of the malignancies.

Reduced levels of IFN-γ and TGF-β1 have also been found in the supernatants of the Vδ2 T cell and epithelial cell co-cultures compared to the supernatants of Vδ2 T or epithelial single cell cultures, respectively.

TGF-β1 is quite ambiguous in its functions in immunity against malignancies. It can regulate cell proliferation, differentiation, migration and can elicit tumour suppressor effects but it can also act as a potent immunosuppressant (Elliott & Blobe 2005; Li *et al.* 2005). Moreover, TGF-β1 acts as a tumour suppressor in normal colon but in the later stages of CRC, it serves as a tumour promoter (Li *et al.* 2005). TGF-β1-mediated downregulation of NKG2D has also been reported on both CD8^{+} T cells and NK cells and the level of the growth factor is higher in the serum of HCC, CRC and melanoma patients (Elliott & Blobe 2005; Ghiringhelli *et al.* 2006; Clayton *et al.* 2008). Therefore, TGF-β1 was a suspected cause of NKG2D inhibition in these experiments. Its involvement in the reduced NKG2D expression was evaluated here by investigating the levels of the growth factor in the Vδ2 T cell/Hep3B cell supernatants compared to the supernatants of Hep3B cells alone. Significantly depleted levels of TGF-β1 were found in the Vδ2 T cell/Hep3B cell co-culture supernatants compared to the Hep3B cell supernatants. This might be due to lower TGF-β1 expression by the Hep3B cells and propose that this may be a result of Vδ2 T cell-mediated inhibition of the growth factor. Such inhibition could hinder the immunosuppressive effects of the epithelial cells on the Vδ2 T cells and could facilitate more effective anti-tumour immune responses.

IFN-γ expression was higher when Hep3B cells were co-cultured with Vδ2 T cells, most probably because the HMBPP/IL-2-expanded Vδ2 T cells are potent producers of this cytokine. However, it was significantly reduced in the co-culture supernatants compared to when Vδ2 T cells were cultured alone suggesting that Hep3B cells caused a decrease in its expression by Vδ2 T cells. Interestingly, IL-10 levels were found to be higher in the supernatants of Vδ2 T cell/Hep3B cell co-cultures compared to the supernatants of Vδ2 T cells alone while they were similar to when Hep3B cells were cultured alone. This suggests that Hep3B cells were producing the majority of the IL-10. From this, it may be proposed that that IFN-γ expression by Vδ2 T cells may be inhibited by Hep3B-derived IL-10, however, further experiments using exogenous IL-10 or neutralising antibodies against IL-10 might provide stronger evidence of this.

Furthermore, intracellular cytokine staining and flow cytometry could also confirm the sources of such cytokines and their expression before and after co-culture.

The putative MICA/B-shedding mechanism of NKG2D downregulation appears to be used by all cell lines assessed in these experiments and it is even more significant in the co-cultures with the HT29 and GRM cells suggesting that these cell lines may have more immunosuppressive potential than Hep3B cells. However, the reduced IFN-γ levels are most evident in the co-cultures with the HCC-derived cells while HT29 cells appear to produce the cytokine. Since the Hep3B cells are derived from a HBV patient and express HBsAg, it is possible that the protein may induce IL-10 production by the Hep3B cells. In Chapter 5, the frequencies of IFN-γ-expressing Vδ2 T cells were shown to be lower after PBMC were cultured in the presence of HBsAg thus suggesting that the protein may inhibit IFN-γ by these cells. From the results obtained with Hep3B cells, it would appear that the suppression of IFN-γ is achieved via the induction of IL-10. If this is true, HBV would not be the only virus to induce IL-10 production. HIV gp120 and HCV NS4 induce IL-10 production by monocytes while CMV produces an IL-10 homologue that can elicit the same biological effects as human IL-10 (Taoufik *et al.*1997; Kotenko *et al.* 2000; Brady *et al.* 2003; Rowan *et al.* 2008). Therefore, HBsAg may elicit immunomodulatory effects on Vδ2 T cells but since this is not apparent in our patient cohort of asymptomatic carriers who are all HBsAg-positive, it may depend on high viral load.

Although HT29 and GRM cells were used initially as control cell lines while our main concern was HBV-associated HCC and the Hep3B cell line, they have proven to be equally interesting in terms of their interactions with Vδ2 T cells. The struggle for immune evasion is apparent in the expression patterns of many of the surface markers on either cell type. CD54 (ICAM-1) expression by HT29 and GRM, but not Hep3B cells, was significantly higher following co-culture with Vδ2 T cells. CD54 is expressed by B cells, dendritic cells, memory and activated T cells and tumour cells (Roebuch & Finnegan 1999; Sun *et al.* 1999; Alexiou *et al.* 2001). The main function of CD54 is to induce a specific reversible cell-cell adhesion that enables intercellular communication and in the normal immune response, it facilitates MHC-restricted antigen presentation to the T cell and subsequent signal transduction to induce T cell stimulation (Roy *et al.* 2001). CD54 expression can be induced by IFN-γ, IL-1, IL-2, IL-6, TNF-α and LPS and inhibited by IL-4, IL-10 and glucocortoids (Roy *et al.* 2001; Dymicka-Pierkarska &

Kemona 2009). Therefore, the absence of CD54 upregulation by Hep3B cells following co-culture with Vδ2 T cells may be due to the presence of IL-10 and the apparent inhibition of IFN-γ and might further enhance tumour survival in HCC. However, in HT29 and GRM cells, CD54 expression appears to be greatly increased following co-culture with HMBPP/IL-2-expanded Vδ2 T cells, fresh PBMC or PHA/IL-2-expanded αβ T cell thus increasing tumour cell recognition and the chances of immune-mediated destruction. CD54 expression has been found to be critical for colon tumour recognition by Vδ2 T cells and, its expression is higher in CRC patients compared to controls but significantly decreased after resection of the tumour. CD54 has also been associated with lesion thickness and metastasis in melanoma (Sun *et al.* 1999; Alexiou *et al.* 2001). Furthermore, the serum levels of CD54 are higher in CRC patients with metastasis, compared to those without metastasis (Alexiou *et al.* 2001; Corvaiser *et al.* 2005; Dymicka-Pierkarska & Kemona 2009). However, these increased serum levels are due to shedding of CD54 which is an immune evasion strategy used by tumours to facilitate metastasis (Sun *et al.* 1998; Dymicka-Pierkarska & Kemona 2009). Soluble CD54 enables this by interfering with NK and lymphocyte activated killer cell activity. It also permits the adhesion of tumour cells with migratory leukocytes thus facilitating the dissociation of individual cells from the primary tumour and the occurrence of 'blood borne metastasis'. Here, the lack of CD54 surface expression upregulation by Hep3B cells may indicate that the CD54 has been shed as yet another immune evasion strategy of the tumour cells. Indeed, serum levels of CD54 have previously been shown to be higher in HCC and soluble CD54 is believed to play a role in the resistance of such tumours to immunotherapy. It can achieve this by blocking LFA-1 thus preventing attachment of CTLs and NK cells to tumour cells and therefore, helping to provide a suitable environment for tumour development and metastasis (Sun *et al.* 1999).

Overall the Vδ2 T cell/Hep3B co-culture experiments suggest that HCC-derived cells employ aggressive mechanisms of immunoregulation in order to evade destruction. The design of an effective immunotherapy must take these mechanisms into consideration. Neutralizing antibodies against IL-10 may restore IFN-γ expression by Vδ2 T cells while metalloproteinase inhibitors are already being tested to inhibit MICA shedding. MICA conjugated mAbs specific for tumour-associated antigens are also being assessed for their ability to restore NKG2D-mediated cytotoxicity (Gonzalez *et al.* 2008). Furthermore, Wrobel *et al.* (2007) suggest that NKG2D suppression could be

overcome by therapies that induce 'forced' TCR-dependent tumour cell recognition which might be achieved by using both aminobiphosphonates and BrHPP. They also suggest that tumours whose recognition by Vδ2 T cells is solely mediated by NKG2D would not be suitable candidates for a Vδ2 T cell-based immunotherapy and our results support this belief. Previous research has also shown that TCR-dependent recognition of F1-ATPase is another means whereby Vδ2 T cells can recognise hepatocytes (Wrobel *et al.* 2007). This may be a preferable option for the treatment of HCC.

More work is warranted before this can be confirmed but the putative subversion strategies of HCC cells must be addressed before any effective and durable cell-based therapy can be developed. However, the treatment of HBV may be a better option which in turn, could limit disease progression and prevent the development of HCC.

Overall, this work has identified a novel mechanism of non-cytolytic control of HBV infection in which NT cells play a key role. It may be proposed that such cells are derived from an inefficient virus-specific CD8+ T cell response which could no longer control HBV replication due to antigenic drift. Such NT cells appear to reduce HBV replication to a level which is subsequently sustained with the help of Vδ2 and Vδ1 T cells and leads to an asymptomatic carrier condition in which little or no liver injury occurs. It is hypothesised that the identification of the potential of innate T cells to control HBV infection can be manipulated to treat patients in which HBV infection is not sufficiently controlled and in whom there is a high risk of liver disease.

# Chapter 8

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