Constraints on the CD8 T cell response in chronic HBV infection

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Declaration

'I, Abhishek Das, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

Abstract

The inflamed liver in chronic hepatitis B virus infection (CHB) is characterised by a large influx of non-virus specific CD8 T cells, which we hypothesize could provide insights into mechanisms of failure of viral control and liver damage in this setting. We compared the effector function of total circulating and intrahepatic CD8 T cells in CHB patients and healthy donors. We demonstrated that CD8 T cells from CHB patients, regardless of their antigen specificity, were impaired in their ability to produce IL-2 and proliferate upon TCR dependent stimulation. By contrast, these CD8 T cells had preserved production of the proinflammatory cytokines IFN-γ and TNF-α.

We provide evidence that this global CD8 T cell impairment of IL-2 production and proliferation may be partially attributable to a) downregulation of the proximal TCR signalling molecule CD3ζ (zeta), secondary to L-arginine depletion within the inflamed hepatic microenvironment and b) exhaustion of CD8 T cells associated with a terminally differentiated phenotype and shortened telomeres following long-term exposure to high level viraemia.

In addition, we implicate IL-10 as a regulatory cytokine in CHB, and show preferential suppression of HBV-specific CD8 T cells compared to responses for other viruses by IL-10 blocking experiments. Circulating IL-10 levels were elevated in patients and correlated temporally with viral load and ALT (surrogate marker of liver inflammation) during spontaneous flares of CHB. B cells were shown to be a potent source of this cytokine upon CpG stimulation, and these 'regulatory' cells, identified as CD24^{high}CD38^{high} transitional, were selectively enriched in CHB and correlated with disease flares. Depletion of this subset rescued HBV-specific responses, implicating a pathogenic role in suppressing T cell function and viral persistence in this setting.

Collectively, our data imply that impairment of CD8 T cell function during CHB is likely multifactorial, and that these polarised CD8 T cells may then both impede viral control, whilst contributing to the pro-inflammatory cytokine environment.

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List of common abbreviations

AIM	acute infectious mononucleosis
AKT	protein kinase B
ALT	alanine aminotransferase
APC	antigen presenting cell
BCR	B cell receptor
Bim	Bcl2-interacting mediator
Breg	Regulatory B cell
CCR7	chemokine (C-C motif) receptor 7
CD	Cluster of differentiation
CD3ζ	CD3zeta
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CHB	chronic hepatitis B virus infection
CMV	Cytomegalovirus
CpG	cytosine and guanine separated by phosphate
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen 4
DC	dendritic cell
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme-Linked ImmunoSorbent Assay
ELISPOT	Enzyme-linked immunosorbent spot
ER	endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
flow-FISH	flow based fluorescence in-situ hybridisation

Foxp3	Forkhead Box P3
GRB2	Growth factor receptor-bound protein 2
HBcAg	Hepatitis B virus core antigen
HBeAg	Hepatitis B virus precore-core antigen
HBsAg	Hepatitis B virus surface/envelope antigen
HBV	Hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	Human leucocyte antigen
hTERT	telomerase reverse transcriptase
HTLV-1	Human T-cell Lymphotropic Virus
IDO	idoleamine 2,3-dioxygenase
IFN-α	interferon alpha
IFN-γ	interferon gamma
Ig	Immunoglobulin
IL-10R	interleukin-10 receptor
IL-2	interleukin-2
INF-A	influenza A
ITAM	Immunoreceptor Tyrosine–based Activation Motif
KLRG-1	killer cell lectin-like receptor subfamily G, member 1
LAT	Linker for activation of T cells
LCK	leukocyte-specific protein tyrosine kinase
LCMV	Lymphocytic Choriomeningitis Virus
LPS	Lipopolysaccharide
LSEC	liver sinusoidal epithelial cell
MDSC	myeloid-derived dendritic cell

MFI	mean fluorescence intensity
MHC	Major histocompatability complex
NK	natural killer
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed death-1
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-Myristate 13-Acetate
PRR	Pattern recognition receptor
RNA	ribonucleic acid
SIV	Simian immunodeficiency virus
Tcm	central memory CD8 T cells
TCR	T cell receptor
Tem	effector memory CD8 T cells
Temra	revertant CD8 T cells
TGF-β	transforming growth factor beta
Th3	CD4+ T helper 3
TLR	Toll-like receptor
TNF-α	tumour necrosis factor alpha
Tr1	CD4+ T regulatory 1
Treg	natural regulatory T cell
TRF	telomeric restriction fragment
WHV	Woodchuck hepatitis virus
ZAP-70	Zeta-chain-associated protein kinase 70

Layout of Thesis

The introduction (*Chapter 1*) of this thesis describes the structure of the hepatitis B virion, and the epidemiology, clinical course and treatment of chronic HBV infection. A broad overview of innate and adaptive immune responses against viral infections is provided, with more focussed sections detailing the immune response during acute and chronic HBV infections.

Chapter 2, materials and methods, contains details of protocols and reagents used.

A large influx of non-antigen specific CD8 T cells is recruited into the livers of patients with liver inflammation and high viral load, however, their role in CHB is unknown. In *chapter 3*, we analyse the functional characteristics of CD8 T cells from the periphery and liver of patients, and demonstrate a global CD8 T cell dysfunction in IL-2 production and proliferation. In the following chapters, potential mechanisms for dysfunction within both global and virus-specific CD8 T cells are studied.

Chapter 4 provides evidence that downregulation of proximal signalling molecules, CD3zeta and CD28, are associated with impaired CD8 T cell function. Furthermore, downregulation of CD3zeta is linked to L-arginine depletion, and this is heightened within the HBV infected liver where we detect higher expression of the enzyme arginase-1, which catabolises L-arginine.

Chapters 3 and 4 have been published this year (Das et al. 2008) and therefore will be presented concisely in this thesis. The introduction and discussion sections for chapters 5 and 6 however are more thorough.

In *chapter 5*, we investigate whether chronic viral stimulation during CHB may drive CD8 T cells prematurely towards a terminally differentiated, immunosenescent state. We show that there is an accumulation of highly differentiated end stage cells in young patients with high viral load, and these cells have the shortest telomere length.

Finally, *in chapter 6*, we analyse whether global CD8 T cell hyporesponsiveness could be partially mediated by the immunosuppressive cytokine IL-10, and whether a subset of transitional B cells may regulate the immune response via production of this cytokine.

1 Introduction

Viruses do not themselves possess the adequate machinery to replicate, and are therefore dependent on their host cells for survival and transmission. It is not in the interest of the host cell however to harbour such microbes, which compete for cellular resources, mediate cell damage and immunopathology and may have oncogenic potential. This conflict of interest is the basis for a dynamic host-virus interaction, the balance of which determines whether viral infection is cleared or persists.

On the one hand, viruses have developed a multitude of evasion strategies to hide from or directly attack the immune response against them. On the other hand, the immune response has co-evolved to mount a series of timed defensive strategies to block viral entry and actively suppress replication. The innate immune response is the first line of defence, and through production of anti-viral type I interferons (IFNs) and NK cell mediated lysis of infected cells, attempts to rapidly control viral replication at the local site of infection. Viral control may also be mediated by other innate mechanisms, for example, by intracellular anti-viral restriction factors belonging to the APOBEC (*apo*lipoprotein *B*mRNA-*e*diting *c*atalytic polypeptide) and TRIM (tripartite motif) family of proteins (Takaori-Kondo 2006;Towers 2007). Professional antigen presenting cells including dendritic cells are primed at this stage, and provide a critical bridge to initiate the following adaptive arm of the response. Although CD4 T cell help and production of neutralising antibodies by B cells are required for an efficient adaptive response, it is generally accepted that CD8 T cells are the main mediators of viral clearance. Since the discovery by Doherty and Zinkernagel that CD8 T cells can recognise foreign viral particles in the context of self-MHC (Zinkernagel & Doherty 1974a;Zinkernagel & Doherty 1974b), many studies have characterised how these cells recognise and combat virus infected cells, through both cytolytic destruction and non-cytolytic clearance of virus with curative cytokines. A strong, multi-specific CD8 T cell response is associated with disease resolution, however a weak mono-specific response can result in viral persistence.

Why the immune response controls infection in some individuals but not in others is as yet unclear. Further studies are required therefore, to understand the underlying basis for immune dysfunction in the setting of chronic viral infection. This will facilitate the development of novel immunotherapeutic strategies which are in great need to reduce the global burden of disease associated with persistent viruses.

1.1 Epidemiology

Chronic hepatitis B virus infection (CHB) affects over 400 million individuals worldwide, and confers a high risk of liver cirrhosis and hepatocellular carcinoma which result in over one million HBV related deaths per year (Beasley 1988;McMahon 2005). The geographical areas of highest disease prevalence include Sub-Saharan Africa, China and parts of South-East Asia, whereas Western Europe and the United States are classed as low-endemic areas. Perinatal transmission from mother to baby is the most common method of virus transmission, however in highly endemic areas, virus may also be spread horizontally through close personal contact with individuals, or through sexual contact. Infection at birth confers a 90% risk of subsequent chronic infection, possibly due to early tolerance of the neonatal immune system by HBV antigens. The risk of chronic infection declines however with increasing age (0-5 yrs risk = 25-30%; >5yrs risk = 5-7%) (McMahon 2005). In the U.K., the incidence of chronic HBV infection is rapidly rising, with an estimated 7000 new cases between 1996 and 2000 (estimation based on mathematic modelling and WHO estimates of chronic disease in different countries). Although a proportion of these cases were secondary to hetero/homosexual encounter or through sharing of intravenous needles, the large majority (96%) were in fact seen in the migrant population (Health Protection Agency 2006). This may be a by-product of globalisation, frequent international travel and immigration, which have increased in recent times.

1.2 Hepatitis B virus

Hepatitis B virus is a hepatotropic and non-cytopathic virus, which is a member of the hepadnaviradae family. These viruses somewhat resemble retroviruses, because they replicate through reverse transcription of an RNA intermediate, the pregenomic RNA. However, unlike retroviruses, hepadnavirions contain DNA and not RNA (Beck & Nassal 2007).

HBV virions (Dane particles) are 40-42nm particles, with an outer lipoprotein envelope encasing an inner nucleocapsid or core. Contained within this inner core is a relaxed-circular, partially double-stranded (full length negative strand, and an incomplete plus strand) DNA genome consisting of approximately 3200 nucleotides, and the polymerase protein required for synthesis of viral DNA (Rehermann & Nascimbeni 2005).

The genome incorporates four overlapping open reading frames; surface, core, polymerase and X genes. The X open reading frame encodes the viral X protein (HBx), and the P coding region encodes the viral polymerase required for RNA encapsidation and DNA synthesis. By initiation of translation at three different in-frame initiation codons in the preS-S region, the genome can encode three different proteins; S protein/HBsAg, M/preS2 protein and the L/preS1 protein. PreS1 may be required for the binding of virus to the host cell. Finally, the preC-C region can encode the HBV core antigen (21kD), a structural unit of the viral capsid, or if translation is instead initiated at the upstream AUG codon, a larger preC protein (24kD) is formed. The extra portion of the preC protein includes a sequence that directs it into the secretory pathway where it is cleaved by proteases in the Golgi to generate the 16-kD HBeAg (Ganem & Prince 2004).

HBV genome
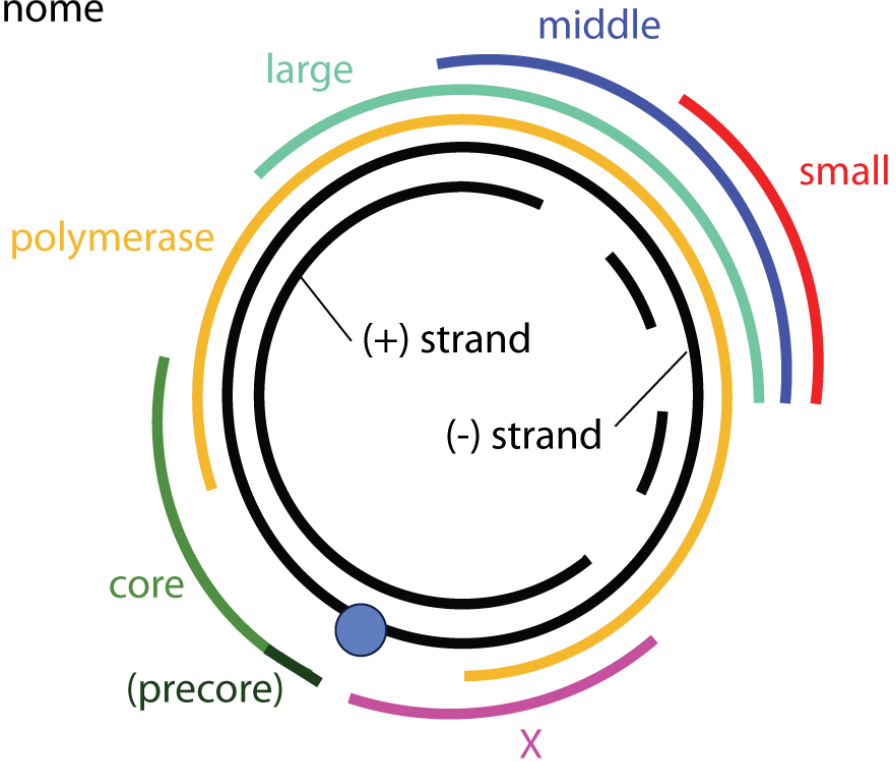
HBV virion
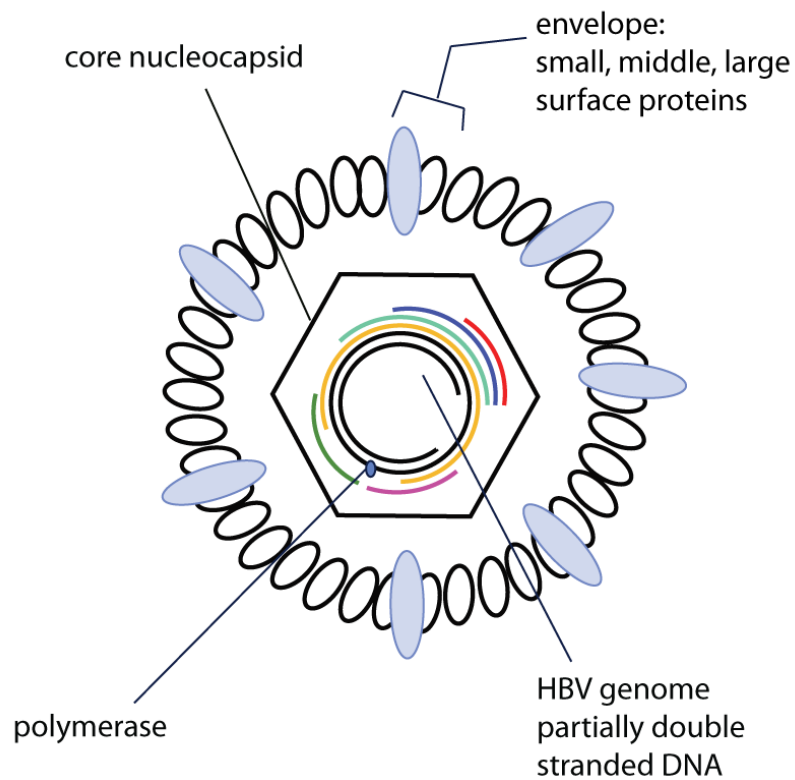
Figure 1a: HBV genome and the HBV virion (Dane particle) structure. Modified from (Ganem & Prince 2004;Rehermann & Nascimbeni 2005).

1.3 Hepatitis B virus replication

HBV virions bind to (as yet unknown) cell surface receptors and are internalised. HBV nucleocapsids then transport the genomic HBV DNA to the nucleus where the relaxed circular DNA is transformed into covalently closed circular DNA (cccDNA), which is the template for the transcription of four viral RNAs of varying lengths by the enzyme RNA polymerase II. Viral RNAs are then transported to the cytoplasm where they are used as mRNAs for the translation of HBV surface, core, polymerase and X proteins. Nucleocapsids are then assembled in the cytoplasm, into which a strand of pregenomic RNA is encapsidated with the polymerase protein, and is reverse transcribed inside the nucleocapsid. Pre-genomic RNA is reverse transcribed to minus-strand DNA, which is then used to synthesize plus-strand DNA by DNA polymerase. Resulting core particles may either bud into the endoplasmic reticulum and be enveloped with L, M and S surface antigens and released as virions, or a smaller proportion are transported back to the nucleus where newly formed DNA genomes are converted to cccDNA, to maintain the pool of transcriptional templates. In addition to whole virions, large amounts of 20nm sphere and filamentous HBsAg particles are also generated, which outnumber virions by a factor of 10^4-10^6. High circulating concentrations of HBsAg are found in microgram quantities in patients with HBV, which is not seen in other human viral infections (Ganem & Prince 2004;Rehermann & Nascimbeni 2005).
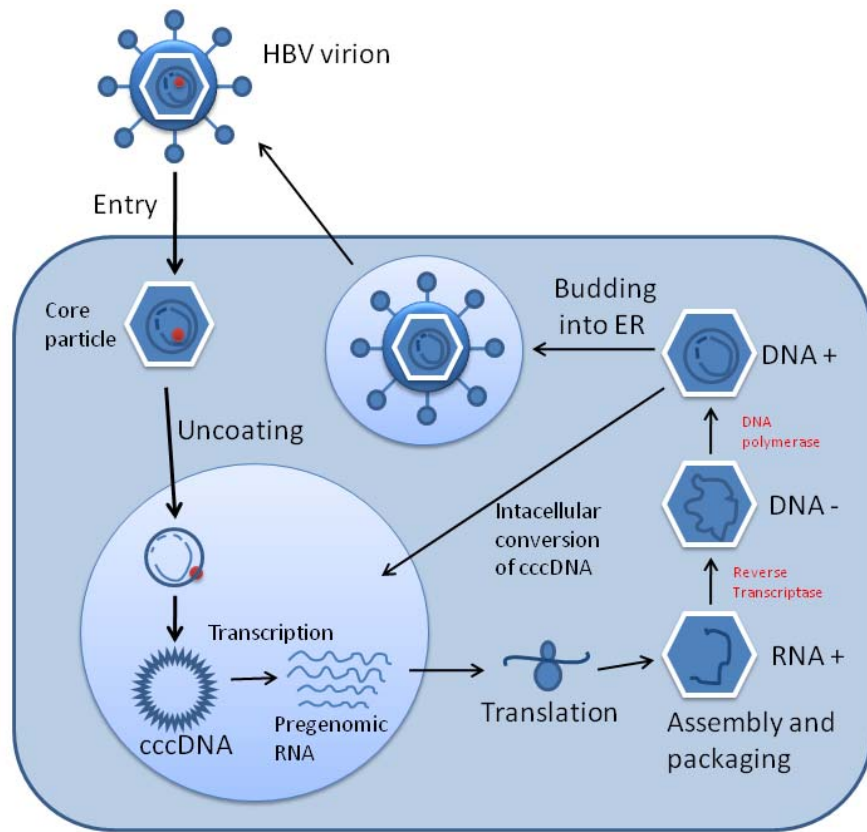
Figure 1b: HBV Replication Cycle. Adapted from (Fung & Lok 2004).

1.4 Animal models of HBV

Study of the pathogenesis and immunology of HBV infection has been hampered by the lack of suitable animal models and tissue culture systems for HBV. Data on acute HBV infection have come mainly from chimpanzees experimentally infected with HBV, or humans accidentally exposed to it, although in the early 1950's, studies were conducted in 172 male inmates of U.S. Federal Penitentiaries experimentally inoculated with pooled plasma samples harvested from patients with hepatitis B (Murray 1955). Mice are not susceptible to HBV infection, however transgenic mouse models in which HBV can be expressed in the liver have proved useful, although they do not spontaneously develop hepatitis in the liver, and require infusion of T cells from non-transgenic mice. Furthermore, in these animals, cccDNA is not produced like in human infection (Wong & Pamer 2003). Recent advances however have been made by study of animal models with related hepadnavirus infections, including pekin duck and woodchuck HBV. Infection of woodchucks with woodchuck hepatitis virus (WHV) can result in persistent infection, chronic hepatitis and hepatocellular carcinoma much like in human CHB. Study of these animals has been facilitated by the set-up of woodchuck breeding colonies, and the constant development of immunological tools to study them. Infection of neonates has also helped in understanding early events which may later determine viral clearance or resolution. In addition, these models may further be used to evaluate the safety and efficacy of novel drugs, vaccines and immunomodulators (Menne & Cote 2007;Menne & Tennant 1999).

1.5 Clinical course of acute and chronic HBV infection

Primary infection with HBV can be either symptomatic or asymptomatic. 95% of adults clear the infection and develop lifelong immunity, however in less than 5% of individuals, persistent infection is established which can progress to chronic hepatitis. 1% of individuals may develop fulminant hepatitis associated with large-scale tissue damage and may require a liver transplant with or without antiviral therapy (Dusheiko & Antonakopoulos 2008). Approximately 20% of patients who develop chronic hepatitis progress to liver cirrhosis, and the chance of developing hepatocellular carcinoma is also increased by 100-fold (Beasley 1988). The development of chronic hepatitis is much higher if infection is acquired by vertical transmission. Babies born to HBeAg positive mothers have a 70-90% risk of contracting the virus and 90% develop chronic hepatitis. If the mother is HBeAg negative however and is able to partially control viral replication, the risk of infection is reduced to 10-40% and rate of chronicity is also reduced (40-70%) (Alter 2003).

Chronic infection has a dynamic course which varies greatly between patients. Patients may be divided broadly into 3 categories of disease; HBeAg positive CHB, HBeAg negative CHB or HBeAg negative inactive carrier state (Dusheiko & Antonakopoulos 2008). HBeAg positive patients are characterised by a high level of HBV replication sustained over a long period of time. They may or may not have raised ALT. Individuals who are infected at birth go through an immunotolerant phase, characterised by elevated HBV DNA ($>10^5$ copies/ml) and presence of HBeAg in the serum, in the absence of liver inflammation (Figure 1c(a)). This may be due to HBeAg mediated tolerance of the immune response, which subsequently fails to control HBV replication, and does not mediate damage to HBV infected cells via cytolytic killing which would normally cause liver inflammation. In most patients, there is a decline of serum viral load titre over time, and HBeAg to anti-HBe seroconversion occurs at a rate of 5-10% per year (Figure 1c (b)). Rate of clearance of HBsAg however, is much more rare at 0.5%/year. As mentioned above, HBeAg negative patients may fall into two groups. The first group are patients with anti-HBe positive chronic hepatitis. In these individuals, absence of HBeAg from the serum is due to viral mutations in the preC region which prevents its expression. These patients are susceptible to active flares of liver disease associated with acute increases of HBV DNA and ALT. Necroinflammation and varying stages of fibrosis can be seen on liver biopsy

(Ganem & Prince 2004). The second group have seroconverted to anti-HBe status, and typically have normal ALT and HBV DNA $<10^5$ copies/ml (Figure 1c (c)) (McMahon 2005). These individuals are termed anti-HBe positive inactive carriers. It may be difficult to distinguish patients in these two groups, and this may require longitudinal follow-up of viral load and ALT as well as analysis of liver biopsy tissue.
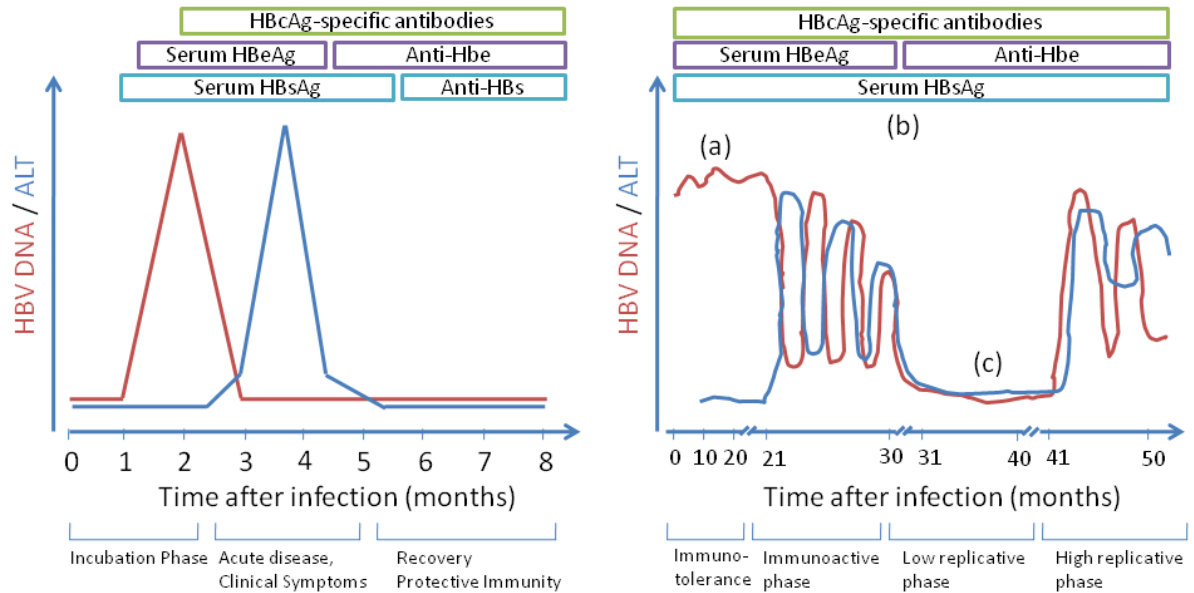
Figure 1c: Clinical course of acute and chronic HBV infection. Fluctuations of serological markers, serum viral load and liver inflammation (ALT) over the typical course of acute and (vertically transmitted) chronic HBV infection. Modified from (Rehermann & Nascimbeni 2005).

1.6 Vaccine and Antiviral therapy

A safe and effective vaccine against the hepatitis B virus surface antigen has been available since 1982 (Lavanchy 2004). Its efficacy has been shown in countries like Taiwan where introduction of the vaccine into routine immunisation schemes has reduced the prevalence of HBV infection by 93% in under 15 year olds (Namgyal 2003). However, despite its success and a recommendation by the WHO in 1991 that all countries implement the vaccine into their routine immunisation schemes by 1997, overall uptake has been poor (116/215 countries took part in 2000), due to a number of limitations (Alter 2003). The main obstacle is cost, which has hindered its introduction in many low income countries such as India and Sub-Saharan Africa, though some progress has been made by the Global Alliance for Vaccines and Immunization (GAVI) in funding vaccination programs in some of the 74 poorest countries in the world which it supports (Global Alliance for Vaccines and Immunization). However additional hurdles remain, including lack of infrastructure in developing nations, poor compliance with the full course of immunisation (3-4 intramuscular doses) and suggested, though uncomfirmed case reports, that link the vaccine to Rheumatoid Arthritis (Duclos 2003) or demyelinating diseases such as Guillian-Barre Syndrome (Khamaisi et al. 2004) that further hinder implementation of vaccination programs.

Despite the availability of a vaccine which can effectively prevent the development of chronic hepatitis in 95% of cases (Lavanchy 2004), there is still a huge burden of patients with chronic HBV infection worldwide for whom the prophylactic vaccine is of no use. For these patients, anti-viral therapy is used to reduce morbidity and mortality; however existing treatment is rarely curative and therefore of limited efficacy, with ongoing problems of toxicity, intolerance, viral resistance and expense. Finite treatment regimens are not suitable for the majority of patients, and the endpoint goal of treatment may be more focussed on preventing progression to liver cirrhosis, hepatocellular carcinoma and end stage liver disease. Due to the heterogenous nature of disease which varies between individuals, treatment regimens vary from monotherapy to combination therapies with different drugs. Licensed drugs include the immunomodulator IFNα, and nucleoside and nucleotide analogues, which interrupt viral replication. Lamivudine, a potent nucleoside analogue reverse transcriptase inhibitor, has been the main first line treatment because it is inexpensive, has a good safety record, and can suppress HBV DNA in the serum reliably

by 2-4 log10. However, drug-resistant viral mutations emerge quickly due to the rapid error prone replication of HBV (Ling et al. 1996). Furthermore, 'immunotolerant' patients who characteristically have a low level of liver inflammation but high viral load, have a poor response to lamivudine. No resistance is developed against IFNα treatment, however this therapy is generally of limited efficacy and is very poorly tolerated. Other commonly used therapies include Adefovir (nucleotide analogue that blocks reverse transcription) and Entecavir; a guanosine analogue which blocks the HBV polymerase at three stages; base priming, reverse transcription of the negative strand from pregenomic RNA, and synthesis of the plus strand of HBV DNA by DNA polymerase. New emerging drugs in the U.K. include Tenofovir (not yet licensed), which has a similar mechanism to Adefovir however is thought to be less nephrotoxic, and may be more suitable for patients with highly replicative HBV or HBV/HIV coinfection (Dusheiko & Antonakopoulos 2008).

Although new emerging drugs and treatment regimes may successfully suppress HBV viral load for longer and with fewer side effects or further clinical sequelae, it is unlikely that this therapy alone will provide a definitive cure which can be delivered cheaply and efficiently to millions of individuals in developing countries suffering with chronic infection today. Clearance of viral infection and disease resolution will probably require a synergistic boost to the host's own immune response, and this approach in addition to suppression of virus load with antiviral agents, may tip the balance in favour of viral clearance. There is a great need therefore to better understand the mechanisms of disease and why the immune system fails in some, but not other individuals. This knowledge will facilitate development of novel immunotherapeutic strategies to aid viral clearance and reduce the global burden of disease- the ultimate aim of research into viral infection.

1.7 The immune response against viral infection

1.7.1 Innate immune response

The immune system is equipped with a sequence of defensive manoeuvres which are revealed at different points over the course of viral infection. The innate response is initiated almost immediately after inoculation, and aims to rapidly contain viral spread. The hallmark of this response is production of Type I IFNs, which can inhibit viral infection but also have immunoregulatory effects including induction of NK cell function (Lanier 2008), Dendritic cell maturation (Dalod et al. 2003) and priming of CD8 T cell responses (Tough et al. 1996). Production of other cytokines such as IL-12, IL-6 and TNF may also contribute to viral control (Pichlmair & Reis e Sousa 2007). NK cell IFNγ production or direct lysis of infected cells too is important during the innate response. These cells may recognise altered levels of MHC-I on infected cells, or become activated by stress-induced molecules (Moretta et al. 2005).

Type I IFNs can be produced by all nucleated cells in response to viral infection. Their role as factors which interfere with viral replication was first demonstrated by Isaacs and Lindenmann in 1957 (Isaacs & Lindenmann 1957). Infection of mice with Lymphocytic Choriomeningitis Virus (LCMV), an RNA virus which in some cases is hepatotropic and can induce acute hepatitis, is a useful model to study both the innate and adaptive immune response, as it is possible to reliably induce either self-limiting or persistent infection by altering the strain/dose of virus or the inoculation procedure. Despite the presence of adaptive immunity, mice that lack normal IFNα and IFNβ signalling pathways were unable to clear virus infection (Muller et al. 1994;van den Broek et al. 1995).

Depending on which cellular compartment the virus occupies within an infected cell, distinct cytosolic or endosomal pathways can 'sense' viral nucleic acids and induce type I IFN production, although leakage of viral nucleic acids between compartments allows for cross-talk. In the cytosolic pathway, viral RNAs are recognised by MDA5 (melanoma differentiation factor-5) and RIG-1 (retinoic acid inducible gene I) resulting in a downstream cascade of events leading to the phosphorylation of transcription factors IRF3 (interferon regulatory factor 3) and IRF7 which induce Type I IFN production. Alternatively, the virus may be taken up into endosomes where their nucleic acids are exposed to Toll-Like Receptors (TLR), a type of Pattern Recognition Receptor which

detect pathogen-associated molecular patterns (PAMPs) found in a variety of bacteria, viruses, fungi and protozoa (Kawai & Akira 2006). In plasmacytoid dendritic cells, major producers of IFNα, detection of viral nucleic acids by TLR 7,8 and 9 leads to MyD88 mediated phosphorylation of transcription factor IRF7 which regulates expression of Type I IFN. TLR's can also be found on the surface of cells including hepatocytes, and TLR4 for example, can induce IFNα and IFNβ via the TRIF pathway (Pichlmair & Reis e Sousa 2007).

Binding of Type I IFNs to their receptor initiates a signalling cascade which upregulates the expression of IFN stimulated genes (ISGs). These genes code for proteins involved in viral control, including the TRIM and APOBEC proteins; restriction factors which target incoming retroviral capsids and deaminate retroviral genomes respectively. Other ISG's include MX1 (myxovirus resistance 1), IFN-inducible double-stranded-RNA-dependent protein kinase (PKR) and IFN-regulatory factor 1, and are equally important in antiviral function (Pichlmair & Reis e Sousa 2007). Apart from their direct effects on gene transcription, type I IFNs are also important in priming the adaptive immune response and initiating NK cell production of IFNγ (Lanier 2008). The role of NK cells in innate immunity will be further discussed in the context of acute HBV infection in a later section. Therefore, early exposure to cytokines, an inflammatory environment and varying doses of viral particles may affect how the adaptive response is primed, and the overall outcome of infection.

1.7.2 Early innate response to HBV

It has been difficult to study the very early immune events after HBV infection in humans, due to the fact that patients usually present with clinical symptoms which only develop 10-12 weeks after inoculation, past the point where the burden of viral load has already been cleared. However, data from chimpanzees experimentally infected with HBV and limited data in humans accidentally infected with HBV have provided some information on the early control of this disease.

In order to survive and be successfully transmitted to another host, most viruses replicate rapidly upon entry in an attempt to overwhelm the immune response. This can be shown in the mouse model of LCMV for example, where inoculation with a high dose or rapidly replicating strains of virus results in impairment of the adaptive virus-specific CD8 T cell

response and leads to persistence of infection (Moskophidis et al. 1993). Fast viral spread is also true for hepatitis C virus (HCV) infection where virus in the serum was detectable 1-2 weeks post inoculation, in 5 health care workers accidentally infected with HCV through needle-stick injury (Thimme et al. 2001), and a similar kinetic of viral load was observed in acutely infected chimpanzees (Major et al. 2004).

Hepatitis B virus however does not behave in this manner, and instead both viral load and HBeAg/HBsAg particles are undetectable within the liver and serum of patients for 4-7 weeks (Guidotti et al. 1999b). Previous studies have speculated that this may be due to inefficient replication in hepatocytes or habitation of immunopriveleged sites apart from the liver such as the brain (Bertoletti & Gehring 2006). Alternatively, one could postulate that suppression of replication could also be immune mediated. IFNα and IFNβ have been shown to non-cytolytically inhibit HBV replication in a transgenic mouse model, and this was associated with a ten-fold decrease in viral capsids containing HBV pregenomic RNA (McClary et al. 2000;Wieland et al. 2000). Furthermore, HBV infected hepatocytes may produce type I IFNs (Chang & Lewin 2007) and PH5CH8 cells (derived from non-neoplastic hepatocytes) produced IFNβ in vitro upon stimulation with poly (I:C) or Sendai virus by TLR3 or RIG-1 mediated pathways respectively (Li et al. 2005a). However, novel gene profiling studies provided strong evidence against this argument, and showed instead that the type I IFN response was absent during the early stages of HBV infection. Wieland et al showed that in serial liver biopsies isolated from 3 HBV infected chimpanzees, there was no intrahepatic gene expression during the entry and incubation phases (Wieland et al. 2004). In contrast, 27 genes were upregulated upon HCV infection, many of which were regulated by the type I IFNs (Su et al. 2002). HCV infection is accordingly associated with induction of a strong Type I IFN response, whereas HBV has been described as a stealth virus as it is able to evade the innate immune response altogether.

After an initial quiescent phase, there is a rapid exponential rise in viral load, to the point that serum levels may reach 10^9-10^{10} copies/ml and almost all hepatocytes may be infected. This initiates an acute phase response characterised by a robust activation of TNF and IFNγ, which is associated with clearance of up to 80% of virus load. This occurs prior to the influx of both virus specific and non-virus specific lymphocytes into the liver, which in chimpanzees and humans, appears 2-3 weeks later and correlates with liver inflammation and clinical symptoms in patients (Guidotti et al. 1999b;Webster et al. 2000). These data

imply two things; 1) clearance of the majority of HBV DNA is non-cytolytic and tissue sparing as it precedes liver inflammation and 2) production of IFNγ at this early stage is not likely to be from CD8 T cells. Candidates for this IFNγ production are NK and NKT cells. NK cells account for a large proportion of resident intrahepatic lymphocytes, and resting mature NK cells constitutively express transcripts for IFNγ (Lanier 2008). In transgenic mouse models, NKT cells have been shown to efficiently inhibit viral replication via IFNγ upon α-galactoceramide stimulation (Kakimi et al. 2000;Kakimi et al. 2001a). Furthermore, study of ex vivo NK cell frequencies in presymptomatic patients (infected during a single source outbreak), revealed that highest circulating numbers of NK cells were detectable during the peak of viral load, although whether NK cell frequencies fluctuate in the liver remains to be defined (Webster et al. 2000). Thus NK cells are a potential source for the initial burst of IFNγ, however what activates them to do so is unclear. Usually type I IFNs are known to activate NK cells in this setting, and may also induce IL-15 production required for their proliferation (Zhang et al. 1998). However, in the absence of type I IFN induction during the incubation phase of HBV, it may be that direct recognition of viral particles or stress signals on infected hepatocytes may be enough to activate these cells.
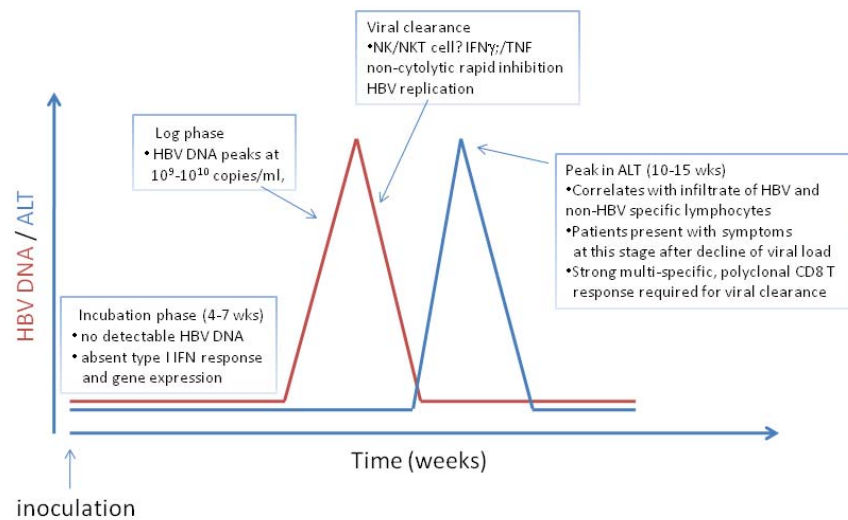
Figure 1d: Kinetics of HBV replication and the immune response during acute HBV infection.

Dendritic cells provide a vital bridge between the innate and adaptive immune responses. They mature in response to innate signals including Type I IFNs and interaction with NK

cells (Bertoletti & Ferrari 2003), and subsequently, are highly efficient at priming CD8 T cell responses in lymph nodes draining the site of infection, within hours of onset of infection. Defective dendritic cell function may therefore perpetuate chronicity by hampering an effective adaptive response, onset of which correlates with clearance of HBV in patients who resolve infection. Whether or not DC function is altered during HBV infection is unclear; some studies have suggested that plasmacytoid and myeloid dendritic cells were functionally impaired during CHB (van der Molen et al. 2004), however the stimulatory effects on T cell priming seemed minimal.

1.7.3 Adaptive immune response

A strong multi-specific CD8 T cell response, with adequate CD4 T cell help and co-ordinated humoral immunity, provides a solid adaptive immune defence against virus infection (Bertoletti & Gehring 2006). The main cellular components of this response include B and T lymphocytes. In this section, I first discuss the development of these cells, with a particular emphasis on development of peripheral B cell subsets, as in chapter 6 we investigate the ability of 'transitional' B cells to modulate the immune response during CHB.

The adaptive immune response to viral infection is then described in the context of HBV infection, and comparisons are made with the immune response against other similar viruses which may develop persistence; particularly HIV and HCV. Prior to this, a brief section describes how viral peptides are recognised by CD8 T cells, in the context of MHC class I mediated presentation. Other topics concerning CD8 T cells; memory development, T cell signalling and CD8 T cell differentiation are covered during the introduction sections of chapters four and five.

1.8 Lymphopoesis

1.8.1 B cell development

B cell development takes place in the foetal liver and in the bone marrow after birth. It is characterised by a series of checkpoints which decide lineage commitment and cell fate. Successful rearrangement of genes at the immunoglobulin heavy and light chain loci is required for successful expression of a B cell receptor. This receptor must be mono-specific to one non-self antigen. Checkpoints such as allelic exclusion and negative selection are in place to ensure this (Martensson & Ceredig 2000).

The earliest cell committed to the B cell lineage is the Pro (progenitor) B cell derived from the Common Lymphoid Progenitor Cell. This lineage commitment is thought to be mediated by transcription factors such as PU.1, E2A and EBF, which suppress lineage inappropriate genes and initiate D to J recombination at the heavy chain locus (Hagman & Lukin 2006;Pongubala et al. 2008). Once the lineage is determined, further maturation stages occur which can be defined by expression of cell surface markers and immunoglobulin gene rearrangement (see figure 1). Hardy et al. made use of multicolour flow cytometry in the early 1990's to divide these cells into fractions A, B and C dependent on expression of markers CD43 (mucin leukosialin), BP-1 and CD24 (heat stable antigen) (Hardy et al. 1991). Rowlink and Melchers further contributed by identifying that CD25 and c-kit are expressed at certain maturation steps (Rolink et al. 1994). Their classification system describes that a Pro B cell progresses through Pre-B1, large Pre-BII, small Pre BII steps before exiting into the blood stream as an immature transitional B cell.

The bone marrow environment is critical for early B cell development. Common Lymphoid progenitors and Progenitor B cells develop on a stromal cell framework and adhere to these cells intially via integrins (VLA-4 binds to VCAM-1). Stromal cells then provide survival signals to the B cells via soluble factors (IL-7 which induces proliferation of pro-B cells) and surface bound stem cell factor which binds to c-kit and also induces proliferation. Blockade of integrin binding intercepts the survival signal blocking further maturation. In addition to external signals, internal factors such as expression of transcription factors are also critically important. Recombinase Activating Gene (RAG 1 and 2) encodes enzymes required for the rearrangement of the immunoglobulin genes.

Pax5, another transcription factor, promotes lineage commitment like E2A and EBF by initiating transcription of CD19, a B cell specific signalling molecule.

B cell development produces a wide repertoire of B cells specific to a variety of antigens. This wide antibody repertoire is possible because of gene rearrangment. Multiple V (variable) D (diversity) and J (joining) genes can combine in the process of VDJ recombination of the Ig heavy chain at the Pro B cell stage. The Ig heavy chain rearrangement occurs first and if successful results in the formation of the mu heavy chain. This is temporarily expressed at the cell surface in conjugation with surrogate light chain (VpreB and lambda5) as the pre-B cell receptor. This is a vital checkpoint. Signalling through the pre-BCR initiates proliferative expansion and inhibits further rearrangement on the Ig heavy chain (Melchers et al. 1995). Immunoglobulin light chain rearrangement occurs later during the Pre B cell stage. Different combinations of heavy and light chains may then combine together to form the B cell receptor. Further diversity is added at any of these recombination stages because of imprecise joining, and the action of enzyme Terminal Deoxynucleotidyl Transferase (TdT) which can add nucleotides at VDJ junctions. These mechanisms exponentially increase the variety of potential B cell receptors, however only B cells which can receive survival signals through their receptors will survive.

Immature B cells exit the bone marrow through the venus sinus into the blood stream. At this stage they are functionally immature 'transitional cells' and have a short half life of 3 days. At these secondary sites, they can undergo positive selection if they encounter an appropriate antigen and have the potential to mature into memory or plasma cells.

1.8.2 Peripheral B cell subsets in mouse and man

Peripheral B cells can be broadly characterised into 3 main groups; transitional, mature (which are both naïve subsets) and memory. In both mice and humans, these subsets can be distinguised from one another by their phenotype and tissue localisation.

Transitional cells are 'functionally immature' B cells which have newly egressed from the bone marrow. In humans they are the major component of the B cell compartment in cord blood (50%) however their frequency declines through infancy. In adults they account for 4% of peripheral B cells (Marie-Cardine et al. 2008). In mice, transitional cells are divided into T1 or T2 cells. T1 (IgM^{bright}, IgD^{neg}, $CD24^{high}$, $CD21^{low}$, $CD23^{low}$) migrate to the white

pulp of the spleen and can be found in the outer PALS (peri-arteriolar lymphatic sheath). T2 (IgM^{bright}, IgD^{bright}, CD24^{high}, CD21^{high}, CD23^{high}) are found in the surrounding lymphoid follicles where mature cells are also present. However, unlike the mature, the T2 cells are confined to these follicles whereas the mature are free to recirculate through lymph and blood (Carsetti et al. 2004;Martensson & Ceredig 2000). Human transitional B cells are thought to have similar tissue distribution and marker expression as in mice. Carsetti et al. recently showed that dependent on expression of two developmentally regulated markers CD24 and CD38, peripheral human transitional cells could be accurately identified by their high expression of both CD24 and CD38. They have also been shown to lack CD27 (mainly present on memory cells) and to co-express IgM, IgD, CD21, CD23 and L-selectin (Carsetti et al. 2004).

Transitional 1 and 2 B cells are precursors for mature B cells, an active process in the spleen of mice. Transition from T1 to T2 to mature B cells is thought to be a linear process. Loder et al have shown that intravenous transfer of T1 cells into RAG 2 knockout mice results in development of T2 and mature B cells by 48 hours (Loder et al. 1999). In addition, it was shown in patients followed up post Haemopoeitic Stem Cell transplant (HSCT) that by 1-2 months post HSCT all detectable CD19+ cells were transitional, but by 9 months these populations declined and the mature fraction increased to 80% of total B cells (Marie-Cardine et al. 2008). However, it cannot be ruled out that T1 cells directly differentiate to mature B cells without a T2 intermediate stage.

Only 1-3% of transitional cells that exit the bone marrow will mature. The exact signals which induce this maturation step are as yet unknown, however there is suggested involvement of antigen and BCR signaling. Loder et al. have shown, for example, that mice with deletions in elements involved in the B cell receptor signaling cascade are arrested at the T1 or T2 developmental stage (Loder et al. 1999). Mature B cells are long-lived IgM+IgD+ cells which are can recirculate in lymph and blood and enter lymphoid follicles of spleen and lymph nodes where they can encounter antigen on follicular dendritic cells.

On recognition of specific antigen, B cells may then enter the long-lived pool as memory or plasma B cells. These cells, unlike the mature, respond rapidly to secondary antigenic stimulation with production of high affinity antibodies. On binding antigen via the B cell receptor in the secondary lymphoid follicle, the antigen is internalised and presented on

MHC II to T cells. This, along with co-ligation of CD40L (on T cells) to CD40 (on B cells) can induce class-switching. B cells may then proliferate in germinal centres and undergo somatic hypermutation to form highly efficient antibody-producing plasma cells. Memory B cells in humans are mostly in the resting state, and are CD24bright, CD38dull and CD27positive.

1.8.3 T cell development

Lymphoid progenitor cells exit the bone marrow and migrate in the blood to the thymus where T cell lymphopoiesis occurs. This involves a series of maturation steps which require interaction with stromal and epithelial cells of the thymic architecture (Zuniga-Pflucker 2004). Early thymocyte progenitors committed to the T cell lineage do not express the T cell receptor (TCR), or the co-repectors CD4 or CD8, hence are termed double negative thymocytes (CD4-CD8-). These cells may be further subdivided into four stages of differentiation based on expression of CD25 and CD44.

DN1 (CD44+CD25-) ----- > DN2 (CD44+CD25+) -----> DN3 (CD44-CD25+)--- > DN4 (CD44-CD25-)
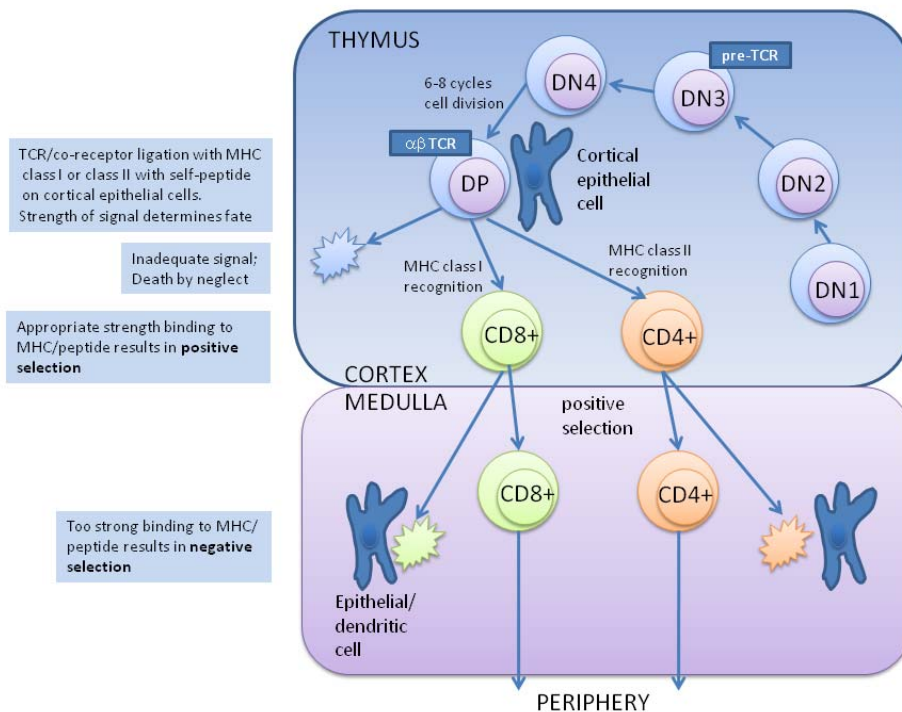
Figure 1e: Development of T cells within the thymus. DN = double negative. DP = double positive. Adapted from (Germain 2002).

DN3 thymocytes express the pre-TCR which is made up of a non-rearranged pre-TCRα chain and a rearranged TCR-β chain. Signalling through this receptor is required for further maturation, and induces 6-8 cycles of cell division in DN4 cells which give rise to double positive (DP) CD4 and CD8 expressing cells which are the precursor pool from which single positive CD4 and CD8 are derived. In DP thymocytes, the pre-TCRα-chain is replaced by a rearranged TCRα-chain, thus allowing for expression of a complete αβ TCR which can then interact with MHC-class I or MHC-class II loaded with self peptides on the surface of cortical epithelial cells (Germain 2002). The strength of this signal interaction determines the fate of the DP thymocyte. If the strength of interaction between the TCR and MHC/self-peptide complex is too low, cells do not receive a survival signal and die by neglect. Those that can bind MHC/self-peptide with sufficient strength are positively selected. If the strength of binding with self peptide and MHC is too high however, these potentially autoreactive cells are later subjected to negative selection upon encounter with dendritic cells in the thymic medulla (Kisielow et al. 1988;von Boehmer et al. 1989). T cells that survive these selection processes may exit the thymus and populate the peripheral T cell pool.

Successful maturation requires the co-receptor CD4 or CD8 to have the same MHC class specificity as the corresponding TCR. There are a number of models as to how this CD4-CD8 lineage commitment might occur. The 'instructional model' proposes that the biochemical signal through the TCR differs depending on whether MHC class I is co-recognised by TCR and CD8, or MHC class II is co-recognised by TCR and CD4. Each qualitative signal provides an instruction which induces the correct lineage commitment, and switches off genes associated with the other pathway (Germain 2002). One idea that has been put forward is that the intensity and duration of the signal through the TCR determines the lineage; short duration results in CD8 T cell development, and prolonged signals induce CD4 T cell development. This may be internally regulated partially by mitogen-activated protein kinase 1 (MAPK1); higher activity of which has been shown to preferentially induce CD4 T cell development (Wilkinson & Kaye 2001). MAPK1 phophorylates LCK at a serine residue, which protects it from becoming dephosphorylated by SHP1. This results in prolonged action of LCK and prolonged TCR signalling which favours CD4 T cell development.

Alternatively, the 'selection model' suggests that DP thymocytes may make a random or stochastic choice as to which lineage to commit to, which does not take into account the specificity of the TCR. This may result in choosing an incorrect co-receptor, which does not provide optimum antigen responses with the TCR of that particular cell. These cells are likely negatively selected during later antigen encounter, at which point they fail to receive survival signals due to inability to express the appropriate co-receptor (Germain 2002).

Recent data have shown that signalling through the Notch/Notch-ligand pathway controls many cell fate decisions during thymopoesis including that of T cell commitment (Maillard et al. 2005). In mammals, there are four highly conserved transmembrane receptors (Notch 1-4) which can interact with five ligands (Jagged 1+2, Delta-like 1, 3 + 4), which are highly expressed in the thymic environment. Binding to the ligand initiates proteolytic processes in the Notch transmembrane domain and release of ICN (intracellular Notch). This translocates to the nucleus and associates with DNA binding protein CSL, and results in eventual transcription of Notch target genes (Tanigaki & Honjo 2007). Deletion of Notch/Notch ligand genes is embryonically lethal, and inactivation of Notch 1 results in complete blockade to T lineage commitment, which cannot be compensated by signalling through the other Notch receptors (Radtke et al. 1999). This pathway is thought to regulate the early T vs. B cell fate decision, commitment to the $\alpha\beta$-T cell lineage over the $\gamma\delta$ T cell lineage, rearrangement of the TCR β gene and the CD4 vs. CD8 cell fate decision (Zuniga-Pflucker 2004).

1.9 MHC Class I presentation pathway

In the normal state, cells present thousands of peptides complexed to MHC class I on their surface, which are derived/processed from virtually all proteins synthesized in the cell. This allows CD8 T cells to constantly 'survey' the intracellular protein content of cells. In the event of a microbial infection, or if intracellular proteins are mutated in cancer, peptides derived from these non-self proteins are also complexed with MHC class I and presented to CD8 T cells (Hammer et al. 2007;Zinkernagel & Doherty 1974a;Zinkernagel & Doherty 1974b). Once CD8 T cells with the appropriate T cell receptor bind to these new complexes, they are alerted to the presence of non-self peptides, and rapidly mount either a cytolytic or non-cytolytic effector response to eliminate the infected or tumour cell (Xu et al. 2001).

The process of antigen presentation begins in the cytoplasm, where recently synthesized proteins are degraded into a mixture of peptides of variable lengths by the multi-catalytic proteosome, or other proteases and chaperones. These peptides are then transported into the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP), where the pMHC I repertoire is customised. This is achieved firstly by formation of the peptide-loading complex (PLC) which promotes the retention of MHC I in the ER, and its association with high-affinity peptides. Within the PLC, associated chaperone proteins help fold the heavy and β2 microglobulin chains of the MHC I molecule together so that it is in a peptide-receptive state (Hammer et al. 2007). Peptides which have then been cleaved to an optimum length by the ER aminopeptidase associated with antigen processing enzyme (ERAAP or EARP1 in humans) may associate into the MHC groove. ERAAP trims extra N-terminal residues from peptides, but does not alter the C-terminal end (Blanchard & Shastri 2008).

During viral infection, CD8 T cells may be primed by dendritic cells or other antigen presenting cells which present viral peptides complexed with MHC class I. These viral peptides may be derived from endogenously produced viral proteins, or from phagocytosed infected apoptotic bodies or endocytosed soluble viral antigens (Xu et al. 2001). During HBV infection, hepatocytes may have the capacity to present viral peptides on MHC class I directly to virus-specific CD8 T cells, however in a TCR-transgenic mouse model it was shown that TCR/MHC/peptide interaction without appropriate co-stimulation resulted in CD8 T cell tolerance (Bertolino et al. 1998).

1.10 Adaptive Immune response against HBV

HBV-specific CD8 and CD4 T cells are critical mediators of viral control in patients with self-limited infection. Viral clearance is dependent on the ability of these T cells to mount a strong, multi-specific response to all viral proteins; failure to do so results in viral persistence and chronic hepatitis (Maini & Bertoletti 2000).

CD8 T cells in particular have been singled out as the main effector cells involved. Thimme et al. showed that in chimpanzees acutely infected with HBV, depletion of CD8 T cells (starting from week 6 when the exponential phase of viral replication is expected to occur) resulted in extended peak levels of HBV DNA. Furthermore, on reappearance of CD8 T cells by week 13, there was a concurrent fifty-fold decrease in HBV DNA levels and this correlated with the appearance of virus-specific T cells and mRNA for CD3, CD8 and IFNγ in the liver. In contrast, depletion of CD4 T cells with a monoclonal antibody at week 6 did not alter duration or course of infection compared to the control antibody (Thimme et al. 2003). However, CD4 T cells have been shown to have important immunoregulatory function during the early course of infection, particularly the production of Th1 cytokines and IL-2, which are required to induce and maintain CD8 T cell function (Penna et al. 1997). Therefore, had these cells been depleted at an earlier stage, one might expect that this could alter the expansion and functional ability of the CD8 T cells. The importance of early CD4 T cell priming of CD8 T cell responses has been shown during primary CMV infection, where early CMV-specific CD4 T cell responses preceding CD8 T cell responses were associated with asymptomatic infection, whereas delayed CMV-specific CD4 responses were associated with symptomatic disease (Gamadia et al. 2003). These data imply, therefore, that inefficient CD4 T cell help of CD8 T cells and humoral responses could lead to viral persistence, although in this setting, CD4 were not required just for help, and could also independently induce CMV clearance through production of IFNγ.

The requirement of CD4 help for an effective cellular immune response against HBV is supported firstly by early studies which consistently found vigorous CD4 T cell responses towards HBV antigens in HBV infected subjects who developed acute self-limiting hepatitis, however this responsiveness was significantly impaired in patients with chronic HBV infection (Ferrari et al. 1990;Jung et al. 1991). Furthermore, strong multispecific CD4 responses with a Th1 profile were found in the blood of patients with acute self-

limited infection (Penna et al. 1997). In these patients, during the incubation phase, expansion of virus-specific CD8 and CD4 T cells was noted prior to viral clearance (Webster et al. 2000).

Consistent with a role for CD4 T cells in protective immunity, dysfunction of CD4 T cells has been shown to impair viral clearance in other examples of chronic infection. Adult CD4 knockout mice failed to clear LCMV infection, whereas in wildtype mice, virus was cleared from most tissues by 2 months (Zajac et al. 1998). During HCV infection, loss of virus-specific CD4 responses (in patients who initially mounted a strong response resulting in virus elimination; HCV PCR negative) was associated with a resurgence of HCV viraemia and recurrence of infection (Gerlach et al. 1999). In HIV infection, poor viral control in viraemic patients with high viral load was associated with an impaired capacity of HIV-specific CD4 T cells to proliferate in response to antigen. Antigen-specific CD4 T cells from aviraemic patients, however, maintained proliferative capacity and were additionally capable of producing IL-2 in vitro (Younes et al. 2003). The frequency of IL-2 producing HIV specific CD4 T cells was further shown by Harari et al. to negatively correlate with viral load (Harari et al. 2004), and preferential loss of IL-2 by HCV specific CD4 T cells was found in patients with chronic HCV, but not in those who resolved infection (Semmo et al. 2005).

In addition to producing IL-2 and supporting the proliferation of cytotoxic CD8 T cells, CD4 T cells are also required to prime humoral immune responses, and may additionally have a regulatory role in HBV infection. Production of HBeAg specific antibodies, for example, is dependent on CD4 T cells, and in acute HBV infection, a strong CD4 response against nucleocapsid antigens occurs at around the same time as clearance of enveloped antigens from the serum (Bertoletti & Ferrari 2003). As regards to their regulatory role, there is conflicting evidence as to the contribution of these cells in HBV (for a full background on regulatory T cells please refer to chapter six). Some studies have found no increase in classical regulatory CD4+CD25+ cells in CHB (Franzese et al. 2005), however others have found an enrichment of these regulatory T cells in the periphery and livers of patients with severe CHB infection (Peng et al. 2008a;Xu et al. 2006). In both the above studies however, depletion of CD4+CD25+ T cells increased the function of HBV-specific T cells.

In addition to T cells, B cells have an important role in inducing a humoral immune response. The importance of antibody production is highlighted by the fact that production of HBsAg-specific antibodies are neutralising (Rehermann & Nascimbeni 2005) and production of anti-envelope antibodies correlates temporally with clearance of HBV (Alberti et al. 1978). Furthermore, HBsAg-specific and HBcAg-IgG antibodies persist for life and provide protective immunity in the host. In addition to antibody production, B cells in mice have also been shown to regulate immune responses through production of cytokines such as IL-10 (Fillatreau et al. 2002;Mauri et al. 2003;Mizoguchi et al. 2002). The capacity for B cells to regulate immune responses during viral infection through production of IL-10 in humans is not yet documented, however has been investigated in this thesis.

1.11 Mechanisms of viral control

In earlier studies, it was assumed that clearance of HBV was proportional to the number of virus-specific CD8 T cells which could recognise viral peptide complexed to MHC-I on infected hepatocytes, and directly lyse these cells. However, it was realised that this could not be the sole mechanism of viral clearance as 1) upto 10^{11} hepatocytes may be infected with HBV (Guidotti et al. 1996), thus cytolytic damage of each cell would result almost certainly in irreversible tissue damage and fulminant hepatitis which is clinically only seen in 1% of patients, 2) hepatocytes have been shown to be relatively resistant to perforin mediated lysis (Kafrouni et al. 2001) and 3) during acute HBV infection in chimpanzees and humans, clearance of the majority of viral load is achieved in the absence of liver inflammation, suggesting that non-cytolytic mechanisms must be in play (Guidotti et al. 1999b;Webster et al. 2000). An elegant study in the transgenic mouse model of HBV revealed that upon adoptive transfer of virus-specific CD8 T cells into the livers of mice, HBV gene expression and replication was abolished with only minimal killing of HBV-infected hepatocytes. This non-cytolytic clearance was mediated by 'curative' cytokines IFNγ and TNF released directly by virus-specific CD8 T cells and also macrophages and T cells, which became activated. These cytokines could activate virocidal pathways in hepatocytes leading to destabilisation of viral RNA and elimination of HBV nucleocapsid particles with replicating genomes. Furthermore, injection of mice with HBsAg-specific H-2^d-restricted CD8 CTL clones derived from HBsAg-primed perforin knockout mice, resulted in disappearance of HBV DNA replicate forms and hepatocellular cytoplasmic HBcAg, without hepatocyte damage or elevation of ALT (Guidotti et al. 1996).

Non-cytolytic clearance of LCMV, another non-cytopathic infection, is also partially mediated by IFNγ production (Guidotti et al. 1999a). However, in this case, there is a clear co-dependence for both cytolytic and non-cytolytic mechanisms. Walsh et al. highlighted the need for cytolytic mechanisms by demonstrating that perforin knockout mice failed to clear certain strains of LCMV infection (Walsh et al. 1994). At the same time, Mueller et al. showed that knocking out the effect of IFNγ by generating IFN-γR knockout mice, resulted in uncontrolled LCMV replication, even though cytolytic function was preserved (Muller et al. 1994).

1.12 Mechanisms of liver pathogenesis

Hepatitis B virus is non-cytopathic, and it is the immune response against it which mediates immunopathology and liver damage. It has been assumed that cytolytic killing of infected hepatocytes by HBV-specific CD8 T cells was responsible for the majority of liver damage, partially based on work in HBV transgenic mice which showed that adoptive transfer of HBsAg specific CD8 T cell lines and clones into the liver resulted in development of acute necro-inflammatory liver disease (Ando et al. 1993;Moriyama et al. 1990). However, the finding that activated HBV-specific CD8 T cells are present in the livers of patients with no inflammation who successfully control HBV replication, may suggest a more complex scenario. In this study, Maini et al. showed that the absolute intrahepatic frequencies of HBV-specific CD8 T cells were similar in patients with or without liver inflammation. However, in the latter group, these HBV-specific CD8 T cells were diluted in amongst a massive non-antigen specific lymphocytic infiltrate, which was not detected in the absence of inflammation (Maini et al. 2000). How this infiltrate is recruited and what role it may have in the liver has been studied in the transgenic mouse model of HBV. In two consecutive studies, it was shown that recruitment of non-antigen specific lymphocytes was mediated in part by IFN-γ induced release of chemokines CXCL9 and CXCL10 from hepatocytes and non-parenchymal cells (Kakimi et al. 2001b), but also adoptively transferred virus-specific CD8 T cells could amplify this recruitment by inducing matrix metalloproteinase activity in GR1+ cells (neutrophils, macrophages, plasmactyoid dendritic cells), which further recruited non-antigen specific GR1- cells (NK/NKT/B/T lymphocytes, macrophages, dendritic cells) into the liver (Sitia et al. 2004). Most importantly, blocking of chemokines or depletion of GR1 cells reduced the influx of non-antigen specific lymphocytes and was associated with reduction in the severity of liver disease without affecting non-cytolytic clearance of the virus. Studies in humans have shown that NK cells may be a candidate cell type for mediating liver pathology, as they are abundant in the cellular infiltrate and account for 30-40% of resident intrahepatic lymphocytes (Norris et al. 1998). In a recent study, Dunn et al showed that these cells could mediate hepatocyte killing through the TNF-related apoptosis-inducing ligand (TRAIL) pathway. Furthermore, induction of cytokines IFNα and IL-8 during HBeAg negative flares of HBV, promoted NK TRAIL mediated apoptosis of hepatocytes, which in vivo could further aggravate liver pathology (Dunn et al. 2007). Other components of the

lymphocytic infiltrate may also contribute to this liver pathology, and the potential role of non-antigen specific CD8 T cells will be discussed further in this thesis.

1.13 Dual role for virus-specific CD8 T cells in viral control and liver damage

Data by Maini et al. showed that the absolute frequency of HBV-specific CD8 T cells was similar in the liver of patients who controlled virus replication with no liver inflammation, and patients with high viral load and liver inflammation (Maini et al. 2000). This suggested two intriguing concepts: 1) the frequency of HBV-specific CD8 T cells does not necessarily correlate with viral control 2) HBV-specific CD8 T cells can mediate viral clearance in the absence of liver inflammation.

With regards to the first point, studies in Human T-cell Lymphotropic Virus (HTLV-1) infection showed that frequency of tetramer positive HTLV-1 specific CD8 T cells was similar between patients with HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) and asymptomatic carriers (Jeffery et al. 1999), despite 16 fold greater proviral load in the former group (Nagai et al. 1998). This suggested that viral control was not related to the frequency of virus-specific CD8 T cells, but rather their responsiveness. This was further supported by use of a mathematical model which predicted that differences in the equilibrium proviral load was related to the rate of CTL proliferation in response to antigen, and the rate that a CTL killed a virus-infected cell (Nowak & Bangham 1996).

The second observation that virus-specific CD8 T cells can control infection without significant immunopathology is supported by data in HCV infected chimpanzees. Cooper et al. showed that intrahepatic HCV-specific CD8 T cells isolated from non-inflamed livers had cytotoxic capacity, however, the same populations isolated from highly inflamed livers had no detectable intrahepatic CTL activity (Cooper et al. 1999). These data suggested that liver damage and viral control may be independent of each other. Theoretical mathematical models have been applied to study the role of virus-specific CD8 T cells during persistent viral infection with HTLV-1. Asquith and Bangham proposed a model whereby the threshold of proviral load could be used to predict whether the virus-specific CD8 T cell response was harmful or resulted in viral clearance. Individuals who mounted a strong, efficient CD8 T cell response against the virus, were able to suppress antigen load below a certain threshold which was sufficient to ligate T cell receptors and induce lysis of

infected cells, but was not sufficient to induce production of IFNγ. However, in poor T cell responders, antigen levels were not sufficiently suppressed, and consequently reached a threshold sufficient to induce pro-inflammatory IFNγ production. This could induce a 'role switch' whereby the net effect of CTL effector function favoured tissue damage and inflammation over anti-viral cytolytic effector function (Asquith & Bangham 2000). This is consistent with a study in a TCR-transgenic mouse model of influenza infection, where high frequencies of virus-specific CD8 T cells were protective at low viral doses, but caused pathology and mortality in mice upon challenge with high viral doses, and this could be blocked through IFN-γ (Moskophidis & Kioussis 1998).

1.14 Characteristics of the virus-specific T cell response during CHB

It is widely accepted that HBV-specific CD8 T cell responses are strong and multispecific in patients with self-limited infection, however are characteristically weak, transient and barely detectable in patients who develop CHB (Bertoletti & Maini 2000). This was first shown in the early 1990's following stimulation with synthetic peptides derived from HBcAg and HBeAg in vitro (Bertoletti et al. 1991;Penna et al. 1991), and has since been confirmed by ex vivo quantification of these cells using tetramers (Maini et al. 2000), intracellular cytokine staining (Webster et al. 2004) and ELISPOT analysis after stimulation with either HLA-A2 restricted viral peptides, or overlapping pools of peptides spanning the entire HBV genome (Boni et al. 2007). Furthermore, microarray studies have confirmed that the gene profile of HBV-specific CD8 T cells differs in patients with CHB and resolved infection, and upregulation of apoptotic genes may contribute to attrition of the HBV-specific response in the former group (Lopes et al. 2008).

In addition to both quantitative and qualitative dysfunction of virus-specific populations in CHB, an epitope hierarchy exists within HBV-specific T cell responses which can be altered if virus persists. During acute HBV infection, ex vivo characterisation of HBV-specific CD8 T cells showed that the frequency of core 18-27 tetramer positive cells was much higher than polymerase 575-583 or envelope 335-343 epitopes (Maini et al. 1999a), although in some cases polymerase and envelope responses may dominate the CD8 T cell response (Webster et al. 2004). In patients who develop chronic hepatitis, above a certain threshold of viral load (HBV DNA>10^7copies/ml), core 18-27 specific responses could not be detected ex vivo or following in vitro expansion, and the frequency of these responses in the liver was shown to correlate inversely with viral load (Webster et al. 2004). Instead,

envelope responses with altered tetramer binding (Reignat et al. 2002) and polymerase responses could sometimes be detected in these individuals.

The ability to visualize virus-specific CD8 T cell populations with HLA Class I tetramers and simultaneously determine their functional capacity with intracellular cytokine staining, has facilitated the study of these cells in a wide range of chronic viral infections in mice (LCMV), primates (Simian Immunodeficiency Virus (SIV)) and man (HCV, HIV). These studies have consistently shown that, akin to CHB, a strong multispecific CD8 T cell response against a variety of viral epitopes is favourable for viral clearance and disease resolution, whereas mono-specific responses associated with functional loss allow viral persistence. Accordingly, marked constraints on virus-specific CD8 T cell responses have been documented when the virus is able to persist.

The observation that high frequencies of virus-specific CD8 T cells can persist in individuals with chronic SIV or HIV infection, but fail to clear the virus, suggested that these cells may be qualitatively inadequate/inefficient. Vogel et al showed that early after infection of monkeys with SIVmac239, the majority of virus-specific tetramer positive CD8 T cells were able to produce IFNγ. However, after four months of viral persistence, the ratio of IFNγ+ to tetramer+ cells was reduced to 0.66, suggesting that these cells became dysfunctional upon viral persistence (Vogel et al. 2001). A similar functional loss has been described during HIV infection, where Shankar et al. showed that less than 25% of tetramer+ HIV-specific CD8 T cells produced IFNγ upon stimulation with the relevant gag or reverse transcriptase peptides, and in addition, freshly isolated CD8 T cells were not cytolytic against HIV infected CD4 T cells (Shankar et al. 2000). This is supported by Appay et al. who demonstrated that HIV-specific CD8 T cells were perforin low and cytolytically impaired compared to CMV-specific CD8 T cells from the same chronically infected individuals (Appay et al. 2000).

Qualitative dysfunction within the HCV-specific CD8 T cell response has also been documented in chimpanzees who failed to resolve acute HCV infection and developed chronicity. In these animals, Cooper et al. observed strong, multi-specific response CTL responses against at least 6 viral epitopes in resolved chimpanzees, however responses were narrow and weaker in those who developed chronic infection (Cooper et al. 1999).

During HCV infection in humans, virus-specific CD8 T cells may be 'stunned' by high viraemia early after acute infection resulting in transient impairment of IFN-γ production (Lechner et al. 2000), or exhibit a longterm 'stunting' of function associated with poor TNF and IFNγ production on stimulation with specific peptides or with mitogens compared to CMV/EBV-specific CD8 T cell responses (Gruener et al. 2001;Klenerman et al. 2002). The prolonged dysfunction during chronic infection may be partially attributable to hyperexpression of PD-1, a negative regulator of the immune response, on both circulating and intrahepatic HCV-specific CD8 T cells. Blockade of PD-1 was shown to recover function in circulating but not intrahepatic HCV-specific CD8 T cells, which were phenotypically even further exhausted (CD127^{low}, CD28^{low}, CTLA4^{high}) (Nakamoto et al. 2008;Radziewicz et al. 2007).

During chronic LCMV infection, Zajac et al. demonstrated that CD8 T cells specific to certain dominant viral epitopes (GP 33-41) persisted indefinitely in mice and had an activated phenotype, however CD8 T cells specific for other dominant epitopes (NP 396-404) were deleted. The persisting GP33-41 specific CD8 T cells were impaired in their ability to release IFNγ and kill infected cells, and this antiviral function was completely abolished in the setting of CD4 deficiency. The level of unresponsiveness of these cells was found to be proportional to the duration of exposure to viral antigens; this could be modulated by inoculating mice with different strains of virus. With short periods of infection (1 week) induced by the Armstrong strain, all GP-33 CD8 T cells were functional, however upon infection with the Clone 13 virus which results in a highly disseminating, persistent chronic infection with high viral load, approximately 80% of GP-33 specific CD8 T cells were rendered unresponsive for a prolonged period. These data indicated that different immunodominant viral peptides may activate T cells to varying degrees resulting in either anergy or deletion to 'silence' the immune response during chronic infection (Zajac et al. 1998). In patients with CHB, where a similar epitope hierarchy exists within HBV-specific CD8 T cells, it is feasible that similar mechanisms may apply, and that core responses could be more susceptible to deletion than envelope or polymerase responses-possibly by excessive tolerance by large quantities of secreted HBeAg in patients with CHB. In support of this, it was found that HBcAg specific T cells were tolerised in HBeAg transgenic mice (Milich et al. 1990).

How persistence of virus could impact the immunodominance of virus-specific responses or impair functional responses was questioned in a follow-up study by Wherry et al, who studied the functional attributes of virus-specific CD8 T cells longitudinally during chronic LCMV infection induced by the Clone 13 strain of virus. They showed that there was a hierarchical loss of effector function within virus-specific CD8 T cells, driven by antigen load; IL-2 production and proliferative capacity were lost first, followed by capacity to produce TNFα then IFNγ. Cells which were functionally 'exhausted' beyond this point were physically deleted. This study not only characterised distinct phases of functional exhaustion defined by effector function, but also provided a valuable model of how persistent viruses may impact effector function and impair viral control (Wherry et al. 2003a).

1.15 Mechanisms of viral persistence

Viruses have developed a number of evasion strategies against the innate and adaptive immune system. 'Hiding' strategies include ability of some viruses to mutate frequently, preferentially occupy immunoprivileged sites, and to persist in a 'latent' state with minimal protein expression. Additionally, HBV may evade Toll Like Receptor detection due to the fact that viral replicative intermediates of ssRNA or viral DNA may be shielded within its nucleocapsid (Bertoletti & Gehring 2006). Other strategies may be more aggressive, and directly target the host's anti-viral defence mechanism. The HSV (Herpes Simplex Virus) Immediate-early protein ICP47 physically associates with TAP in cells, inhibits peptide translocation to the ER, and therefore prevents peptide loading of MHC class I molecules (Fruh et al. 1995). Similarly HIV nef protein can reroute surface MHC class I into clathrin-coated pits for endosomal degradation (Collins et al. 1998), and HCMV genes US3 and US6 code for proteins which retain class I molecules in the ER and block TAP mediated peptide translocation respectively (Tortorella et al. 2000). These various strategies which interfere with presentation of viral peptides by MHC-I are mechanisms to evade the potent cytolytic and non-cytolytic anti-viral CD8 T cell response.

In addition viruses use a host of additional strategies; inhibition of apoptosis of virus infected cells, expression of MHC class I homologues to evade NK cell mediated lysis and direct suppression of cytokine pathways including the early innate cytokines IFNα and IFNβ (Tortorella et al. 2000). It is likely that HBV has evolved to incorporate more than one of these evasion mechanisms to escape immune control.

1.16 Possible mechanisms of HBV persistence

1.16.1 Immune tolerance by virus/viral antigens

Chronic HBV infection is characterised by persistence of high dose antigen over many decades. In the mouse model of LCMV, high dose antigenic load has been shown to progressively drive CD8 T cells towards functional exhaustion and deletion (Wherry et al. 2003a). Therefore the quantitative and qualitative deficiencies observed in HBV-specific CD8 T cells, particularly in patients with high viral load, may be partially attributable to sustained attrition following repeated cycles of TCR mediated activation without appropriate rest cycles. Alternatively, tolerance of these immune responses over time could be secondary to exposure to viral antigens. One feature unique to HBV infection is the production and secretion of large amounts of surface and core antigens during viral replication which do not contain the HBV genome. HBeAg, the secretory form of the core antigen, in particular has been shown to have no role in viral replication, and instead may be required to induce tolerance in utero (Milich et al. 1990). This is supported by epidemiological data which show that children born to HBeAg negative mothers were less likely to develop chronic infection (Alter 2003). A tolerogenic role for viral antigens is also documented in patients with HCV. The HCV core protein can inhibit T cell proliferation by binding to gC1q receptor (Kittlesen et al. 2000) and may have an immunomodulatory role as it can also bind to the cytoplasmic tail of Lymphotoxin-β receptor, involved in development of peripheral lymphoid tissue and germinal centres (Matsumoto et al. 1997). Since the expression of these receptors is not confined to virus-specific cells, it is likely that tolerance by these antigens may provide a non-specific mechanism for T cell suppression affecting the global CD8 T cell population.

1.16.2 Viral escape

Mutation of immunodominant viral epitopes is a common evasion strategy to bypass immune detection, however this usually requires the virus to be under selection pressure by a strong narrowly focussed cytotoxic T cell (CTL) response. Because HCV polymerase lacks proof-reading capacity, and HIV reverse transcriptase is similarly error prone, high viral replication, in the setting of a strong CTL response, generates many possible mutations. However, this evasion strategy is uncommon during chronic HBV infection, where there is a characteristically weak CD8 T cell response, and low selection pressure on

the virus. Despite this, Bertoletti et al. showed that HBV isolates from two chronically infected patients had naturally occurring mutations within the immunodominant core epitope. Complexing of this variant peptide with MHC class I could then antagonise binding of the wildtype core peptide/MHC I with the T cell receptor. This could reduce the CTL response against the immunodominant wildtype core epitope, and may also allow preferential survival of cells expressing the variant peptide (Bertoletti et al. 1994). The ability of HBV to mutate can also be shown in patients on lamivudine treatment, in whom it is extremely common for viral mutations to develop within 3-5 years due to the rigorous selection pressure applied by antiviral therapy (Dusheiko & Antonakopoulos 2008).

1.16.3 Regulation of immune responses

The role for CD4+CD25^{high}Foxp3+ regulatory T cells in chronic HBV infection is as yet unclear, however, some studies have suggested that they are present at higher frequencies in the livers of patients with severe HBV infection (Xu et al. 2006), and depletion of these cells in vitro recovers HBV-specific CD8 T cell function (Franzese et al. 2005;Stoop et al. 2005). In addition to the classical CD4+CD25^{high}Foxp3+ regulatory T cell, we explore here a role for a novel subset of 'transitional' B cells, which have been shown to modulate immune responses through production of IL-10 (Fillatreau et al. 2002;Mauri et al. 2003;Mizoguchi et al. 2002), although it is likely that these cells are part of a regulatory framework through which their effects could be amplified. Thus, there is a potential for these cells to impair HBV-specific CD8 T cell responses in vivo through production of immunosuppressive IL-10 or contact dependent mechanisms.

1.16.4 Liver environment

The liver is a multi-purpose organ and one of its major roles is to process food antigens derived from the gut. In order to avoid immune responses against these non-pathogenic antigens, the liver is naturally an immunotolerant site (Calne et al. 1969;Crispe 2003). Recent studies have shown that this tolerance may partially be maintained by a constitutive production of IL-10 by resident Kupffer cells in response to gut derived bacterial antigens (Tu et al. 2008b). In addition, activated CD8 T cells have been shown to be preferentially sequestered to the liver where they undergo activation induced cell death (Crispe et al. 2000). Antigen-specific CD8 and CD4 T cells may be primed in the liver by hepatic stellate cells (Ito cells), hepatocytes and liver sinusoidal epithelial cells (LSECs). Ito cells

are liver resident, and were shown to resemble professional antigen presenting cells as they could present peptide on MHC-I and MHC-II to antigen-specific CD8 and CD4 T cells respectively, which mediated protection against bacterial infection (Winau et al. 2007). Priming by LSECs and hepatocytes on the other hand had a tolerogenic influence on T cell function. Naïve CD4 T cells primed by LSECs failed to produce Th1 cytokines (Knolle et al. 1999), and priming of naïve CD8 T cells in the liver by hepatocytes, which have a lower expression of surface MHC class I and lack co-stimulatory molecules found on professional antigen presenting cells, induced only transient cell activation and eventual CD8 T cell death by apoptosis (Bertoletti & Gehring 2006;Bertolino et al. 1998). Interestingly, upregulation of the pro-apoptotic molecule Bim in HBV-specific CD8 T cells from patients with chronic but not resolved infection (Lopes et al. 2008), was speculated to perhaps be a result of defective intrahepatic cross-presentation of antigens to CD8 T cells, as Bim is required for deletion of auto-reactive T cells following cross-presentation of soluble antigen (Davey et al. 2002). Thus a combination of Bim-mediated deletion, inefficient priming of effector responses by hepatocytes, and exposure to immunosuppressive IL-10 may contribute to impairment of CD8 T cell responses within the liver and perpetuate viral persistence.

2 Materials and Methods

2.1 Study Cohort

Patients were recruited from three sites; Mortimer Market Clinic (London), the Royal Free Hospital (London), and Adenbrooke's Hospital (Cambridge). Full ethical approval was obtained and each patient gave informed consent. HBV DNA viral load was determined by the Roche Amplicor Monitor Assay, and a commercial enzyme immunoassay was used to detect HBeAg/ anti-HBe antibody status (Murex Diagnostics). All patients were negative for antibodies to HIV-1 and HIV-2 (Ortho Diagnostics). All patients on antiviral therapy were excluded from the study unless otherwise stated. Healthy volunteers, both staff and students at University College London, kindly donated blood for the study.

Chronic hepatitis B virus is a heterogenous disease, and serological markers can be used to classify patients into different disease groups. Presence of HBV surface antigen (HBsAg) for a period greater than six months is diagnostic of chronic HBV infection. HBeAg, the secretory form of the HBV core antigen, is a marker of active HBV replication. Patients can clear HBeAg from the serum and this correlates temporally with the appearance of anti-HBe antibodies in the serum. HBeAg to anti-HBe seroconversion occurs early in patients with acute HBV infection, but may be delayed by years to decades in those with CHB, and occurs at a rate of 5-10% per year. Additionally, a group of patients with 'HBeAg negative hepatitis' do not have HBeAg present in their serum because of a viral mutation in either the basal core promoter or preC region of the HBV genome which prevents its expression. Patients with CHB may be further subdivided by their HBV DNA (IU/ml) and ALT levels, where ALT is a marker of liver inflammation (>50 IU/ml is abnormal). Patient tables with details of clinical characteristics can be found in the results sections of the relevant chapters.

Patients who have resolved after past infection with HBV can be identified by the presence of anti-HBs and IgG anti-HBc antibodies in their serum. In patients who are immune following vaccination, only anti-HBs antibodies are detectable.

2.2 Separation of Peripheral Blood Mononuclear Cells from whole blood

Venous blood was collected in EDTA tubes, layered on top of Ficoll-Hypaque at a 2:1 ratio, and centrifuged for 22 minutes at 2000 RPM with no brake. The top plasma layer

was then discarded, and the visible PBMC layer was removed with a Pasteur pipette into RPMI 1640 medium for washing. After two wash cycles, cells were resuspended in RPMI complete medium with 10% fetal bovine serum for counting.

2.3 Cell Counting

A 10μl aliquot of cells was diluted in 90μl of trypan blue and the suspension was counted on a Neubauer haemocytometer under a light microscope. The number of cells per millilitre of the original cell stock was determined by the following formula:

Number of cells/ml = number of cells counted in central grid **x** dilution factor (10) **x** 10^4

2.4 Freezing/Thawing of cells

Peripheral blood mononuclear cells that were not used for direct ex vivo experiments were resuspended at a concentration of 5 million cells/ml in freezing medium (90% fetal bovine serum and 10% DMSO) and rapidly aliquoted into 1.5ml cryovials (Nunc). These were stored at –80°C in Mr. Frosty Chambers (Fisher Scientific, UK) for a maximum of 7 days, following which they were transferred to liquid nitrogen tanks for long-term storage.

When required, cryovials were thawed in a 37°C waterbath and the cell suspension was rapidly transferred to RPMI medium to buffer the toxic effects of DMSO. Following two wash cycles each time spinning the cells for 8 minutes at 1600rpm, decanting the medium and resuspending the cells in RPMI, cells were then ready for counting.

2.5 Processing Liver samples

Liver samples were obtained from patients with chronic HBV infection or patients with non-viral liver disease undergoing diagnostic liver biopsies or from resected tissue. Liver tissue was suspended in RPMI 1640 (Sigma) and macerated with a plunger from a 25ml syringe and a scalpel in a Petri dish. The cell suspension was then passed several times through a 70μm cell strainer (BD biosciences), each time washing the filter with fresh RPMI to minimise cell clumping and loss. The cell suspension was then washed three times and resuspended in RPMI complete medium with 10% fetal bovine serum for counting. Lymphocytes were identified under a high magnification by their size, shape and granularity.

2.6 Antibodies and Reagents

CD107a FITC	555800	BD Pharmingen
CD19 Pe-Cy7	25-0199-73	eBioscience
CD19 PerCP	345790	BD
CD24 PE	555428	BD Pharmingen
CD27 FITC	340424	BD
CD28 FITC	555728	BD Pharmingen
CD3 PerCPCy5.5	332771	BD
CD38 FITC	555459	BD Pharmingen
CD45RA APC	MHCCD45RA05	Caltag Laboratories
CD8 APC	555369	BD
CD8 biotin	0264	Coulter Immunotech
CD8 PE	R0806	DAKO
CD8 Pe-Cy5	555368	BD
CD8 PerCPCy5.5	341050	BD
HLA-A2 FITC	MCA2090F	Serotec
IFN-γ APC	341117	BD
IFN-γ FITC	IC285F	R&D
IFN-γ PE	IC285P	R&D
IL-10 APC	554707	BD Pharmingen
IL-2 FITC	IC202F	R&D
Ki67 FITC antibody set	556026	BD Pharmingen
Mouse IgG1-PE	PN IM0670	Coulter Immunotech
Normal affinity C18-27-specific tetramer PE	Alison Turner, Paul Klenerman, Oxford	
Streptavadin Cy3	CLCSA1010	Cedarline lab.
TCR ZETA PE	PN IM3169	Coulter Immunotech
TNF-α PE	559321	BD Pharmingen

anti-CD28	555725	BD
anti-CD3 (OKT3)	Kind gift of Peter Beverley and Arne Akbar	
anti-IL-10 Receptor (CDw210)	556011	BD Pharmingen
β-Mercaptoethanol	31350010	Gibco
Bovine Serum Albumin	A-7906	Sigma
Brefeldin A	B7651	Sigma
BS3 Bis(Sulfosuccinimidyl) suberate	21580	Perbio Science
CD19 MicroBeads	130-050-301	Miltenyi Biotec
CFSE	V12883	Molecular Probes
Copper(II) sulfate pentahydrate	C-7631	Sigma
CpG ODN 2006	tlrl-hodnb	InVivoGen
Cytoperm/Cytofix	554722	BD
DMSO	D2650	Sigma
EDTA	E-7889	Sigma
Essential amino acids	11130.036	Gibco
Fetal Bovine Serum	10108-165	Invitrogen
Ficoll-Paque PLUS	17-1440-03	GE Healthcare
Formaldehyde	F8775	Sigma
Formamide	444475W	BDH
Functional grade anti-IL-10	16-7108-85	eBioscience
γ-^{33} P-ATP	NEG602H	Perkin Elmer
HBcAg	kind gift from	Rhein Biotech
Hepes	15630056	Gibco
Human IL-10 ELISA Kit	950.060.096	Diaclone
Human IL-10 High Sensitivity Elisa kit	850.880.096	Diaclone
Human IL-2 Quantikine Immunoassay	D2050	R & D
Human T cell nucleofector Kit	VPA-1002	AMAXA Biosystems

human-CD8 T cell isolation kit II	130-091-154	Miltenyi Biotec
IL-2 Human	11 011 456 001	Roche
Ionomycin	I0634	Sigma
L-arginine	A8094	Sigma
Manganese chloride	M3634	Sigma
Monensin	M5273	Sigma
Non-essential amino acids	11140.035	Gibco
Penicillin/streptomycin	15070.063	Gibco
Phorbol 12-Myristate 13-Acetate	P8139	Sigma
Phosphate Buffered Saline	P4417-100 TAB	Sigma
PMSF	P7626	Sigma
PNA probe	4330098	Applied Biosciences
Potassium sodium tartrate tetrahydrate	S-2377	Sigma
Protease inhibitor cocktail	P8340	Sigma
RPMI 1640	R8758	Sigma
RPMI 1640 modified w/o L-arginine	81230-1000M2268	JRH Biosciences
Saponin	S-4521	Sigma
Sodium Chloride 5M solution	S-5150	Sigma
Sodium Hydroxide	S-5881	Sigma
Sodium pyruvate	11360.039	Gibco
Sodium tungstate dehydrate	T-2629	Sigma
Telomerase PCR ELISA[PLUS]	12013789001	Roche
TRAPEZE telomerase Detection Kit	S7700	Chemicon
Tris	T1503-1Kg	Sigma
TRIS-HCl 1M Stock	T-2538	Sigma
Trucount Tubes	346334	BD
Trypan Blue	T8154	Sigma
Tween 20	161-0781	BIO-RAD
Zenalb 20 (Human Albumin 20%)		Bio Products Lab.

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2.7 Detection of IFN-γ/IL-2/TNF-α production

PBMC were incubated at 37°C in a humidified incubator with 5% CO_2 for 16 hours in the presence of either:

1. *Reagents which act via the T cell receptor:* 1μg/ml **anti-CD3mAb** [OKT3 clone kindly provided by Peter Beverley and Arne Akbar] and 5μg/ml **anti-CD28mAb** (BD biosciences).

2. *T cell receptor bypassing reagents:* **Phorbol Myristic Acetate** (PMA, 3ng/ml) and **Ionomycin** (1μg/ml)

3. *Negative control:* RPMI complete medium with 10% fetal bovine serum
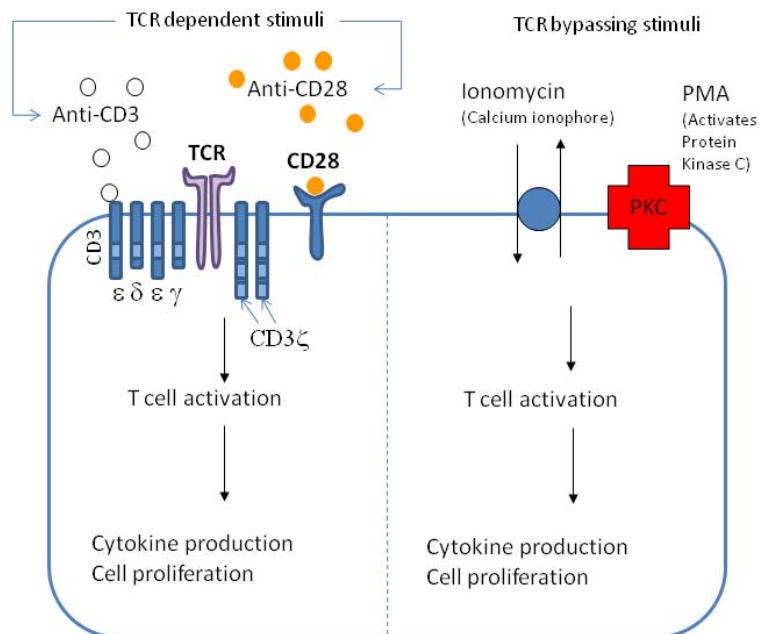
Figure 2a: Stimulation of T cells with either TCR-dependent or TCR-bypassing mitogens.

Brefeldin A (10μg/ml, Sigma-Aldrich) was added 1 hour into the incubation time to suspend protein transport from the endoplasmic reticulum to the Golgi apparatus, hence preventing extracellular release of cytokines. PBMC were then washed in PBS, stained with anti-CD8–Cychrome (PE-Cy5) mAb (30 minutes) and then permeabilized and fixed with Cytoperm/Cytofix (BD Biosciences) at 4°C (30 minutes). After a further wash in PBS, cells were dual stained with anti-IFN-γ-PE mAb (R&D systems) and anti-IL-2-FITC mAb (R&D systems) or anti-TNF-α-PE mAb (BD Pharmingen) alone in PBS containing 0.1% saponin at 4°C for 30 minutes. After 2 further washes cells were fixed (PBS, 1% formaldehyde, 1% FBS) and acquired immediately on a FacsCalibur flow cytometer using Cell Quest Software.

2.8 CFSE proliferation assay

PBMC were washed 3 times in PBS, resuspended at 1 x 10^{6} cells/ml in PBS and incubated for 10 minutes at 37°C with 0.5 μM carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes). An equal volume of FBS was added to quench the reaction. Cells were subsequently washed twice in RPMI complete medium supplemented with 10% fetal bovine serum and then transferred into a 96 well plate with pre-coated anti-CD3 mAb (1μg/ml). Soluble anti-CD28 was added (5μg/ml) in addition. At day 6, cells were harvested, stained with anti-CD8–Cychrome (PE-Cy5) and then acquired on the flow cytometer.

2.9 CD107 degranulation assay

PBMC were stimulated for 16 hours at 37°C in the presence of anti-CD3 and anti-CD28 in addition to anti-CD107a-FITC. Monensin was added 1 hour into the incubation time. Cells were then surface stained with anti-CD8-Cychrome (PE-Cy5) (BD biosciences) and acquired on the FACS Calibur Cytometer.

2.10 Stimulation with synthetic viral peptides

The following peptides representing HLA-A2 restricted viral epitopes were used in this study:

Peptide	amino acid sequence
HBV env 183-191	**FLLTRILTI**
HBV env 335-343	**WLSLLVPFV**
HBV env 338-347	**LLVPFVQWFV**
HBV env 348-357	**GLSPTVWLSV**
HBV pol 455-463	**GLSRYVARL**
HBV pol 502-510	**KLHLYSHPI**
HBV core 18-27	**FLPSDFFPSV**
HCMV pp65 495-504	**NLVPMVATV**
EBV BMLF-1 259-267	**GLCTLVAML**
INFLUENZA A MP 58-66	**GILGFVFTL**

Table 2.1: Details of viral peptides used in this study. All peptides were obtained from Proimmune.

Overnight stimulation

PBMC were stimulated with 10μM peptide for 12 hours and incubated at 37°C in the presence of Brefeldin A (added 1 hour into the incubation).

Short-term culture

1μM peptide (or a pool of the seven HBV peptides) were added to 0.25x10^{6} PBMC in a 96 well plate with 50 IU IL-2 in RPMI complete medium for 10 days at 37°C. IL-2 and medium were refreshed on day 4 of culture. On day 10, PBMC were restimulated with 1μM peptide for 5 hours, in the presence of Brefeldin A (added 1 hour into the incubation).

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2.11 Detection of CD28 and intracellular CD3ζ

PBMC isolated ex vivo were plated (2.5 x10^5/well) in a 96 well round bottomed plate and incubated at 4^oC for 30 minutes with saturating concentrations of anti-CD3 PerCP mAb, anti-CD8 APC mAb and anti-CD28 FITC mAb. After a wash step in PBS, cells were fixed and permeabilized according to the manufacturers protocol with Cytoperm/Cytofix for 30 minutes (4^oC) after which intracellular staining was carried out using anti-TCRζ-PE mAb (Coulter Immunotech) or its corresponding isotype control, IgG1-PE mAb in PBS containing 0.1% saponin to permeabilise the cells. Washed cells, resuspended in a solution of PBS, 0.1% formaldehyde and 10% Fetal Bovine Serum, were acquired on a Becton Dickinson Facs Calibur using Cell Quest Software and data files were analysed using FloJo software (Tree Star).

2.12 Transfection with chimeric receptors

The CD3ζ/CD28 fusion chimeric receptor was used in this study. The receptor was cloned into an expression vector derived from pQBI-AdCMV5 (QBIOgene) and subsequently plasmid DNA preparation was done with p500 columns (Qiagen). Plasmid DNA was kindly gifted to us by Dr. Alastair Lawson and Dr. Helene Finney (Celltech).

5 x 10^6 PBMC were washed in PBS then resuspended in 100μl of nucleofector solution for T cells. 3-6μg of plasmid DNA (or PBS as a negative control) was added. The suspension was subsequently electroporated using program U-13 in the nucleofector device (Amaxa Biosystems), and then removed into prewarmed RPMI complete with 10% fetal bovine serum. Transfected cells were rested overnight at 37^oC, and the proportion of cells expressing the chimeric receptor was determined using anti-CD33 (FITC) by flow cytometry.
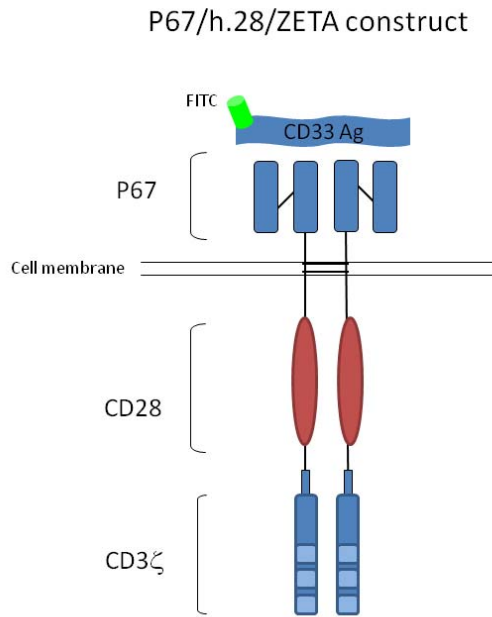
Figure 2b: The CD3ζ/CD28 fusion chimeric receptor.

2.13 Detection of IL-2 production by ELISA

Transfected cells were rested overnight at 37°C, following which 2.5×10^5 cells were seeded into appropriate wells in a 96 well flat bottomed plate pre-coated with either CD33 antigen (5μg/ml), anti-CD3 (1μg/ml), both anti-CD3 and CD33 antigen together, or neither as a negative control. Soluble anti-CD28 was added to the appropriate wells (5μg/ml). Cells were then left in the incubator at 37°C for 48 hours following which supernatants were harvested. Enzyme linked immunosorbent assays were then conducted in duplicate using the supernatants collected, according to the manufacturer's instructions (human IL-2 quantikine immunoassay, R&D systems).

2.14 Detection of CD3ζ following culture in L-arginine free medium

PBMC (2.5×10^5) were seeded into a 96 well round bottomed plate and incubated at 37^oC for 6 days in either L-arginine free medium (GRH biosciences) or L-arginine free medium supplemented with L-arginine (Sigma-Aldrich) (0.2g/l). CD3ζ expression in CD8 T cells was then measured using the protocol detailed above.

2.15 Proliferation of HBV-specific CD8 T cells with or without L-arginine

In order to examine the proliferative capacity of HBV-specific CD8 T cells, PBMC were stained for CFSE as above, then stimulated with HBV c18-27 peptide, and on day 7, HBV-specific CD8 T cells were detected by costaining with anti-CD8-Cychrome (PE-Cy5) and an HLA-A2/c18-27-specific tetramer (PE) (kindly provided by Alison Turner and Paul Klenerman, Oxford). PBMC were cultured in L-arginine free medium, with or without the addition of L-arginine at the range of concentrations indicated.

2.16 Detection of serum L-arginine levels

L-Arginine levels were determined by electrospray mass spectrometry. Samples (50µl) were deproteinized with acetonitrile, chromatographed (acetonitrile:water, 1:1, with 0.025% formic acid) on a Teicoplanin guard column 10mm x 2.1mm ID (Chirobiotic T, ASTEC Ltd, Congleton, UK), and analysed using a SCIEX API4000 (Applied Biosystems, Warrington, UK) in positive ion multiple reaction monitoring mode. Arginine quantitation was done by R. Neil Dalton and Charles Turner of the WellChild Laboratory, Evelina Children's Hospital at St Thomas's Hospital, London.

2.17 Detection of arginase activity in serum

Arginase-I breaks down L-arginine to urea and ornithine. In this assay, a saturating concentration of substate (L-arginine) was added to a serum sample and the amount of urea produced in a given amount of time was used as a measure of the arginase activity in that sample. 1µM Urea made/hour/L serum is often abbreviated to IU/L serum as the units for measurement of arginase activity.

Solutions:

To initiate reaction:

- $MnCl_2$ manganese chloride solution (Sigma) 10mM
- TRIS HCL buffer: 0.1M TRIS, 1M HCL, 1% Saponin (Sigma) pH 7.4
- L-arginine solution (Sigma) 50mM

Stop solution: 0.22g Tungstic acid

              9ml water

              100μl concentrated Hydrochloric Acid

              100μl concentrated Sulphuric Acid

20μl of serum was combined with 20μl of $MnCl_2$ solution, 150μl of Tris Buffer and 100μl of L-arginine solution. Tubes were closed and mixed rapidly after addition of L-arginine, and then incubated for 30 minutes at 37^oC following which the reaction was stopped with a Tungstic acid solution. Epindorph tubes were then spun at 5000 RPM for 10 minutes in a centrifuge precooled to 4^oC to pellet the unwanted particulate matter. 200μl of clear solution was then carefully removed into COBAS tubes for detection of urea on the COBAS INTEGRA 400 (Roche). To subtract background serum urea levels, each experiment was exactly duplicated with the exception that 100μl water was added in place of L-arginine substrate.

2.18 Detection of arginase activity in liver tissue

Liver tissue was weighed then added to TRIS/HCL buffer containing protease inhibitor cocktail (Sigma) and trypsin inhibitor phenylmethylsulphonyl fluoride (PMSF) in a 1:4 ratio at the bottom a glass mortar. A pestle was then used to macerate the liver into a cell suspension. This was transferred to an epindorph, exposed to three 15 minute freeze thaw cycles at -80^oC and then centrifuged at 5000 RPM for 10 minutes to pellet the cellular debris. The clear homogenate was then removed for determination of arginase activity as described above.

2.19 Detection of total liver protein content by Biuret test

The Biuret test can be used to measure protein content. In principle, Copper II ions are reduced to Copper I ions which subsequently bind to peptide bonds in an alkaline solution and this results in a colour change which can be quantified by a spectrophotometer. The magnitude of the colour change is proportional to the amount of protein present.

Biuret solution formulation: 0.75g Copper(II) sulfate pentahydrate (Sigma)

3g Potassium sodium tartrate tetrahydrate (Sigma)

16.66g Sodium Hydroxide

500ml distilled water

In order to prepare a standard curve, 20% human albumin solution (Zenalb) was serially diluted two fold to produce 8 standards ranging from 200mg/ml down to 1.56mg/ml. 15μl of standard or liver homogenate sample was then added in duplicate to individual cuvettes, following which 1ml of biuret solution was added to every tube. Samples/standards were left at room temperature for 1 hour to allow colour change which was then quantified on a spectrophotometer.

Chapter 5 Materials and Methods

2.20 Determination of phenotypic subsets within global CD8 T cells

PBMC were surface stained with the following antibodies for 30 minutes at 4°C; anti-CD27-FITC, anti-CD8-PE, anti-CD3-PerCPCy5.5 and anti-CD45RA APC. Cells were then washed twice in PBS containing 1% FBS, and resuspended in a solution of PBS, 0.1% formaldehyde and 10% FBS ready for acquisition on a Becton Dickinson Facs Calibur using Cell Quest Software. To determine CD3ζ expression within the different subsets, a slightly modified protocol was used whereby cells were instead surface stained with anti-CD27-FITC, anti-CD45RA APC and anti-CD8PerCP, then intracellularly stained with TCRζ PE as described above.

2.21 Quantification of telomere length in CD8 T cells with fluorescence in situ hybridisation (Flow FISH)

Telomeres cap the ends of chromosomes and in humans are made of repeating hexameric TTAGGG sequences. Flow FISH incorporates a technique whereby a fluorescently labelled protein nucleic acid telomeric probe is hybridised onto the telomeric repeats. The flouresence intensity can then be measured on a per cell basis and is a quantitative measure of the length of the telomeres. By co-staining for markers CD8 and CD27, telomere length can be compared between subsets in any individual, or between different individuals.

2×10^{6} PBMC were washed in RPMI, then stained for the following surface markers:

- 1° layer : anti-CD8 biotin (20 minutes at room temperature), x1 wash in PBS

- 2° layer: anti-streptavadin Cy3 (20 minutes at room temperature), x1 wash in PBS

- anti-CD27 FITC mAb (20 minutes at room temperature) x 1 wash in PBS

Cells were then fixed for 30 minutes at 4°C with 1mM BS3 (Perbio Science) following which the reaction was quenched with 1ml of 50mM TRIS in PBS (pH7.2) for 20 minutes in the dark at room temperature. After a further two washes, cells were resuspended in hybridisation buffer and this suspension was equally redistributed to 3 new FACS tubes so that triplicate tubes for each individual could be run to minimise error. 0.75μg/ml protein nucleic acid telomeric probe $(C_3TA_2)_3$ conjugated to Cy5 was added, and cells were heated to 82°C in a waterbath for 10 minutes to allow denaturation of telomeric DNA. Cells were

then rapidly snap cooled and left at room temperature for 2 hours in the dark to allow hybridisation to occur.

Samples were finally washed twice in post-hybridisation buffer, and twice in PBS to remove excess probe and analysed on the flow cytometer. In addition to running triplicate samples, an internal control was run for every experiment; PBMC from a healthy donor whose telomere length had been predetermined both by southern blotting for TRF length and by flow FISH.

Mean fluorescence intensity values were averaged from triplicate samples and used as a quantitative measure of telomere length. In addition, these values were plotted against a standard curve to obtain absolute values of TRF length.

Hybridisation buffer: 7ml formamide

       0.2ml 1M stock Tris HCL

       2.5ml 4% Bovine Serum Albumin

       0.3ml 5M NaCl

Post Hybridisation buffer: 7ml formamide

       0.1ml 1M Tris HCL

       0.25ml 4% Bovine Serum Albumin

       0.1ml 10% Tween 20

       0.3ml 5M NaCl

       2.25ml distilled water

2.22 Telomere length measurement in virus-specific CD8 T cells

Determination of telomere length in virus-specific populations used the above protocol, however, with the following exceptions;

1. PBMC were first stimulated with HLA-A2 restricted viral peptides representing the immunodominant epitopes to CMV/EBV or HBV for either 12 hours at a 10μM concentration (ex vivo), or for 10 days at 37°C at a 1μM concentration.

2. PBMC were not surface stained with anti-CD27 FITC

3. Cells were permeabilised with cytoperm/cytofix instead of BS3

4. Following permeabilisation, PBMC were additionally intracellularly stained with anti-IFNγ FITC in PBS containing saponin to allow detection of virus-specific CD8 T cells. This incubation step was for 30 minutes at 4°C, following which cells were washed twice and then resuspended in hybridisation buffer.

2.23 Measurement of telomerase activity

2.23.1 Purification of CD8 T cells

CD8 T cells were isolated by negative selection using the human-CD8 T cell isolation kit II (Miltenyi Biotech) as per the manufacturer's instructions. Briefly, PBMC were incubated with a cocktail of biotin conjugated antibodies against markers of non-CD8 T cells, including CD19, CD4, CD123, CD56, Glycophorin A and TCR gamma/delta. Cells were then subsequently stained with anti-biotin microbeads which bind to the antibody-tagged cells. Within the magnetic field of the MACS separator, these magnetically tagged non-CD8 T cells were attracted to stick to the column through which they passed, and untouched CD8 T cells were free to pass through and be collected.

2.23.2 Stimulation of CD8 T cells and Ki67/Trucount analysis

Collected CD8 T cells were then either snap frozen (0.1×10^6) for ex vivo analysis of telomerase activity, or stimulated for 3 or 5 days with anti-CD3 (1ug/ml) in combination with irradiated PBMC (40 Grays gamma radiation) at $37^{\circ}C$ in a humidified 5% CO_2 incubator to induce telomerase activity. Since telomerase activity is predominantly induced in cycling lymphocytes and is low in resting cells, two additional wells of CD8 T cells were seeded to allow for co-staining of CD8 with Ki67, a marker of cell cycling, and enumeration of the absolute number of CD8 T cells with Trucount beads (BD biosciences). Thus, upon the PCR stage of telomerase detection, samples were adjusted according to 500 Ki67+ CD8 T cells per reaction.

2.23.3 Detection of telomerase activity

Telomerase activity was determined by a gel based TRAPeze system (Chemicon), modified from the PCR based telomeric repeat amplification protocol, or alternatively with the Roche PCR ELISA Plus TRAPeze Kit as per the manufacturer's instructions. Snap frozen samples were first thawed on ice and incubated with CHAPS lysis buffer. Samples were then centrifuged at 12,000rpm for 20 minutes to precipitate cell debris, and cell extracts were collected for determination of telomerase activity which involved a two step process.

1) Telomerase present within the sample cell extract added telomeric repeats onto the 3' end of a substrate oligonucleotide (TS)

2) The product was then amplified by PCR

This generated a ladder of products with 6 base increments starting at 50 nucleotides. Total Product Generated (TPG) was then used as a quantitative measure of telomerase activity. The positive control used an extract from a telomerase positive tumour cell line. The negative control involved adding lysis buffer to the PCR mix in place of cell extract.

For quantitative detection of the amplified product, the TS primer was initially end labelled with radioactive γ-^{33}P-ATP. Radioactively labelled TS primer was then added to reaction buffer, dNTP mix, TRAP primer mix, Taq Polymerase and distilled water to make up a master mix, to which sample extract was added. After the PCR amplification step, loading

buffer was added to the PCR reaction tube, and 25µl of mixture was loaded onto a 12% polyacrylamide gel in 0.5X TBE buffer. This was run at 400V for approximately 1 hour 40 minutes. After electrophoresis, gels were removed and dried, and placed onto X-ray film in cassettes overnight. Telomerase activity was determined using a GS-800 Bio-rad densitometer using the Quantity one software.

Chapter 6 Materials and Methods

2.24 Quantification of IL-10 in serum and supernatant

2.24.1 Serum

IL-10 levels in serum were quantified with the Human IL-10 High Sensitivity Elisa Kit (Diaclone) as per the manufacturer's instructions. The range of detection for this kit was between 1.56pg/ml – 50pg/ml.

2.24.2 Supernatant

PBMC from patients and controls were stimulated for 5 hours with PMA and ionomycin, following which supernatants were collected and stored at -80°C. IL-10 content in batch collected supernatant samples was then quantified with the Human IL-10 Elisa Kit (Diaclone).

2.25 Stimulation and phenotypic analysis of B cells

PBMC were stimulated with CpG oligonucleotide type B (InVivoGen), a human TLR9 ligand, for 96 hours at 37°C. For the last four hours of stimulation, PMA and ionomycin were added to restimulate the cells, in addition to Brefeldin A to prevent export of cytokines out of the cell. Cells were then surface stained for markers CD19, CD24 and CD38, fixed and permeabilised, then intracellularly stained with anti-IL-10 APC for 50 minutes at room temperature. After two further washes, cells were fixed in PBS containing FBS (1%) and formaldehyde and immediately acquired on the flow cytometer. (In addition to stimulation with CpG, in some cases PBMC were stimulated with HBcAg (10μg/ml) with or without irradiated Chinese Hamster Ovary (CHO) cells expressing CD40L which has been shown to induce IL-10 production in B cells by binding to CD40 on their surface. In these preliminary experiments, PBMC were additionally intracellularly co-stained with anti-TNF PE, to investigate the differential induction of this cytokine in patients vs. healthy donors.

On analysis with Flojo software, dependent on expression of surface markers CD24 and CD38, CD19+ B cells were categorised into 3 phenotypic subsets; mature, memory and transitional (CD19+CD24^{high}CD38^{high}). IL-10 production could then be analysed within total B cells (CD19+) or within each of these subsets.

In some patients, serially stored PBMC were available at multiple timepoints before, during and after flares of liver disease. In these individuals, PBMC were stained ex vivo for phenotypic markers, and the frequency of the different peripheral B cell subsets was analysed longitudinally. In addition, PBMC were stimulated with CpG as described above for 96 hours, following which longitudinal fluctuations of IL-10 producing CD19+ B cells and phenotypic subsets could be analysed.

2.26 Positive selection of B cells with magnetic beads

CD19+ cells were depleted from PBMC by positive selection within a magnetic field. The magnitude of virus-specific CD8 T cell responses, expanded for 10 days with viral peptides was then compared between PBMC with or without B cells present.

2.27 Depletion of transitional CD24^{high} CD38^{high} B cells by flow based cell sorting

PBMC were surface stained with anti-CD38 FITC (BD Pharmingen), anti-CD24 PE (BD Pharmingen) and anti-CD19 Pe-Cy7 (eBioscience) for 20 minutes at room temperature in the dark. After a wash in MACS buffer containing BSA (0.5%) and EDTA, samples were resuspended in buffer in polypropylene tubes suitable for use on the BD FACSAria Cell-Sorting System. Manual gates were drawn to allow selective depletion of the CD24^{high}CD38^{high}CD19+ transitional B cells. These highly pure transitional cells were then collected in separate tubes to the PBMC depleted fraction. Additionally, whole PBMC stained with antibodies were passed through the machine untouched as a control. In later experiments, magnitude of virus-specific CD8 T cell responses could then be compared between whole PBMC, and those depleted of transitional cells.
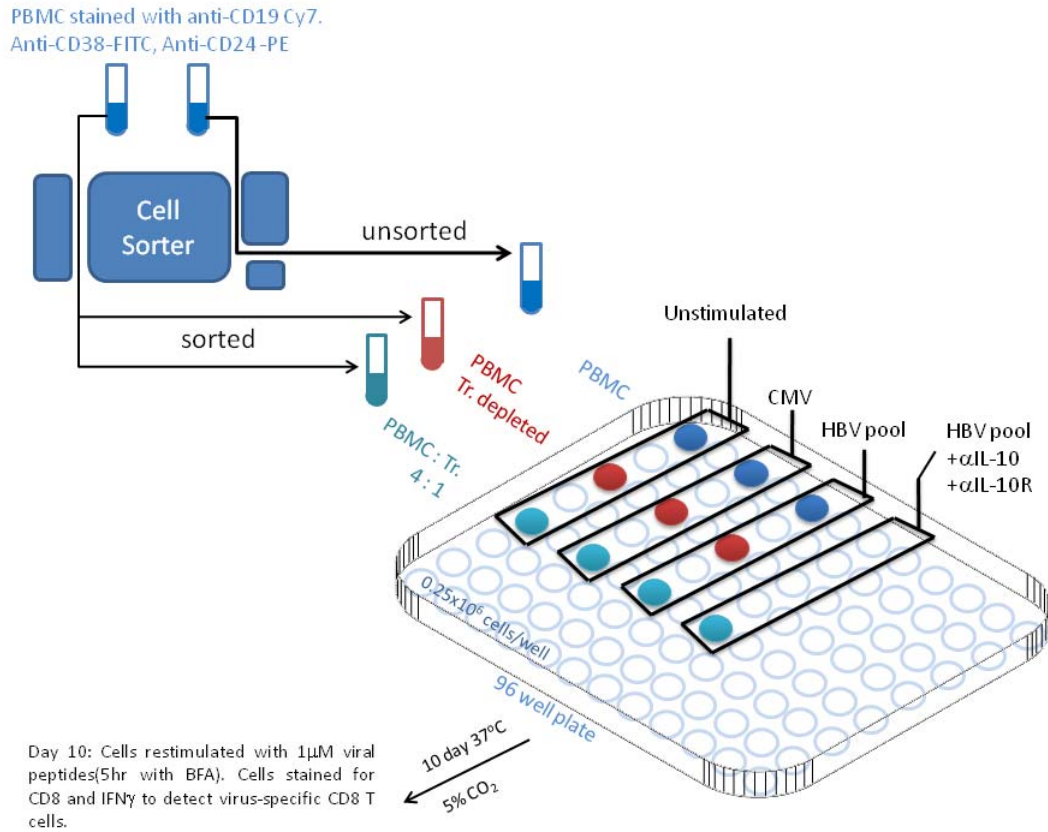
Figure 2c: Diagram to show the stimulation protocol for sorted and unsorted cells.

2.28 Short term virus-specific stimulation with IL-10/IL-10R blockade

PBMC from HLA-A2+ individuals were seeded in duplicate into a 96 well plate (0.25×10^6/well) in the presence of 1µM viral peptide (see table 2.1) and 50 U/ml IL-2, with or without the addition of either a) anti-IL-10 (ebioscience) 5µg/ml b) anti-IL-10R (eBioscience) 10µg/ml or both together. Medium was refreshed on day 4 with further addition of exogenous IL-2 (50U/ml) and anti IL-10 (2.5µg/ml) or its receptor (5µg/ml). On day 10, PBMC were pulsed for a further 5 hours with 1µM peptide in the presence of Brefeldin A (10µg/ml) and then stained with anti-CD8 APC, anti-CD3 PerCPCy5.5 and intracellularly stained with anti-IFNγ-FITC as described above.

2.29 Co-culture of PBMC with transitional cells

To investigate whether transitional cells could suppress virus-specific CD8 T cell responses in vitro, transitional cells were either depleted or added back with PBMC at a 1:4 ratio, and the effect on the magnitude of virus-specific CD8 T cell responses was determined. In addition, to test whether suppression of virus-specific CD8 T cell responses by transitional B cells could be mediated by immunosuppressive IL-10, in some conditions anti-IL-10 and anti-IL-10R were added in addition to transitional cells to block the effect of IL-10 (Figure 2c.)

PBMC were stimulated with a pool of 7 HLA-A2 restricted HBV peptides, or a control CMV peptide.

The magnitude of virus-specific CD8 T cell responses was then compared between these various conditions. For each condition, 0.25×10^6 cells were plated with 1uM control peptide/ pool of 7 HBV peptides for 10 days with the addition of IL-2 on day 0 and day 4 to aid survival of cells. Cells were restimulated on day 10 with 1μM peptide for 5 hours in the presence of Brefeldin A, and virus-specific CD8 T cell populations were detected by staining with anti-CD8-PerCP and intracellular anti-IFN-γ-APC.

2.30 Statistical analysis

Statistical significance was calculated in all cases by the non-parametric Mann-Whitney U test. A p value of <0.05 was deemed significant.

3 Functional skewing of the global CD8 T cell population in chronic HBV infection.

Background

Chronic Hepatitis B virus (CHB) infection remains a major global health threat today affecting over 400 million individuals worldwide (Ocama et al. 2005). Immune clearance is mediated primarily through a strong virus-specific CD8 T cell response, however through production of pro-inflammatory cytokines TNF/IFN-γ and lysis of infected hepatocytes, these cells also contribute to liver damage. CD8 T cells therefore play a dual role within the HBV infected liver (Bertoletti & Maini 2000).

In patients with uncontrolled CHB infection, but not in those who resolve, a large non-antigen specific lymphocytic infiltrate is recruited into the liver (Maini et al. 2000), which comprises a high proportion of CD8 T cells (Doherty & O'Farrelly 2000). What role these cells serve in the HBV inflamed liver is as yet unknown. Within the virus-specific CD8 T cell population, marked qualitative (Reignat et al. 2002) and quantitative defects (Boni et al. 2007;Maini et al. 2000;Webster et al. 2004) have been described, however there is little known about the characteristics of the global CD8 T cell population and their role in viral control and or liver immunopathogenesis. Previous reports have shown that T cells isolated from patients with CHB displayed an impaired response to mitogens and recall antigens, and that responses could be recovered on reduction of viral load with lamivudine (Boni et al. 1998;Livingston et al. 1999). This non-antigen specific global T cell defect is thought to be particularly evident within the immunotolerant liver environment, where CD8 T cells have been shown to proliferate very poorly and are prone to activation induced cell death (Crispe 2003). Here we define the functional profile of non-antigen specific CD8 T cells in patients with CHB, and in further chapters, investigate mechanisms for the defects seen.

Selective defects in virus-specific CD8 T cell function have been described in a number of chronic viral infections and observed to be progressively lost in a predictable hierarchy according to the duration and strength of antigenic stimulation and availability of CD4 help (Kaech et al. 2002;Wherry et al. 2003a). Loss of CD8 T cell-derived IL-2 is an early defect that has recently been found to result in poor viral control and disease outcome in HIV infection (Trimble et al. 2000;Zimmerli et al. 2005). Functional defects affecting non-antigen-specific CD8 T cells would need to invoke factors other than excessive antigenic drive through MHC/peptide interactions. Bystander activation and effects of the large

quantities of circulating HBV antigens are possible candidates. In addition, the liver microenvironment, where the non-antigen-specific CD8 T cells accumulate in HBV infection, has long been recognised to be immunotolerant (Calne et al. 1969). In active HBV infection, intrahepatic T cells would be exposed to a high production of HBV antigens and recurrent hepatic inflammation. Putative factors affecting non-antigen-specific T cells include high levels of pro-inflammatory cytokines, hepatocyte expression of tolerising ligands (Crispe 2003), depletion of essential nutrients (Bronte & Zanovello 2005) or accumulation of toxic metabolites (Fallarino et al. 2006;Frumento et al. 2002).

Through studying the characteristics of non-antigen specific CD8 T cell dysfunction, we may gain a better insight into mechanisms of dysfunction within virus specific CD8 T cells, study of which is hampered by extremely low circulating frequencies in patients and difficulty in isolating cells from liver samples. In addition, it may also provide a valuable insight into the role of non-antigen specific CD8 T cells within the HBV inflamed liver.

In patients with CHB, we found a skewing of effector function in all CD8 T cells, regardless of their specificity. Compared to healthy donors, CHB patients had circulating and intrahepatic CD8 T cells with poor IL-2 production and proliferative potential, however maintained their capacity to produce IFNγ and TNF. Our findings point to a dual pathogenic role for non-antigen specific CD8 T cells within the liver, contributing to both liver inflammation and poor viral control.

Results

3.1 Global CD8 T cells in CHB are impaired in their ability to produce IL-2 upon T cell receptor stimulation

In patients with high viral load and liver inflammation, there is a large influx of non-virus specific CD8 T cells into the liver, not seen in the absence of liver inflammation (Maini et al. 2000). Although these cells account for a significant proportion of the infiltrating lymphocytes, little is known about what role these cells might play in viral control and/or disease immunopathology in the setting of chronic HBV infection. In order to better understand their role, we first set out to characterise the functional properties of the global CD8 T cell subset in healthy donors and patients with chronic HBV infection.

PBMC were isolated from peripheral blood from patients or controls, and then stimulated with either TCR dependent mitogens (anti-CD3/anti-CD28) or TCR bypassing mitogens (PMA/ionomycin). Ability to produce a panel of cytokines was then assessed by intracellular cytokine staining. One of the cytokines measured was IL-2, which is required for proliferation of virus-specific CD8 T cells upon antigen encounter. Loss of CD8-derived IL-2 is an early defect that has recently been found to result in poor viral control and disease outcome in HIV infection (Trimble et al. 2000;Zimmerli et al. 2005).

Representative data for CD8 T cell IL-2 production is shown in figure 3.1(a) for a healthy donor, patient with low viral load ($<10^6$ IU/ml HBV DNA) and a patient with high viral load ($>10^6$ IU/ml HBV DNA). Cross-sectional data is summarised in figure 3.1(b), where patients have been subdivided according to their viral load, ALT (surrogate marker of liver inflammation) or HBeAg status (marker of active viral replication). Upon a TCR dependent stimulus (anti-CD3 and anti-CD28), there was a significant impairment in CD8 T cell IL-2 production in CHB, with a mean 3 fold decrease in patients with high viral load compared to the controls (p<0.001, Mann Whitney test). There was a trend to progressive impairment with high viral load ($>10^6$ IU/ml) and raised alanine transaminase (ALT >50 IU/L) (3.1(b)). In contrast, upon a TCR independent stimulus with ionomycin and PMA, no significant difference in IL-2 production was detected between groups, raising the possibility that this defect may be selectively associated with dysfunction in the T cell receptor or its signalling pathway (fig 3.1(c)).

	Overall CHB	Low viral load	High Viral Load	Healthy Donor
N	58	35	23	28
Median age (range)	35 (23-69)	35 (23-69)	36 (23-63)	28 (22-44)
Sex (M:F ratio)%M	(41:17) 70%	(25:10) 71%	(16:7) 70%	(14:14) 50%
Median serum HBVDNA (IU/ml) (range)	250000 (BLQ*-340x10^6)	1400 (BLQ-945700)	78x10^6 (1.1x10^6-340x10^6)	-
Median ALT (IU/l) (range)	50 (21-498)	36 (21-498)	73 (24-363)	-
HBeAg status (% positive)	11%	10%	87%	-

Table 3.1 Clinical Characteristics of patients and controls used for Chapters 3 and 4. BLQ, DNA positive but below limit of quantification, i.e. <50 IU/ml.
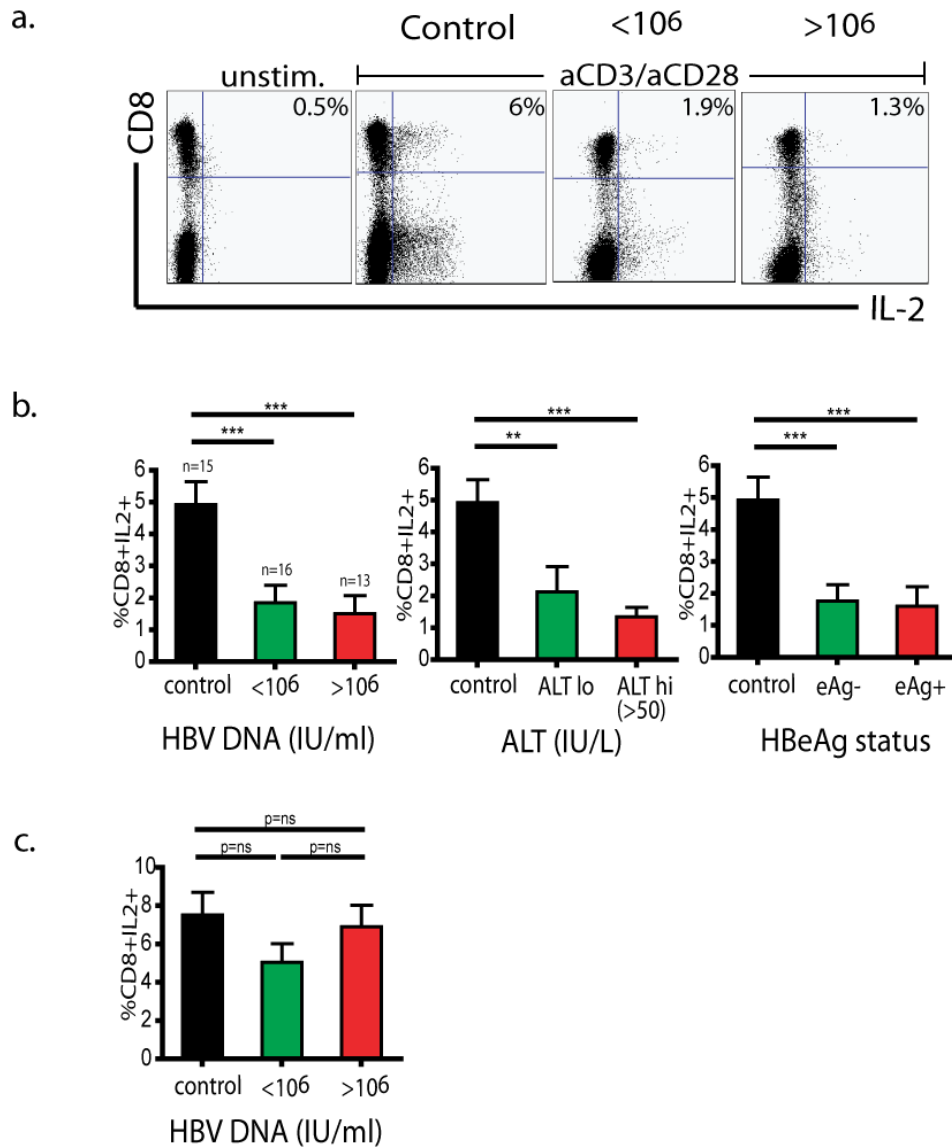
Figure 3.1: Global CD8 T cell dysfunction in IL-2 production upon T cell receptor stimulation. PBMC were stimulated with mitogens OKT3 (anti-CD3, 1ug/ml) and anti-CD28 (5ug/ml) and IL-2 production was determined by intracellular cytokine staining. a) Representative dot plots show IL-2 staining by CD8 T cells in a representative healthy control, patient with low viral load ($<10^6$ IU/ml) and a patient with high viral load ($>10^6$ IU/ml). Cross-sectional data is shown in (b), where subjects have been categorised depending on their HBV DNA levels, ALT (marker of liver inflammation) or HBeAg status (marker for active viral replication). %CD8+IL-2+ on the Y axis= 'percent CD8+IL-2+/total CD8+. c) Levels of CD8 T cell IL-2 in the different groups following TCR independent stimulation with PMA and ionomycin. (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, non-parametric Mann-Whitney U test).

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3.2 Impaired CD8 T cell IL-2 production is associated with poor proliferative capacity

The impairment of CD8 T cell IL-2 production would be expected to limit T cell clonal expansion potential, particularly within the liver, where CD4 help is restricted (Norris et al. 1998). To test this, we measured the proliferative capacity of CD8 T cells upon mitogenic stimulation by CFSE dilution. PBMC stained with CFSE dye, were stimulated with a TCR dependent stimulus (anti-CD3/anti-CD28) for 6 days, after which they were harvested and the proportion of CFSE low cells (those that have divided) were detected by flow cytometry. Consistent with their impaired IL-2 production and/or a direct block of cell cycle progression, fewer CD8 T cells from patients with CHB divided, and those that did underwent fewer divisions, than those from controls (representative examples fig. 3.2(a)). The proportion of CD8 T cells able to undergo at least one division was significantly reduced in CHB patients with high viral load and expression of HBeAg, and showed a negative correlation with liver inflammation within the patient group (r^2=0.59, p=0.043), (data not shown). Impaired CD8 T cell division was seen upon stimulation of whole PBMC, indicating that the available CD4 T cell help could not compensate for the CD8 T cell defect in these patients. Compatible with this, reduced IL-2 production by CD4 T cells was also noted in patients with HBV infection compared to healthy controls (data not shown). Interestingly, only patients with high viral load showed a significant reduction in proliferating CD8 T cells compared to controls, despite both patients with low and high viral load having significant impairment in CD8+ IL-2 compared to controls (fig 3.2.b). In these patients, it is likely that additional factors such as differentiation status (see chapter 5), propensity to exhaustion (Wherry et al. 2003a), replicative senescence, and growth cycle arrest through L-arginine depletion (Rodriguez et al. 2007), in addition to IL-2 loss, could tip the scales towards proliferative dysfunction.
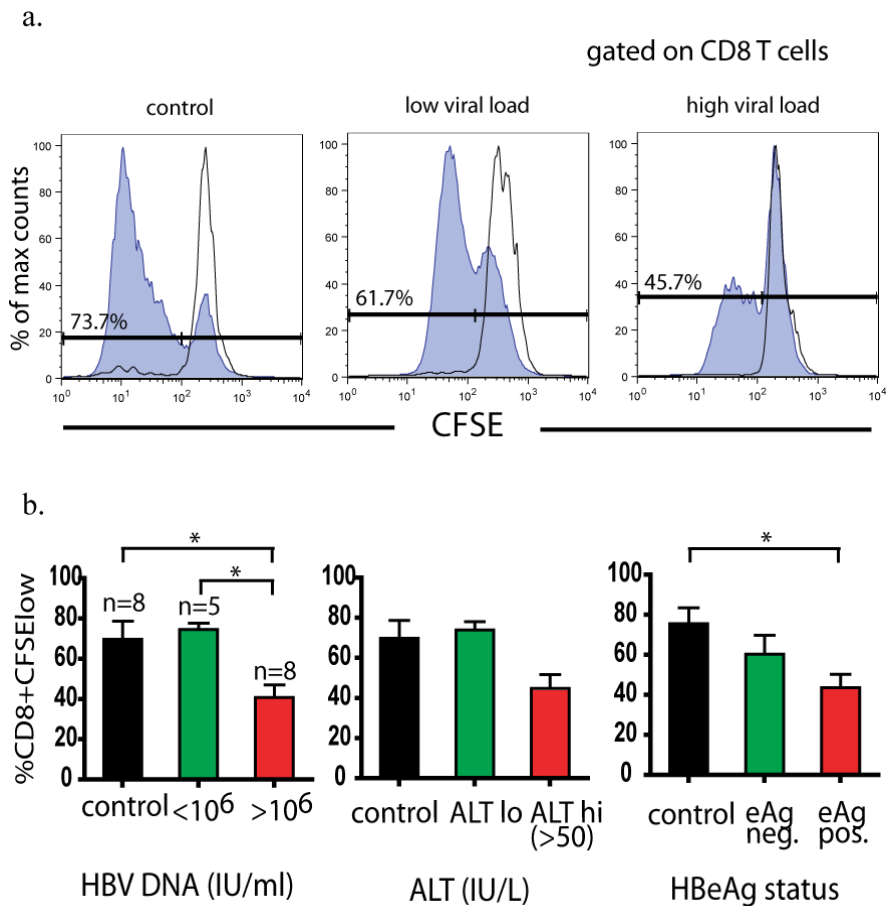
Figure 3.2: Impaired CD8 T cell IL-2 production is associated with impaired proliferation.
a) Representative histograms to show proliferation of CD8 T cells (as measured by
proportion of CFSE low cells) upon a TCR dependent stimulus in a control, and in patients
with low and high viral load. (blue histogram = anti-CD3/anti-CD28 stimulus 6 days; solid
black line = unstimulated). b) Cross-sectional summary of data, stratified by disease
variables HBV DNA, ALT and HBeAg status. (*=p<0.05, non-parametric Mann-Whitney
U test).

3.3 CD8 T cells from patients with high viral load maintain their capacity to produce proinflammatory cytokines and lyse cells

By contrast to IL-2 production, CD8 T cell production of IFN-γ was maintained in patients with high HBV load, liver inflammation and expression of HBeAg compared to healthy carriers or healthy donors (Figure 3.3 top panel). Similarly, TNF-α production and cytolytic potential (measured by surface expression of CD107) were maintained in CHB, and tended to be higher in patients with active disease (Figure 3.3 middle and bottom panels). Using PMA and ionomycin as a TCR bypassing signal, no differences in CD8 T cell effector function could be detected between CHB patients and controls (data not shown). This pointed to the skewed CD8 T cell effector function resulting from aberrations within the T cell receptor associated signalling machinery. This was supported by the fact that cytokine skewing was maintained upon substituting anti-CD3/CD28 stimulation with plate-bound anti-CD3 alone or anti-CD3 in combination with irradiated APC.

CD8 T cells with this altered IFN-γ:IL-2 ratio have recently been noted in other chronic viral infections such as HIV and LCMV and found to correlate with poor viral control and disease progression (Wherry et al. 2003a;Zimmerli et al. 2005).
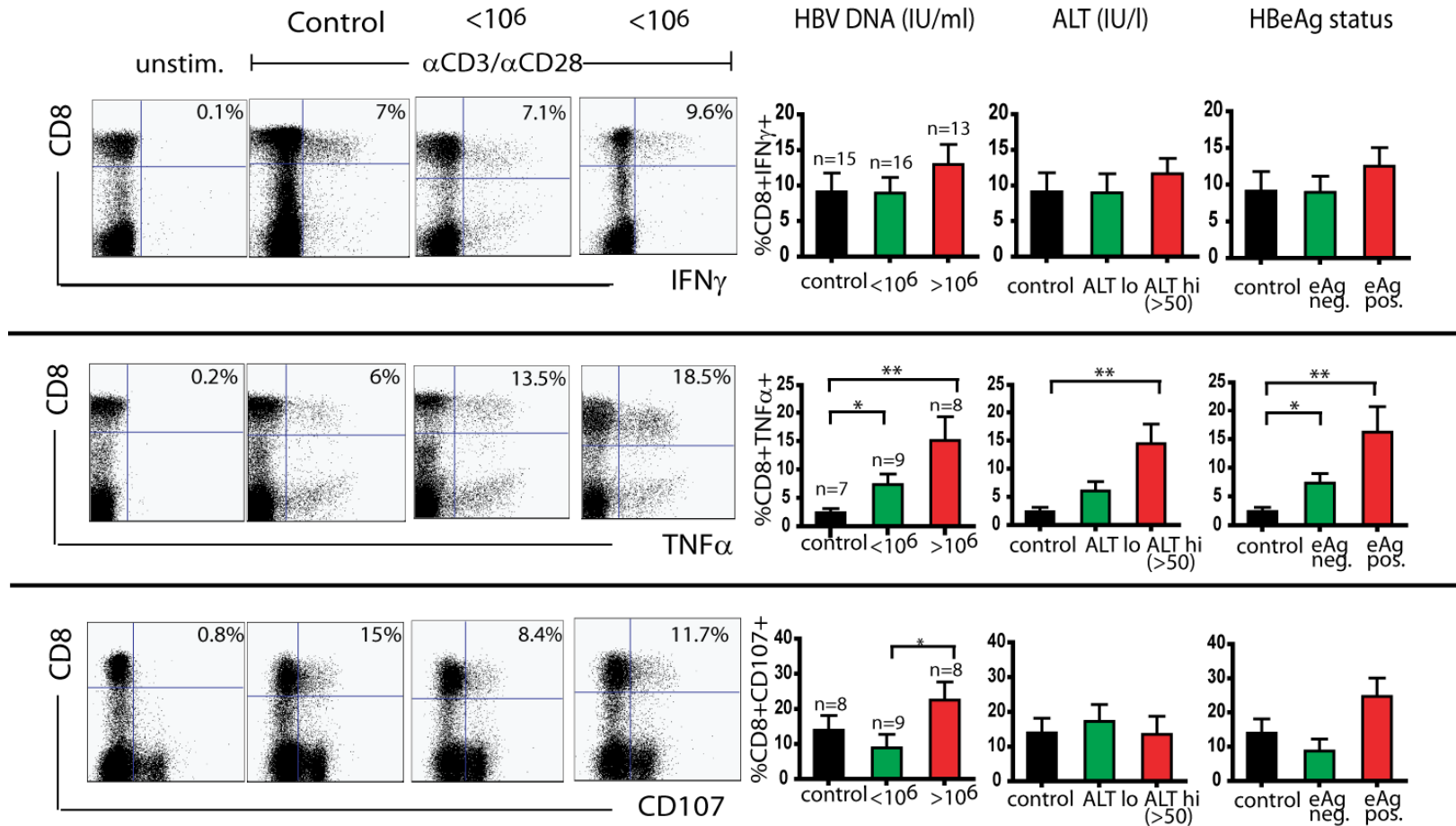
Figure 3.3: CD8 T cells from patients with high viral load maintain capacity to produce proinflammatory cytokines and lyse cells. Left panel: Dot plots to show production of IFN-γ, TNF-α and CD107 (marker of cytolysis) in CD8+ T cells from different patient groups upon anti-CD3/anti-CD28 stimulation. Summary data for each cytokine is shown on the right hand panel. %CD8+IFNγ+ on the Y axis= 'percent CD8+IFNγ+/total CD8+. (*=p<0.05, **=p<0.01, non-parametric Mann-Whitney U test).

3.4 Skewed IFN-γ:IL-2 ratio in CHB is regardless of virus specificity

HBV-specific CD8 T cells are markedly depleted in patients with CHB (Maini et al. 2000;Webster et al. 2004), such that circulating responses are barely detectable, even when overlapping peptides covering the whole genome are applied (Boni et al. 2007). Based on this finding, it is logical to assume that the majority of the CD8 T cells we are studying in the periphery of these patients are non-HBV specific. In order to confirm that the qualitative dysfunction we observed was not restricted to the HBV-specific CD8 T cells but also extended to other virus specificities, PBMC from HLA-A2 positive donors were stimulated directly ex vivo with peptides representing immunodominant HLA-A2-restricted viral epitopes from CMV, EBV and influenza A in addition to HBV (figure 3.4(a)). Virus-specific CD8 T cells from healthy donors produced predominantly IFN-γ but had a clear population of dual IFN-γ:IL-2-responding cells, whereas those from CHB patients only produced IFN-γ (Figure 3.4(a). The percent of IFN-γ positive to dual IFN-γ:IL-2 positive peptide-specific CD8 T cells was plotted as a ratio, following subtraction of background readings from controls without peptide. As shown in figure 3.4(b), regardless of the virus specificity, there was a significantly higher IFN-γ:IL-2 ratio in patients with active HBV infection compared to healthy donors (p<0.01). Since CMV infection has also been reported to skew the repertoire of T cell responses (Almanzar et al. 2005) and was therefore a potential confounding factor, we also compared exclusively CMV-specific responses in a subset of CHB patients and controls who were known to be CMV seropositive. The proportion of CD8 T cells able to produce IL-2 in response to the immunodominant HLA-A2 restricted NLV epitope from pp65 showed a stepwise reduction from healthy donors to low and high level CHB patients (Figure 3.4(c)). These data further confirm that functional impairment is present in the generalised T cell population in patients with CHB, regardless of antigen specificity.
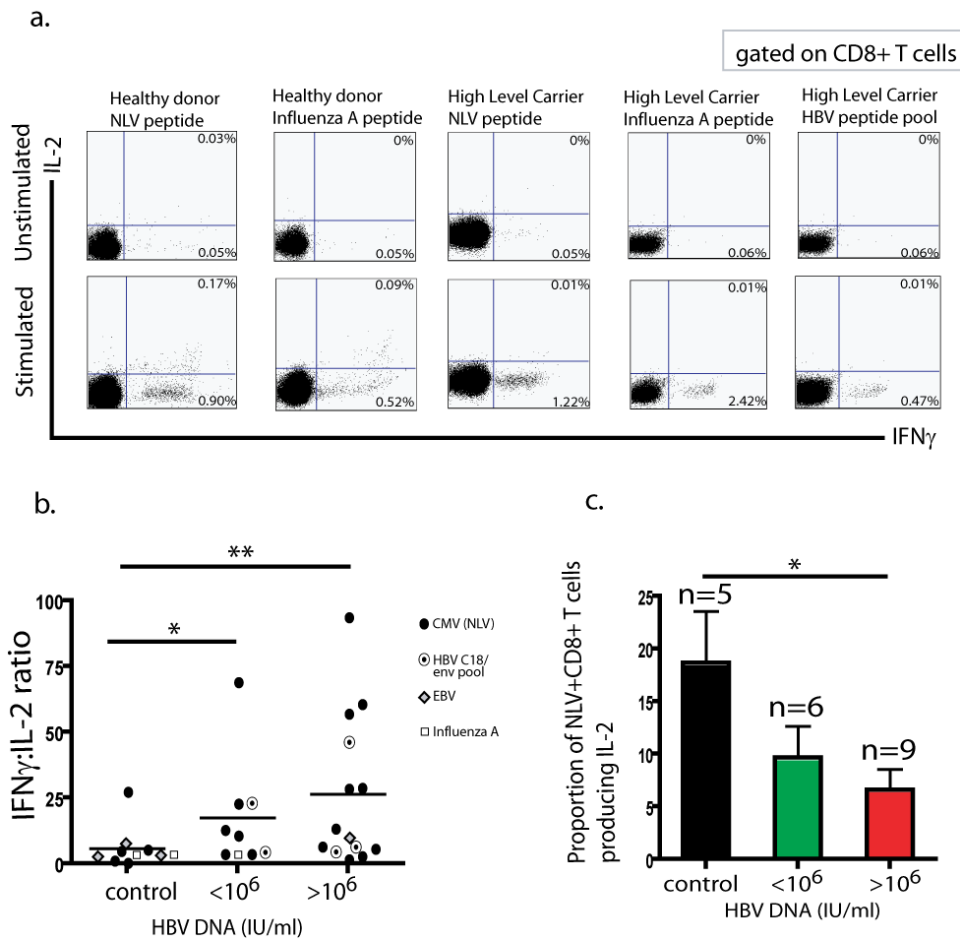
Figure 3.4: Skewed IFN-γ:IL-2 ratio in CHB is regardless of virus specificity. Determination of IL-2 and IFNγ production by CD8 T cells following stimulation with HLA-A2 restricted viral peptides. a) Representative flow cytometric dot plots to show distribution of IFN-γ and IL-2 positive responder cells following ex vivo stimulation with peptides (for INF-A, CMV, HBV envelope pool) in a healthy donor and in a high level carrier. All ex vivo responses were in the range of 0.1-4% following subtraction of background readings. A response was determined as any reading>mean+ 2SD of background. b) Cumulative data to show mean IFN-γ:IL-2 ratio following stimulation with peptides for controls, and patients with low (<10^6IU/ml) and high (>10^6IU/ml) viral load. c) Histogram to show proportion double positive (IFNγ+IL2+/IFNγ+) CD8 Tcells in controls and patients following stimulation with the NLV peptide of CMV ex vivo. Mean and standard error are shown. (*=p<0.05, **=p<0.01, non-parametric Mann-Whitney U test).

3.5 Intrahepatic CD8 T cells in CHB have impaired IL-2 production compared to those from patients with non-viral liver disease

CD8 T cells are enriched in the liver compared to the periphery (Norris et al. 1998), with CD8 T cells constituting 60-90% of intrahepatic T cells. In order to better understand what role this population may play in HBV-associated inflammation and/or viral control, intrahepatic CD8 T cells were isolated and studied. Liver tissue was obtained from routine diagnostic biopsies or resected tissue from six patients with HBV-related liver disease and six controls without HBV infection. Control liver samples included liver tissue with normal architecture and uninflammed histology taken distal to a metastasis, and tissue from patients with other inflammatory liver diseases (see Table 3.2). Following isolation, intrahepatic PBMC were stimulated directly ex vivo with T cell receptor dependent mitogens, and IFN-γ and IL-2 production was determined by intracellular staining (representative examples, Figure 3.5(a)). In non-HBV infected patients, three populations of responder cells could be seen; IFN-γ single positive, IFN-γ:IL-2 double positive and IL-2 single positive. In each of the 6 CHB infected donors however, intrahepatic CD8 T cells almost exclusively produced IFN-γ, resulting in a significantly raised mean IFN-γ:IL-2 ratio compared to the non-HBV infected group (p<0.01) (Figure 3.5(b)). Indeed this is further shown in figure 3.5(c), where it is apparent that there is a selective loss of CD8+ IL-2 producing cells within lymphocytes isolated from HBV livers, however these cells maintain their ability to produce IFN-γ. These data revealed a dysfunction in global intrahepatic CD8 T cell IL-2 production in CHB.
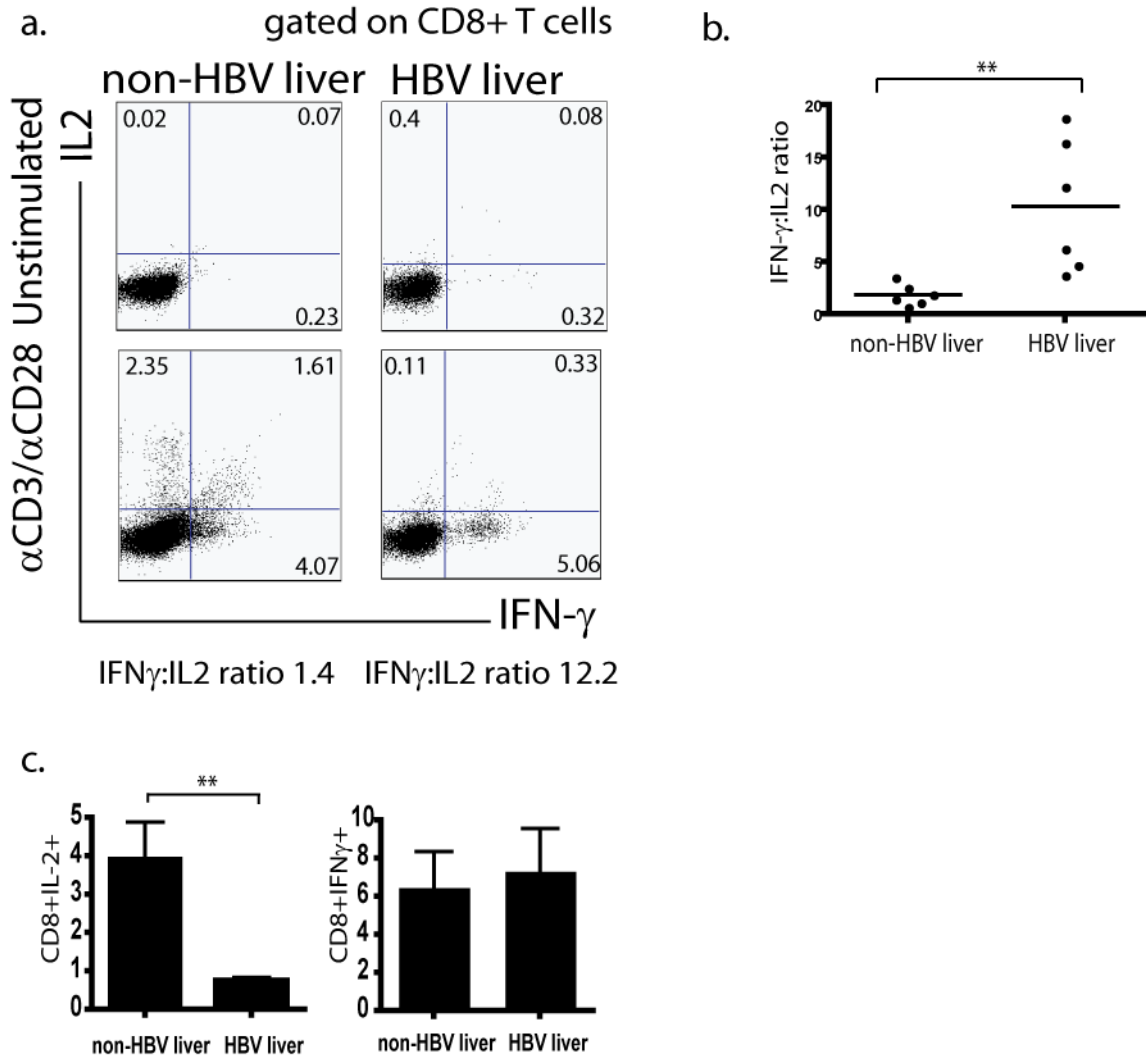
Figure 3.5: Intrahepatic CD8 T cells in CHB have impaired IL-2 production compared to those from patients with non-viral liver disease. a) Intrahepatic CD8 T cells were isolated from liver biopsies and cytokine production was determined by intracellular staining following αCD3/αCD28 stimulation. Representative dot plots show IL-2 and IFN-γ production by CD8 T cells for a typical patient with chronic HBV and a patient with non-viral liver pathology. b) Following mitogenic stimulation, the ratio of IFN-γ:IL-2 production by intrahepatic CD8 T cells was determined for 6 patients with chronic HBV, and 6 patients without chronic HBV infection. Ratios are plotted in the figure (b) and mean IL-2 and IFN-γ production for the two groups are shown separately in (c). CD8+IL-2+ on the Y axis= 'percent CD8+IL-2+/total CD8+. (**=p<0.01, non-parametric Mann-Whitney U test).

Subject	Pathology	ALT (IU/L)	HBeAg	Sex	Age
H1	CHB mild fibrosis	45	NEG	Male	31
H2	CHB mild fibrosis	100	POS	Male	23
H3	CHB moderate fibrosis	246	POS	Male	42
H4	CHB cirrhosis	498	NEG	Male	34
H5	CHB no fibrosis	48	NEG	Male	43
H6	CHB mild fibrosis	63	NEG	Male	43
C1	Non Primary Liver Carcinoma[*]	140	N/A	Female	34
C2	Non Primary Liver Carcinoma[*]	26	N/A	Female	56
C3	Cholangiocarcinoma	41	N/A	Female	34
C4	Non Primary Liver Carcinoma[*]	34	N/A	Male	60
C5	Non Primary Liver Carcinoma[*]	17	N/A	Female	67
C6	Non Primary Liver Carcinoma[*]	209	N/A	Male	66

Table 3.2 Clinical characteristics of patients and controls for liver samples. * resection specimens adjacent to non-primary liver carcinoma; all tissue was of normal architecture.

Discussion

Chronic hepatitis B virus infection is characterised by an intrahepatic influx of non-antigen specific CD8 T cells that fail to suppress viral replication and may instead amplify liver damage initiated by HBV-specific CD8 T cells (Ando et al. 1993;Bertoletti & Maini 2000;Maini et al. 2000). To better understand their role in the liver, we characterised the functional properties of global CD8 T cells isolated from both the periphery and liver of patients with CHB infection. We found that the global CD8 T cell population (including CD8 T cells specific for HBV and for other chronic viruses) were skewed towards IFNγ/TNF production and were impaired in their ability to produce IL-2 and proliferate. This functional bias could contribute to both the failure of viral control and the pathogenesis of resultant liver inflammation, suggesting a dual role for non-antigen specific CD8 T cells.

In patients with high viral load who fail to control HBV infection, the circulating frequencies of virus-specific CD8 T cells ex vivo are barely detectable, thus restricting the study of these cells. By scrutinising the global CD8 T cell population instead, we have identified a functional bias within these cells which may contribute to the failure of virus-specific CD8 T cells to survive and proliferate sufficiently to control HBV replication.

Previous reports have demonstrated that HBV-specific CD8 T cells have impaired capacity for clonal expansion (Boni et al. 2007;Maini et al. 2000;Webster et al. 2004) and a greater susceptibility to Bim-mediated apoptosis (Lopes et al. 2008). In this study we showed that global CD8 T cells were impaired in their capacity to produce IL-2. Autologous IL-2 production has been reported to drive replication of CD8 T cells in settings of impaired CD4 help, such as in the liver environment where CD4 are depleted relative to the peripheral blood (Lopes et al. 2008;Norris et al. 1998), and during HIV infection where there is a CD4 lymphopenia (Lopes et al. 2008;Zimmerli et al. 2005). Thus, an intrahepatic T cell deficiency in IL-2 could both hamper HBV-specific CD8 T cell proliferation, and simultaneously confer a greater propensity for these cells to undergo apoptosis through the IL-2 dependent Bcl-2 pathway (Lopes et al. 2008). A generalised defect in the ability to mount efficient T cell responses in patients with CHB is supported by the long-standing clinical observation that these patients are significantly less likely to reject a liver transplant than patients with other chronic liver diseases (Adams et al. 1991;Farges et al. 1996). Although this global dysfunction in IL-2 might also be expected to compromise the

ability of CD8 T cells to control concurrent intrahepatic infections with other persistent non-cytopathic viruses, we suggest that the HBV-specific response may be most affected by this global CD8 T cell impairment, due to their additional attrition by mechanisms including PD-1 induced anergy (Boni et al. 2007) and Bim mediated apoptosis (Lopes et al. 2008).

We observed that global CD8 T cells capable of IFNγ and TNF release were maintained in CHB patients with high viral load and liver inflammation. These cytokines may have a dual role in the HBV inflamed liver. In the transgenic mouse model, IFNγ and TNF have been shown to mediate non-cytolytic clearance of HBV, and are therefore required for viral control (Guidotti et al. 1996). On the other hand, both these cytokines are pro-inflammatory and may additionally cause immunopathology (Bertoletti & Maini 2000). Constitutive expression of IFNγ in the liver of transgenic mice induces chronic hepatitis (Toyonaga et al. 1994). Liver damage in this setting is partially attributable to IFN-γ mediated induction of chemokine release (CXCL9 and CXCL10) from hepatocytes, which attract a non-antigen specific cellular infiltrate into the liver (Kakimi et al. 2001b), including NK cells which have been shown recently to induce TRAIL-mediated apoptosis of hepatocytes during CHB (Dunn et al. 2007). Blockade of chemokine signalling reduces the severity of liver disease. In addition, IFNγ may also increase the susceptibility of the liver to TNF mediated damage (Morita et al. 1995). TNF has been demonstrated to mediate potent hepatocyte destruction, particularly when expressed in a cell-bound form (Maeda et al. 2003) and its effects may be heightened through the capacity of the HBx protein of HBV to sensitize hepatocytes to TNF-mediated apoptosis (Su & Schneider 1997). Thus bystander CD8 T cell production of pro-inflammatory cytokines may further drive the non-antigen specific lymphocytic infiltrate and propagate liver inflammation.

Functional biasing of CD8 T cells towards IFNγ and TNF production, with a failure to produce IL-2, has previously been reported in terminally differentiated CD8 T cells isolated from healthy donors, and in 'exhausted' virus specific CD8 T cells from patients with chronic viral infections. Hamann et al. characterised the functional and phenotypic profile of global CD8 T cells, and showed that terminally differentiated revertant cells, phenotypically defined as CD27-CD45RA+CD8+, were IL-2 negative but still capable of producing IFNγ and TNFα on PMA/ionomycin stimulation (Hamann et al. 1997). An accumulation of terminally differentiated virus specific CD8 T cells have been reported

during human chronic viral infections, and associated with failure of virus control. Wherry et al showed in a mouse model of LCMV, that CD8 T cells could be driven by persistent high dose antigen towards functional exhaustion. This loss of function was progressive; capacity to produce IL-2 and to proliferate was lost first, but ability to produce TNF and IFNγ were not compromised until later stages of exhaustion (Wherry et al. 2003a). CD8 T cells from patients with HBV in our study had the functional profile of CD8 T cells in the 'partial exhaustion 1' phase, characterised by loss of IL-2 production and proliferative capacity, but maintenance of IFNγ and TNF production (Shin & Wherry 2007). A similar functional profile has been attributed to virus specific CD8 T cells in other chronic viral infections including, Mouse Hepatitis Virus (MHV) (Bergmann et al. 1999), HIV (Trimble & Lieberman 1998) and HCV (Radziewicz et al. 2007;Urbani et al. 2006). Thus, accumulation of functionally exhausted virus-specific CD8 T cells secondary to persistent HBV load could potentially explain the skewed functional profile of CD8 T cells in patients with CHB. However, since HBV-specific CD8 T cell populations are scanty in the periphery, a global biasing of CD8 T cell phenotype and function would require the additional influence of bystander factors. Possible contributors include cytokines IFNα and TNF, which are known to induce CD28 loss on global CD8 T cells (Chiu et al. 2006;Fletcher et al. 2005;Lewis et al. 2004) and are elevated in patients with CHB (Dunn et al. 2007;Sheron et al. 1991), high quantities of circulating HBeAg which is thought to have immunoregulatory properties (Milich et al. 1990), and ligation by tolerizing ligands expressed on hepatocytes (Crispe 2003).

From our data, we have found that upon stimulation with PMA and ionomycin, there was no difference in the proportion of IL-2/IFNγ positive CD8 T cells in healthy donors and patients with low and high viral load. Therefore, although an enrichment of highly differentiated CD8 T cells (which have been shown to be IL-2 low on PMA/ionomycin stimulation (Hamann et al. 1997)) could partially explain the functional bias, clearly other factors are at work. Notably, we have observed that in patients with high viral load, in whom there was the lowest IL-2 production by CD8 T cells, this defect was mediated through T cell receptor dependent stimulation. Therefore, in chapter 4, we have investigated the role of T cell signalling machinery dysfunction in CD8 T cells, as another potential mechanism for the defects in effector function and proliferation described.

Through study of the non-antigen specific CD8 T cells in patients with CHB, we were able to identify a functional biasing in patients with high viral load. This provides an insight into possible mechanisms of dysfunction within virus-specific CD8 T cells, direct study of which is hampered by extremely low circulating ex vivo frequencies. We have demonstrated that CD8 T cells from patients with CHB have impaired IL-2 and proliferative capacity, regardless of their specificity. Such functional skewing could impair their survival and anti-viral potential, particularly for persistent non-cytopathic viruses in the CD4 depleted liver environment. Their capacity for IFNγ/TNFα production could contribute to the pro-inflammatory milieu, thereby exacerbating mechanisms underlying their dysfunction. Our data suggest that chronic exposure to the microenvironment of an inflamed liver could impair T cell effector functions critical to the control of this virus. A major limitation to the success of therapeutic vaccination in the setting of persistent virus infection such as HBV is thought to be the impaired T cell proliferative capacity. Approaches to remedy these T cell defects in patients with CHB could therefore form an important adjunct to future immunotherapeutic strategies.

4 Global CD8 T cell downregulation of proximal TCR-associated signalling molecules, CD28 and CD3ζ, associated with depletion of amino acid L-arginine in CHB.

Background

A global CD8 T cell defect in IL–2 production and proliferation upon a T cell receptor (TCR) dependent, but not TCR-bypassing stimulus, pointed towards dysfunction within the T cell receptor signalling machinery. In this chapter, we examine aberrant expression of proximal TCR associated signalling molecules CD3ζ and CD28, as putative mechanisms for impaired CD8 T cell effector function.

In other diseases of chronic inflammation and high load antigenic persistence analogous to the situation in CHB, selective defects in global CD8 T cell function have been associated with downregulation of CD3ζ, in some cases with a superimposed CD28 downregulation. These include autoimmune disorders (Krishnan et al. 2005;Tsuzaka et al. 2003), malignancy (Nakagomi et al. 1993) and chronic viral (Trimble et al. 2000;Trimble & Lieberman 1998), and bacterial (Bronstein-Sitton et al. 2003) infections. The CD3ζ chain exists as a disulphide linked homodimer and is a proximal T cell receptor associated signalling molecule (Baniyash 2004). Upon T cell receptor ligation, SRC family kinases phosphorylate ITAM residues on the intracytoplasmic domain of the CD3ζ and these then serve as docking sites for adaptor proteins such as ZAP-70 which potentiate further downstream signalling events. CD3ζ is structurally and evolutionarily distinct from other CD3 components as it has 3 ITAM residues on its intracytoplasmic tail (as opposed to 1 on other components) therefore it can most efficiently amplify and propagate a signal into the cell rendering it a rate limiting step in T cell activation (Pitcher et al. 2003;Sussman et al. 1988).
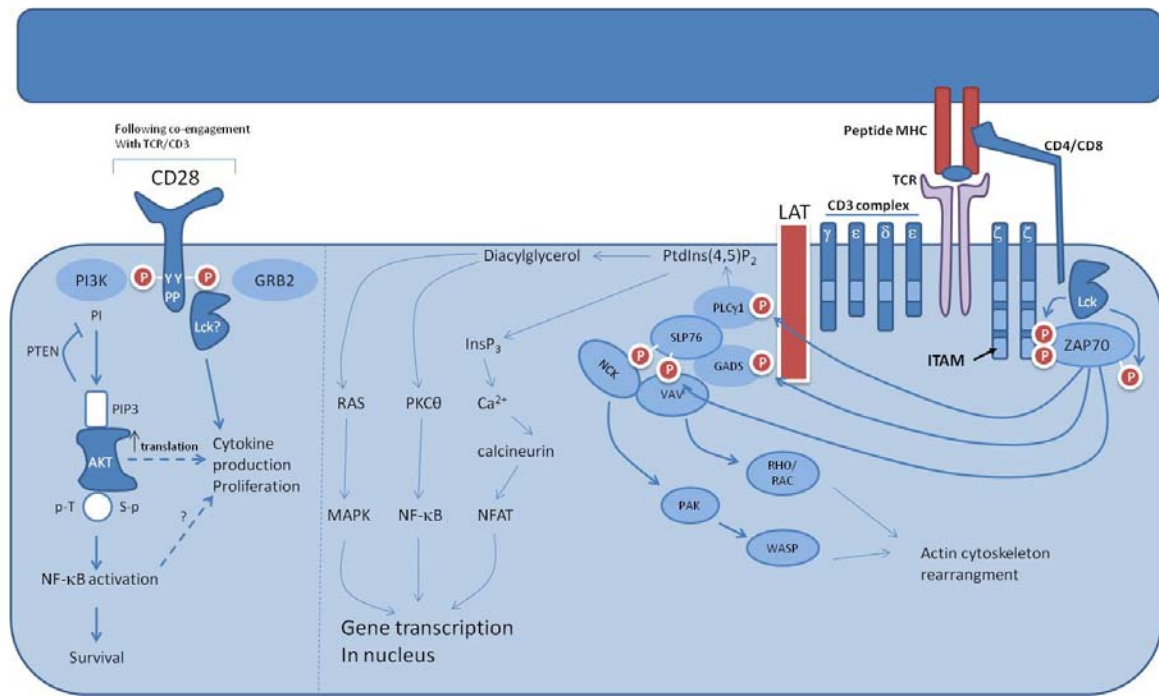
Figure 4a: Signalling through CD28 and the T cell receptor. *(Modified from Baniyash Nature Reviews Immunology 2004 and Alegre Nature Reviews Immunology 2001).* (Left) Ligation of the co-receptor CD28 induces phosphorylation of tyrosine on the intracytoplasmic chain, recruitment of PI3K and GRB2, and resultant cascades which lead to activation of factors including AKT. The biological result is release of IL-2 and upregulation of anti-apoptotic BCL-X$_L$. (Right) Ligation of the T cell receptor results in phosphorylation of ITAM residues on CD3ζ, recruitment of ZAP-70 and phosphorylation of additional adaptor proteins including LAT (linker for activation of T cells) and SLP-76 (SRC homology 2 (SH2)-domain-containing leukocyte protein of 76kDa). A cascade of further downstream signalling events results in gene transcription within the nucleus. Cross-talk between pathways may be mediated by CD28 activation of LCK (Holdorf et al. 1999) or recruitment of CD28 signalling elements to LAT and subsequent enhancement of TCR mediated signalling (Acuto & Michel 2003).

Along with CD3ζ, downregulation of CD28, a proximal co-stimulatory molecule required for efficient signalling, has also been described in viral infections (Maki et al. 2004;Trimble et al. 2000) and associated with T cell anergy.CD28 is a 44kDa glycoprotein which binds CD80 and CD86 on antigen presenting cells and provides a second costimulatory signal which reduces the threshold for TCR activation (Alegre et al. 2001). Similar to TCR triggering, ligation of CD28 results in phosphorylation of tyrosine residues on its intracytoplasmic tail by SRC family kinases LCK and FYN. However, these tyrosine residues are not incorporated within ITAM residues (as they are for CD3ζ) and there is no subsequent recruitment of ZAP-70 and activation of the LAT-SLP76 complex. Instead CD28 signalling leads to downstream activation of proteins including PI3K, ITK, VAV1, AKT and the TEC protein tyrosine kinases which are all involved in the TCR signalling pathway. Based on this observation and microarray studies which showed that CD28 co-ligation with TCR signalling up/downregulated thousands of genes, but CD28 agonists alone elicited few changes in gene expression (Diehn et al. 2002), Acuto et al. suggested that CD28 may have primarily a quantitative role in amplifying TCR signalling. The convergence of CD28 and TCR pathways in this case was hypothesized to be mediated through LAT, which could provide a scaffold onto which CD28 signalling elements could bind and enhance further TCR mediated downstream signalling cascades (Acuto & Michel 2003). The biological consequences of CD28 co-stimulation include potent stimulation of IL-2 production (Lucas et al. 1995), promotion of cell cycle progression (Bonnevier & Mueller 2002) and upregulation of anti-apoptotic BCL-$_{XL}$ (Boise et al. 1995), which are all beneficial for CD8 T cell expansion and survival. Loss of CD3ζ, with or without CD28 downregulation, could therefore impair IL-2 production and proliferation, and subsequently hamper viral control.

Both these proximal molecules are upstream elements which are required to initiate signalling, and thus play a rate-limiting role in controlling the efficiency of TCR signal transduction and consequent generation of effector function. In this chapter, we show that both CD3ζ and CD28 are downregulated in CD8 T cells from patients with CHB, and that upon reconstituting their expression by transfection of CD8 T cells with chimeric receptors, the defect in IL-2 production can be reversed. In addition, we provide evidence that CD3ζ downregulation is further exacerbated within intrahepatic CD8 T cells, and this may partially be due to local depletion of the non-essential amino-acid L-arginine, which is

required for the production of CD3ζ. L-arginine levels were found to be significantly lower in the serum of patients with liver inflammation, and we suggest could be even more depleted within the HBV inflamed liver, where we have shown that the enzyme arginase-1, which catabolises L-arginine, is more highly expressed than in livers with non-viral liver disease.

Exposure to inflammatory cytokines, viral particles and a nutrient depleted environment within the HBV inflamed liver could potentiate downregulation of CD3ζ and CD28. Aberrant T cell signalling could thus provide a partial mechanism for the non-antigen specific CD8 T cell defect in IL-2 production and proliferation. This in turn could hamper the capacity for HBV-specific CD8 T cells to expand and survive, and attenuate viral control.

Results

4.1 CD3ζ downregulation in CD8 T cells from patients with chronic HBV infection

Based on our observation that CD8 T cells from patients with chronic HBV display an IL-2 and proliferative defect selectively upon TCR-mediated stimuli but not TCR-bypassing stimuli, we hypothesized that this impaired effector function could be associated with dysfunction within the T cell signalling pathway. In other chronic infectious diseases such as HIV, a CD8 T cell defect in IL-2 has been partially attributed to selective downregulation of CD3ζ, a proximal TCR associated signalling molecule (Boise et al. 1995;Trimble et al. 2000). This molecule can be downregulated independently of other CD3 signalling molecules, which maintain their expression on the cell surface. In order to investigate whether CD3ζ downregulation could be occurring in CD8 T cells from patients with chronic HBV, we isolated PBMC from subjects (control n=16, patients with low level HBV (viral load<10^6) n=20; patients with high level HBV (viral load >10^6) n=12) and CD3ζ expression was determined by intracellular cytokine staining. CD3ζ expression was determined after gating on CD3ε+CD8+ T cells, to confirm that downregulation of CD3ζ was an isolated defect, and not secondary to downregulation of the entire CD3 complex (anti-CD3ε binds an extracellular domain on the CD3ε chain, therefore does not interfere with binding of CD3ζ to its intracellular determinant). CD3ζ expression after gating on CD3ε+CD8+ cells is shown for a representative patient with CHB and healthy donor in fig. 4.1a.

Subject	Pathology	ALT (IU/L)	HBeAg	Sex	Age
H1 [a]	CHB mild fibrosis	45	NEG	Male	31
H2 [a]	CHB mild fibrosis	100	POS	Male	23
H3 [a]	CHB moderate fibrosis	246	POS	Male	42
H4 [a,b]	CHB cirrhosis	498	NEG	Male	34
H5 [a]	CHB no fibrosis	48	NEG	Male	43
H6 [a]	CHB mild fibrosis	63	NEG	Male	43
H7 [b]	CHB[d]	40	-	Male	60
H8 [b]	CHB[d]	56	-	Male	66
H9 [b]	CHB[d]	110	-	Male	56
H10 [b]	CHB[d]	79	-	Male	44
H11 [b]	CHB[d]	45	-	Female	48
H12 [c]	CHB cirrhosis	31	-	Male	54
H13 [c]	CHB fibrosis	67	NEG	Male	49
H14 [c]	CHB cirrhosis	54	NEG	Male	53
H15 [c]	CHB cirrhosis	47	NEG	Male	50
H16 [c]	CHB + Hepatocellular Carcinoma	19	NEG	Male	61
C1 [a,b]	Non Primary Liver Carcinoma[e]	140	N/A	Female	34
C2 [a]	Non Primary Liver Carcinoma[e]	26	N/A	Female	56
C3 [a,b]	Cholangiocarcinoma	41	N/A	Female	34
C4 [a]	Non Primary Liver Carcinoma[e]	34	N/A	Male	60
C5 [a,b]	Non Primary Liver Carcinoma[e]	17	N/A	Female	67
C6 [a]	Non Primary Liver Carcinoma[e]	209	N/A	Male	66
C7 [b]	Hepatitis C virus infection	54	N/A	Female	41
C8 [b]	Non Primary Liver Carcinoma[e]	30	N/A	Female	60
C9 [b]	Non Primary Liver Carcinoma[e]	30	N/A	Male	54
C10 [b]	Nonalcoholic steatohepatitis	108	N/A	Female	32
C11 [c]	Healthy transplant donor	-	N/A	Male	56
C12 [c]	Alcoholic cirrhosis	19	N/A	Male	63
C13 [c]	Primary sclerosing cholangitis	138	N/A	Female	29
C14 [c]	Primary sclerosing cholangitis	69	N/A	Female	60
C15 [c]	Primary sclerosing cholangitis	-	-	Female	-

[a] Used for functional ICS (Chapter 3)

[b] Used for CD3ζ staining (Figure 4.2)

[c] Used for arginase activity (Figure 4.11)

[d] CHB, histology unavailable

[e] resection specimens adjacent to non-primary liver carcinoma; all tissue was of normal architecture

Table 4.1 Clinical characteristics of patients and controls for liver sample (extended table).
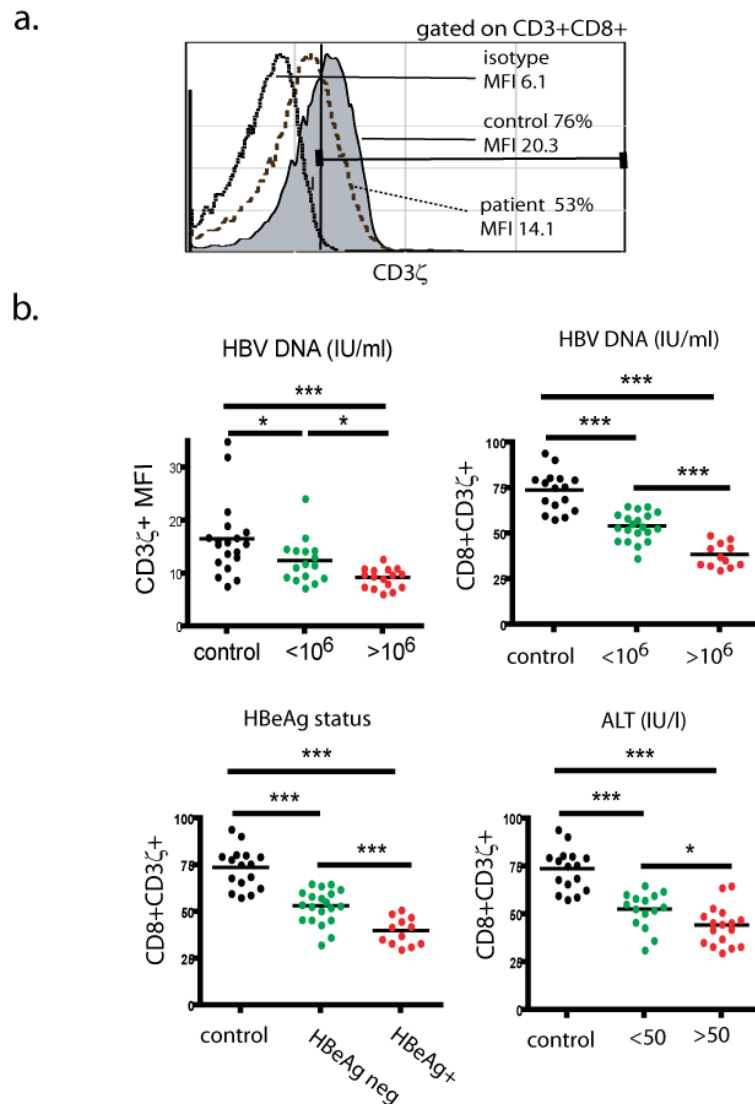
Figure 4.1: Global CD8 T cells from patients with chronic HBV have downregulated CD3ζ. PBMC were surface stained for CD3ε and CD8, fixed and permeabilised, then stained for intracellular CD3ζ or its isotype. A tight lymphocyte gate and secondary gate over CD3ε+CD8+ cells has been applied. A marker was applied at the edge of the isotype control (solid black line) and proportion CD8 T cells to the right of this line represented the CD3ζ+ fraction. a) Representative flow cytometric histogram illustrating CD3 expression (as a percentage and as mean fluorescence intensity:MFI) for a chronic HBV infected patients with high viral load ($>10^6$ IU/ml) (dashed line) and low viral load ($<10^6$ IU/ml) (grey). b) CD3ζ levels, shown as both MFI and percentage of CD3ζ+ in the total CD8 are shown against viral load (above), and percentage values are further broken down by HBeAg status and ALT (below). CD8+CD3ζ+ on the Y axis= 'percent CD8+CD3ζ+/total CD8+. (*=p<0.05, ***=p<0.001, non-parametric Mann-Whitney U test).

To determine whether CD3ζ downregulation correlated with disease status, patients were categorised by their viral load ($<10^6$ or $>10^6$ IU/ml), HBeAg status or by their alanine aminotransferase level (raised >50 IU/l). Patients were classed as ALT high if their ALT was raised at time of sampling, or had been raised in the previous 6 months. This allowed more accurate categorisation, as it is common for patients to have cyclical changes of ALT over time. As shown in figures 4.1b, we observed that CD3ζ expression in CD8 T cells correlated inversely with each of the three parameters. With increasing viral load, we observed a progressive decrease in CD8+CD3ζ+ expression. Patients with the highest viral load had the lowest CD3ζ expression in their CD8 T cells, with a mean reduction of 40% compared to controls. Expression of CD3ζ was also reduced in CD4 T cells from these patients, but to a lesser degree than the CD8 T cells (data not shown), as noted previously in HIV infection (Trimble & Lieberman 1998).

Interestingly, we observed a degree of inter-patient variability in CD3ζ expression even within the control group. This heterogeneity may have been partially attributable to whether or not individuals had intercurrent infections at the time of sampling, predetermined variation of zeta secondary to genetic polymorphisms of the CD3ζ gene (Gorman et al. 2008), and the differentiation status of CD8 T cells (discussed further in chapter 5).

4.2 CD3ζ downregulation is greatest in intrahepatic CD8 T cells

CD3ζ downregulation has been attributed to sustained exposure to inflammatory cytokines and antigenic stimulation; we therefore hypothesised that it would be more pronounced in the intrahepatic compartment, the site of HBV replication and associated pathology. Intrahepatic lymphocytes were isolated from biopsy tissue obtained from 6 patients with CHB and 7 patients with non-viral liver disease (Table 4.1). CD3ζ expression was then compared between intrahepatic and peripheral T cells for each individual (representative example in Figure 4.2a). CD3ζ expression was lower in intrahepatic compared to peripheral CD8 T cells for all HBV infected patients with a mean of 24% fewer CD3ζ+ cells in the liver compared to the periphery (fig 4.2b). In the 7 patients with non-viral liver disease, the difference in CD3ζ expression between peripheral and intrahepatic CD8 T cells was significantly less. These data suggest therefore that the inflamed liver in patients with CHB, may further potentiate CD3ζ downregulation.
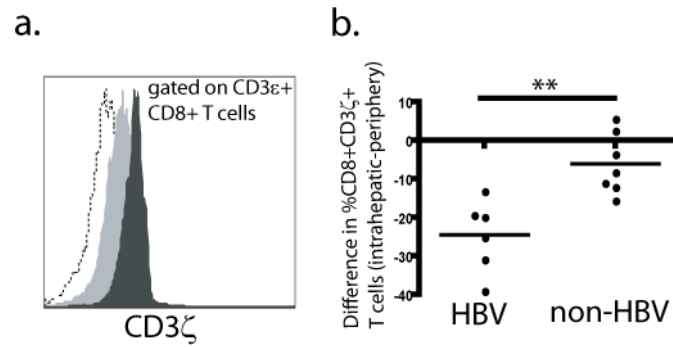
Figure 4.2: CD3ζ downregulation is more marked within intrahepatic CD8+ T cells.
Peripheral blood and intrahepatic lymphocytes (isolated from biopsy tissue) were obtained from 6 patients with chronic HBV and 7 patients with non-viral liver disease. CD3ζ expression was determined by intracellular cytokine staining. a) Histogram to illustrate differential expression of CD3ζ by peripheral (dark grey) and intrahepatic (light grey) CD8 T cells from a CHB donor (dotted line=isotype). b) The difference in CD3ζ expression between intrahepatic and peripheral CD8 T cells is plotted for each patient with HBV/non-HBV related liver disease. (**=p<0.01, non-parametric Mann-Whitney U test).

4.3 CD28 is also downregulated in CHB patients

CD28 is a co-stimulatory molecule on the cell surface which facilitates efficient T cell signalling. Previous studies have suggested that CD28 and CD3 signalling pathways when activated synchronously, converge together and have a synergistic effect in inducing IL-2 production in T cells (Acuto & Michel 2003;Hombach et al. 2001). Like CD3ζ, CD28 downregulation has been described in chronic viral infections such as HIV and CMV and has been associated with cell anergy and dysfunction in IL-2 production (Almanzar et al. 2005;Trimble et al. 2000). In order to investigate whether CD28 downregulation could be playing a role in chronic HBV, we compared surface expression of CD28 on CD8 T cells in patients and controls. Co-staining of CD28 with CD3ζ revealed that CD8 T cells that were CD28 negative in CHB were more likely to have downregulated CD3ζ than their CD28 positive counterparts, but these two proximal signalling molecules were not always down-regulated together (fig 4.3a). The percentage of CD28 positive CD8 T cells was lower in patients with CHB compared to controls (representative examples are shown in fig. 4.3b). CD28 expression was significantly lower in HBeAg patients with high viral load and elevated ALT compared to those with low viral load or healthy donors (4.3c). As CD28 expression is known to be affected by CMV status and age (Almanzar et al. 2005), data was reanalysed with only age matched CMV seropositive individuals and the same trend was seen (data not shown).
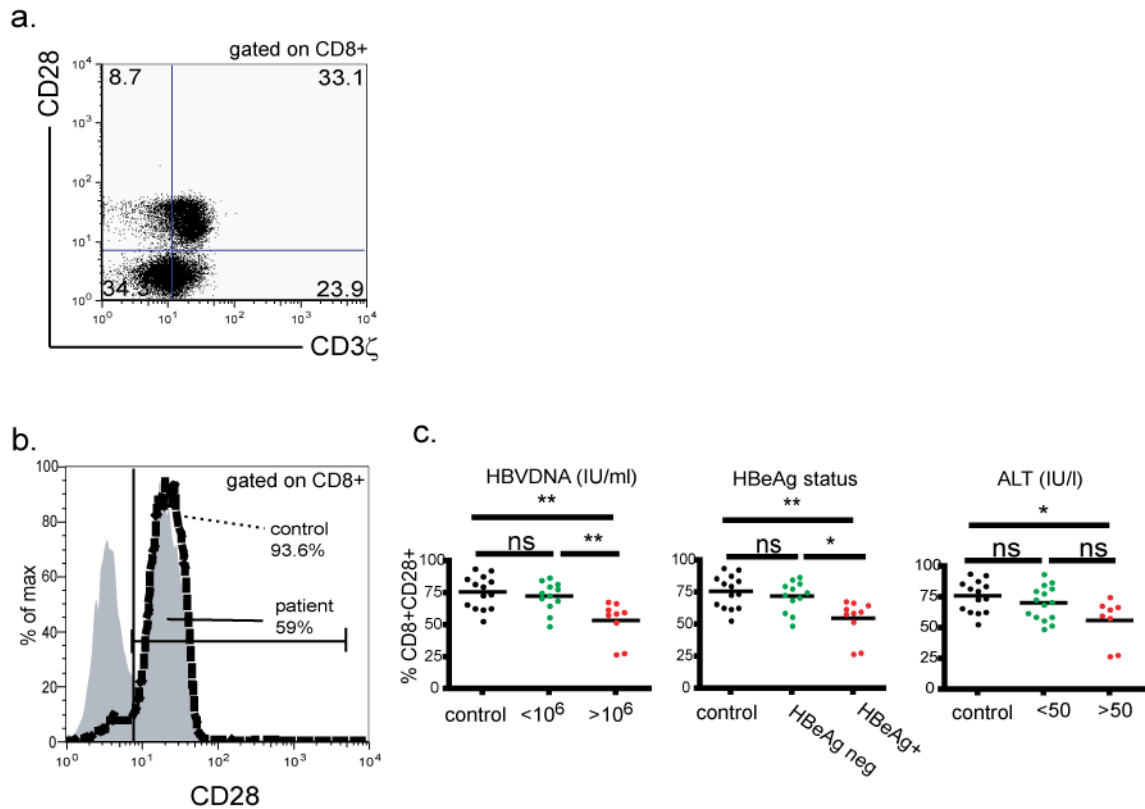
Figure 4.3: CD28 is downregulated in CD8 T cells from patients with high viral load. Representative dot plot to show the co-expression of CD28 and CD3ζ in CD8 T cells in a patient with high viral load. b) Flow cytometric histogram to show the proportion of CD8 T cells which are CD28+ in a patient with high viral load (grey) and a healthy donor (dotted line). c) Cumulative data for CD28 expression is shown against viral load, HBeAg status and ALT level. %CD8+CD28+ on the Y axis= 'percent CD8+CD28+/total CD8+. (*=p<0.05, **=p<0.01, non-parametric Mann-Whitney U test).

4.4 Transfection of peripheral blood leucocytes with a dual CD3ζ/CD28 chimeric receptor replenishes signal molecule expression and IL-2 production

We observed that CD8 T cells from HBeAg positive patients with high viral load and liver inflammation had the lowest expression of CD3ζ and CD28 and were unable to produce IL-2. To test whether a direct link existed between aberrant signalling and functional skewing within these cells, we sought to reconstitute expression of CD3ζ and CD28 signalling molecules and measure the functional consequences. To achieve this, we transfected PBMC from patients with CHB with plasmid DNA encoding a chimeric receptor with intracellular CD3ζ and CD28 domains. Following transfection, we observed that within 4 hours, uptake of the receptor was in the region of 60% for most patients (fig 4.4). Following an overnight rest, transfected PBMC were then stimulated with three types of stimuli; anti-CD3 and anti-CD28 (TCR-dependent stimulus); CD33Ag which could only bind to the extracellular domain of the chimeric receptor, therefore acting like an antigen specific stimulus, or both stimuli together. The same experiments were repeated on untransfected cells which had also been passed through the AMAXA transfection system, however PBS was added in place of the DNA. When PBMC were stimulated through the TCR or via CD33Ag we saw similar levels of IL-2 production. Addition of both stimuli together, further increased IL-2 production. Interestingly, production of IL-2 by the dual stimulus exceeded the combined additive effect of adding either the TCR dependent or antigen specific stimuli separately (fig 4.4). This may suggest that the newly introduced CD3ζ and CD28 may not only function following the antigen specific stimulus, but may also be recruited to T cell receptor signalling following CD3/CD28 stimuli. Previous reports have suggested that activation of Lck by CD28 could enhance tyrosine phosphorylation events induced by TCR engagement (Holdorf et al. 1999). Furthermore, Acuto et al have proposed a model whereby proteins activated through CD28 signalling could be recruited to the adaptor protein LAT which is activated during signalling through the T cell receptor, and thus amplify signal transduction through the TCR. This 'kinase promiscuity' could therefore enhance TCR mediated signalling (Acuto & Michel 2003). No effect was seen by adding CD33Ag alone with the untransfected cells, however they were able to produce IL-2 following CD3/CD28 stimulation suggesting that they were not anergic following the transfection process.
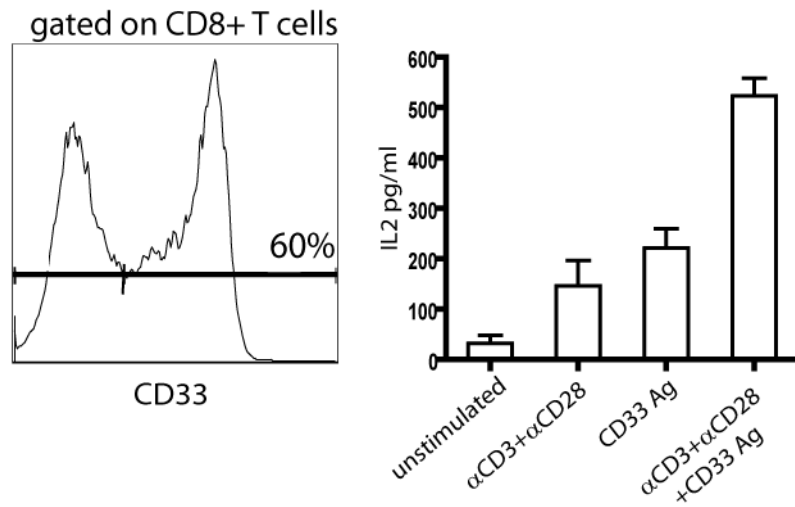
Figure 4.4: Transfection of peripheral blood leucocytes with a dual CD3ζ/CD28 chimeric receptor replenishes signal molecule expression. a) Histogram shows transfection efficiency in a representative patient following transfection of a chimeric receptor with an extracellular CD33-binding domain, and intracellular CD3ζ and CD28 domains. b) Following antigen-specific stimulation through the chimeric receptor, T cell receptor (anti-CD3/anti-CD28) or both together, IL-2 production was determined by ELISA. Mean and SEM of IL-2 production is shown for 3 individuals following the different stimuli.

4.5 Functional reconstitution of CD3ζ in CD8 T cells from CHB patients is partially dependent on L-arginine

We observed that upon resting of PBMC overnight in complete medium, there was significant upregulation of CD3ζ to levels observed in healthy donors (p<0.001), but not CD28 expression (p=ns), compared to CD8 T cells ex vivo (fig 4.5.1 a+b). This was accompanied by a functional reconstitution in IL-2 production, as shown for a representative patient (fig 4.5.1c). The fact that functional reconstitution accompanied a selective upregulation of CD3ζ but not CD28 within CD8 T cells from patients with CHB, suggested that CD3ζ dysfunction may be more important in the setting of CHB infection.

Furthermore, we hypothesized that constituents of the culture medium may have contributed to the substantial upregulation of CD3ζ we observed over a relatively short culture time. One potential candidate was the non-essential amino acid L-arginine, depletion of which is associated with CD3ζ downregulation in both mouse and human studies (Rodriguez et al. 2003;Zea et al. 2004). To investigate whether L-arginine levels modulate CD3ζ expression in T cells of patients with CHB in vitro, PBMC were cultured overnight in medium with or without L-arginine (0.2g/L) and CD3ζ expression was subsequently determined. We observed that after culture in L-arginine reconstituted medium, there was significant upregulation of CD3ζ (MFI 94.3) compared to directly ex vivo (MFI 28.8). However, when L-arginine was depleted from the medium, there was less upregulation of CD3ζ (MFI 71.7) (fig.4.5.2). In addition to measuring CD3ζ expression, proliferative capacity of CD8 T cells was determined after 6 days of culture with a TCR-dependent stimulus in medium with or without L-arginine. We observed that proliferation of CD8 T cells in L-arginine free medium was lower than for CD8 T cells cultured in medium with L-arginine and that in the presence of L-arginine, CD8 T cells both proliferated more and also underwent a further mean division (fig 4.5.3). Therefore, these data suggest that CD8 T cells from patients with CHB are unable to reconstitute CD3ζ fully without replenishment of L-arginine. In addition to loss of proliferative function due to CD3ζ downregulation, L-arginine deprivation has also been directly linked to growth arrest at the G0-G1 phase of the cell cycle (Rodriguez et al. 2007), compatible with the striking loss of CD8 T cell proliferation we observed. Once these cells were removed from

factors present in the HBV-infected patient, they were susceptible to functional reconstitution.
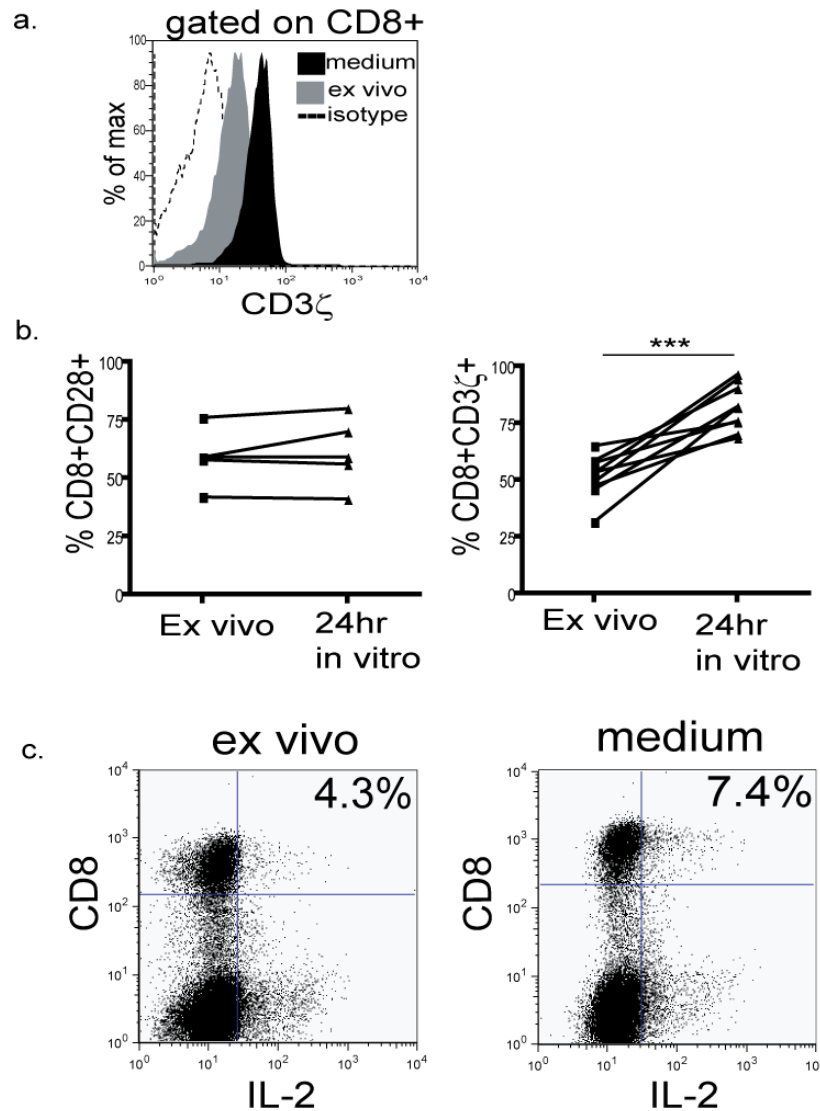
Figure 4.5.1: Reconstitution of CD3ζ but not CD28 upon incubation in medium is associated with recovery of IL-2 production. a) Histogram illustrates effect of overnight culture in medium on CD3ζ levels (dark grey histogram) compared to when measured directly ex vivo (light grey histogram). Isotype control is shown as the dotted line. b) Cumulative data to show change in CD28 (p=non-significant) and CD3ζ expression (p<0.0001, non-parametric Mann-Whitney U test) following in vitro culture compared to ex vivo in 5 and 9 patients respectively. c) IL-2 production was determined in parallel before and after culture and representative dot plots are shown.
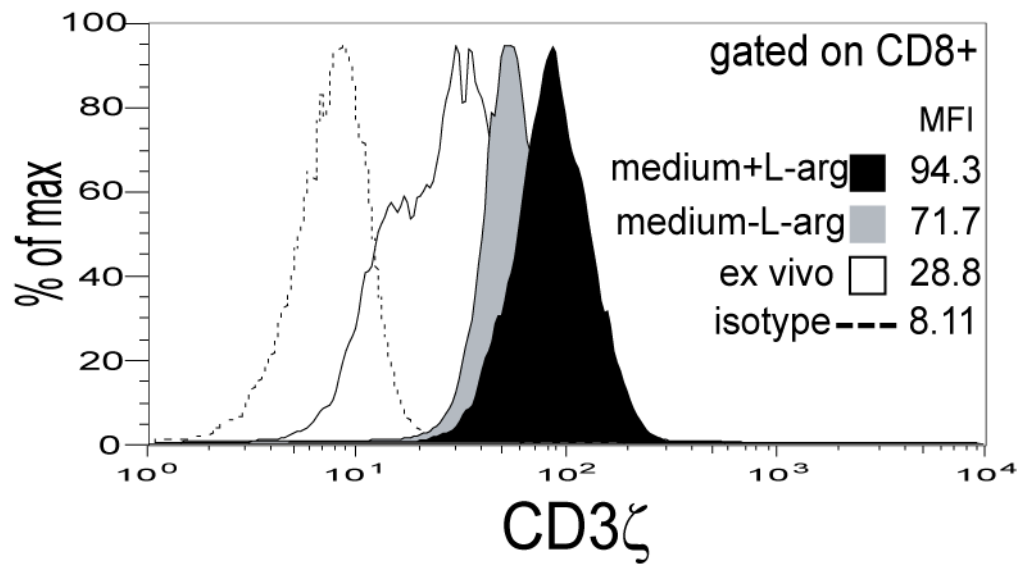
Figure 4.5.2: L-arginine amino acid is partially responsible for selective upregulation of CD3ζ. CD3ζ expression in CD8 T cells was determined directly ex vivo, and then again after overnight culture in medium with or without L-arginine (0.2g/l).
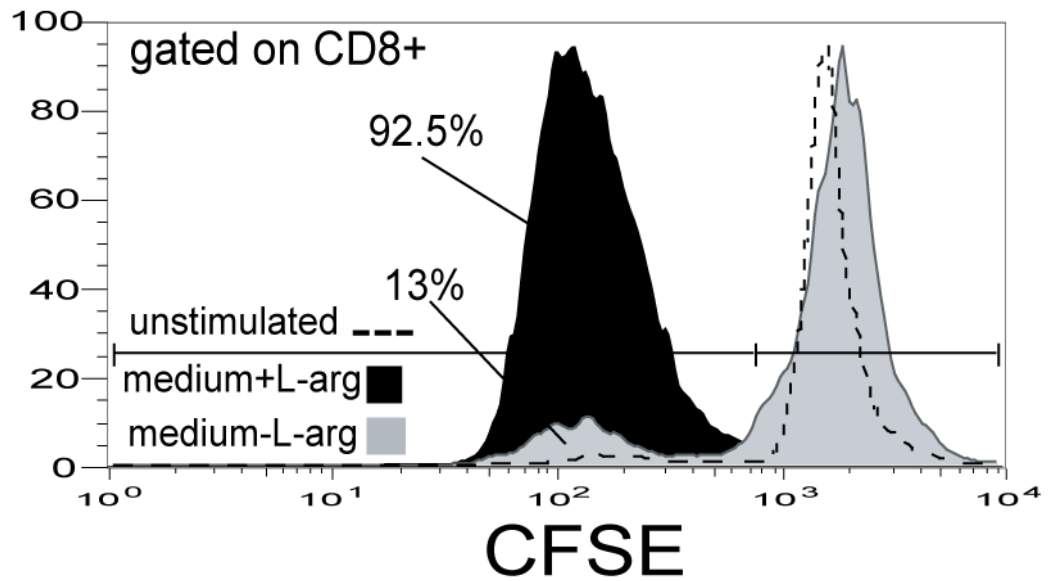
Figure 4.5.3: Depletion of L-arginine in vitro abrogates proliferation of global CD8 T cells upon T cell receptor stimulation. To investigate whether the presence of L-arginine affects CD8 T cell proliferation, PBMC were preincubated with medium with or without L-arginine for 24 hours, stained with CFSE, and then resuspended in their respective mediums in the presence of a TCR-dependent stimulus for 6 days. A gate has been applied on CD8 T cells.

4.6 HBV specific CD8 T cells are unable to proliferate/expand when L-arginine is depleted during short term in vitro culture

To determine what effect L-arginine depletion would have on HBV-specific CD8 T cells which are critical for viral control, PBMC were isolated from HLA-A2 positive patients who had resolved infection, and were stimulated with HBV core18-27 peptide to initiate clonal expansion of HBV-specific cells for 7 days in medium with or without L-arginine present. The expanded population was detected with a core18-27 specific tetramer on day 7, and proliferative capacity of these cells was also determined by their dilution of CFSE dye, which was added at the beginning of culture. As shown in figure 4.6a, in the presence of L-arginine (0.2g/L, which represents the normal amount found in complete medium), a large core18-27 tetramer+ CD8 T cell population was detectable, and 80% of those cells had undergone at least one division as determined by CFSE. In contrast, only a minority of HBV-specific CD8 T cells had divided by one week in arginine-depleted medium and in every case there was inhibition of proliferation compared to responses in the presence of arginine (fig 4.6b). Upon supplementation of L-arginine back into L-arginine depleted medium, we observed a dose dependent recovery in the magnitude of core18-27 specific CD8 T cell responses (fig 4.6c).
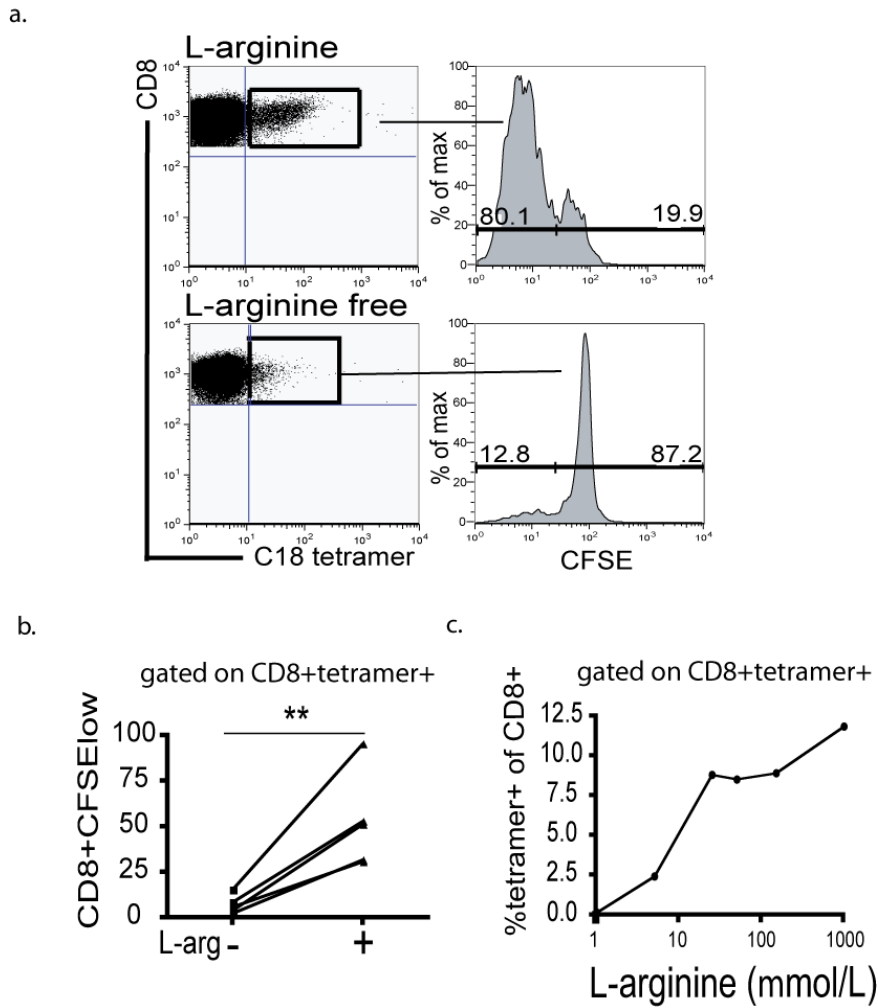
Figure 4.6: HBV specific CD8 T cells are unable to proliferate when L-arginine is depleted during short term in vitro culture. a) To determine the effect of L-arginine on proliferation of HBV-specific CD8 T cells, PBMC from HLA-A2+ patients who had resolved HBV infection were stimulated with core 18-27 peptide for 7 days and detected with an HLA-A2/c18-28-specific tetramer. Representative dot plots depict the HBV-specific CD8 T cell population expanded at one week, along with the number of divisions these populations have undergone, determined by CFSE dilution, in the presence (upper panel) or absence (bottom panel) of L-arginine. b) Cumulative data illustrate the effect of depleting L-arginine on the percent of tetramer positive populations dividing by one week in 5 patients (**=$p<0.01$, non-parametric Mann-Whitney U test). c) HLA-A2/core 18-27 tetramer positive CD8 T cells were compared following 7 days peptide stimulation of PBMC incubated in a range of L-arginine concentrations (0μM, 5μM, 25μM, 50μM, 150μM, 1mM).

4.7 In vivo depletion of serum L-arginine in patients with CHB

To gauge whether L-arginine depletion could potentially impact CD8 T cell signalling and function in vivo, we initially compared serum L-arginine levels in healthy donors and patients with CHB. A significant reduction in circulating L-arginine was observed in patients with high liver inflammation (ALT>100 IU/L), compared to those with normal or mildly elevated ALT and healthy donors (Figure 4.7). Furthermore, the dose range of L-arginine concentrations detected in the serum of patients was equivalent to that we had shown to be sufficient to impair expansion of HBV-specific CD8 T cell responses from resolved individuals in vitro. The concentration of extracellular L-arginine is likely to be lower in the liver. To investigate this, we determined L-arginine concentrations from liver homogenates from patients with CHB vs. non-viral liver disease. Although the homogenisation process is known to release high quantities of arginase-1 from hepatocytes which can rapidly deplete L-arginine levels, we still managed to detect a significantly lower L-arginine concentration within liver homogenates from patients with CHB compared to patients with non-viral liver disease (data not shown).
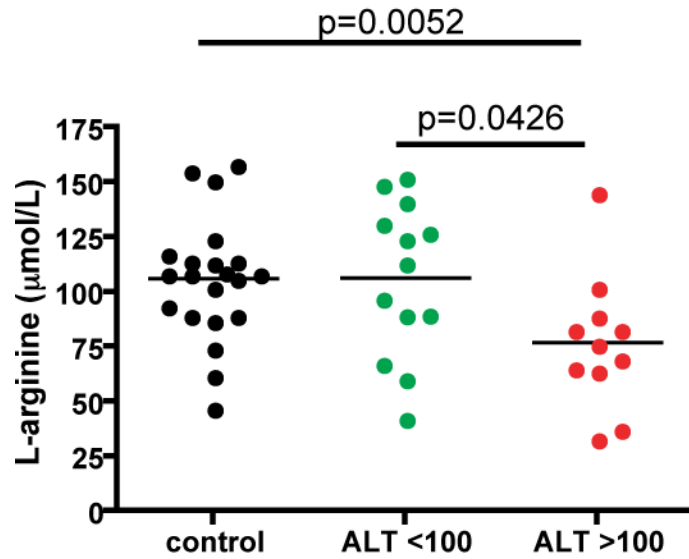
Figure 4.7: Circulating L-arginine is reduced in patients with ALT. Serum L-arginine levels were compared in healthy donors (n=21), patients with low-moderate liver inflammation (ALT<100 IU/L, n=13) and patients with high liver inflammation (ALT>100 IU/L, n=11). (control vs. high ALT p<0.01; low ALT vs. high ALT p<0.05).

4.8 Arginase activity correlates with flares of liver disease and is elevated in the HBV inflamed liver

Depletion of L-arginine within patients with CHB could suggest an increased rate of metabolism of this amino-acid during CHB. Arginase-1 is an enzyme which can catabolise L-arginine to urea and ornithine. It is expressed almost exclusively in the liver making it a candidate for L-arginine breakdown within the inflamed intrahepatic environment, however it can be induced in other cell types also following exposure to cytokines and agents such as LPS (Morris, Jr. 2002). Within the HBV infected liver, myeloid suppressor cells (Bronte & Zanovello 2005) and macrophages exposed to Th2 cytokines such as IL-10 (Modolell et al. 1995), which we have shown is raised during HBeAg negative flares of HBV (Chapter 6), may additionally contribute to arginase activity.

To determine whether HBV related inflammation resulted in upregulation of arginase activity which could account for L-arginine depletion and resultant CD8 T cell dysfunction, we measured arginase activity in both serum and liver homogenates from patients with CHB. Arginase activity was determined by incubating serum/liver homogenate samples with a saturating concentration of substrate L-arginine, and measuring the amount of urea output over a fixed period of time. For liver samples, arginase activity was normalised to the protein content of liver tissue. Ex vivo serum arginase levels showed a non-significant trend to be higher in CHB patients with active liver inflammation but showed considerable variability within patient groups and controls (data not shown). To remove the confounder of inter-patient variability, arginase activity was measured longitudinally in three patients undergoing eAg negative flares of liver disease, characterised by rapid fluctuations of disease activity over a condensed timeframe (fig 4.8). In these individuals, we observed large fluctuations of arginase activity, with peaks correlating with flares of disease activity as measure by ALT or viral load. Since arginase-1 is mainly expressed in the liver, these fluctuations could reflect increased release from the relevant tissue source, as is the case in pregnancy (arginase release from placenta) (Kropf et al. 2007) and during orthoptic liver transplantation (Roth et al. 1994). On measurement of arginase activity from liver homogenates ex vivo, we observed significantly elevated activity in liver extracts derived from patients with CHB (n=5) compared to those taken from patients with non-viral liver disease (n=5) (fig 4.9).
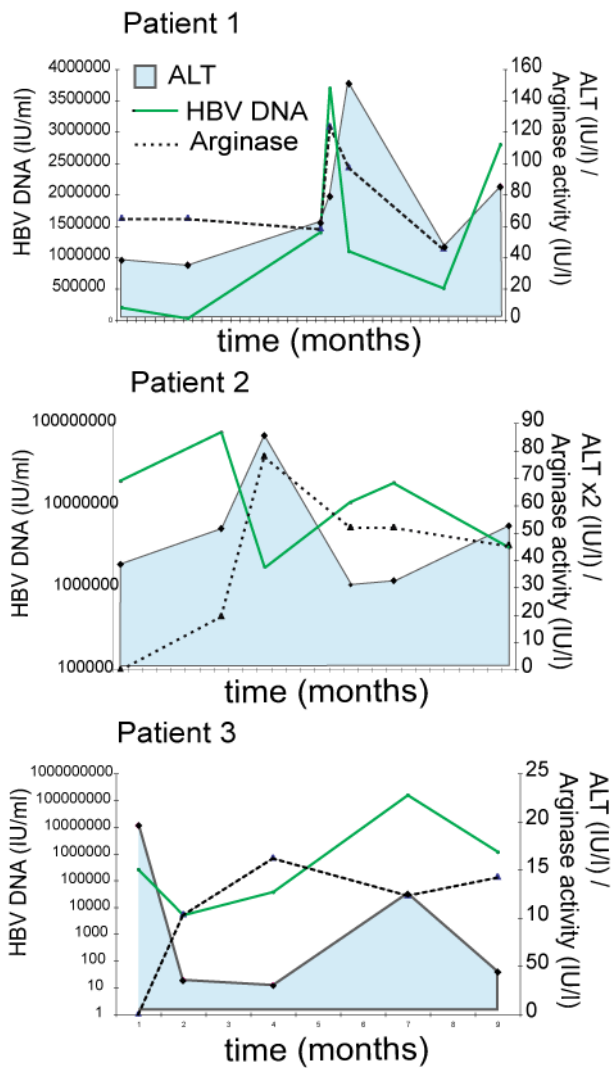
Figure 4.8: Serum liver type arginase-1 activity is elevated during chronic flares of liver disease. Graphs depict longitudinal fluctuations in serum arginase activity (dashed line, IU/L) in association with viral load (solid green line, HBV DNA IU/ml) and ALT (turquoise shaded histogram, IU/L) for 3 patients with flares of CHB.
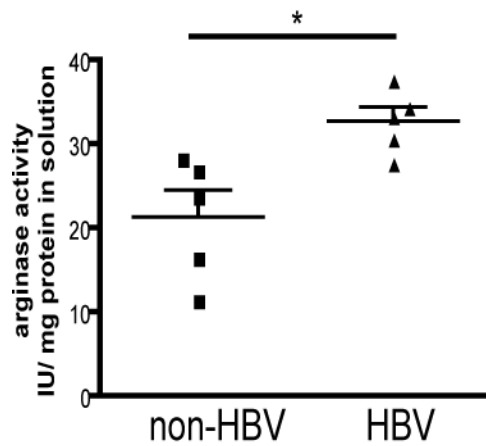
Figure 4.9 Arginase-1 activity in the liver is higher in patients with CHB compared to those with non-viral liver disease. The arginase activity of liver homogenates is shown for 5 individuals with CHB, and 5 individuals with non-viral liver pathology. (*=p<0.05, non-parametric Mann-Whitney U test).

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Discussion

We observed a defect in IL-2 production by CD8 T cells in patients with CHB upon a T cell receptor dependent stimulus, however not upon a TCR bypassing stimulus. This led us to hypothesize that aberrant signalling through the TCR complex, may in part be responsible of this selective defect. In other settings of chronic inflammation and persistent antigenic stimulation, analogous to CHB, downregulation of CD3ζ with or without CD28 has been associated with a CD8 T cell dysfunction in IL-2 production. These proximal TCR associated molecules are upstream initiators of signalling cascades, and play a rate-limiting role in efficient T cell signal transduction. In this chapter, we showed that both CD3ζ and CD28 were downregulated in CD8 T cells from patients with CHB compared to healthy donors and that this was greatest within CD8 T cells isolated from HBeAg positive patients with high viral load and liver inflammation. Transfection of PBMC with chimeric receptors with intracellular CD3ζ and CD28 domains was able to restore IL-2 production. Moreover, CD3ζ expression was found to be even lower within intrahepatic CD8 T cells, suggesting that factors within the HBV inflamed liver may further potentiate CD3ζ downregulation and exacerbate the global functional defects. From our in vitro experiments, we showed that depletion of the non-essential amino acid L-arginine, both attenuated upregulation of CD3ζ and impaired expansion of global and HBV-specific CD8 T cells in a dose dependent manner. In support of this, we noted that L-arginine was depleted within the serum of patients with CHB (with liver inflammation), and that arginase-1, which is almost exclusively expressed in the liver and catabolises L-arginine, was upregulated selectively in liver extracts from patients with CHB.

Mechanisms for CD3ζ downregulation have been described previously at both the protein level (caspase 3 mediated degradation (Krishnan et al. 2005)) and at the mRNA level (mutations in the gene encoding CD3ζ (Tsuzaka et al. 2003)). Within the HBV infected liver, multiple mechanisms could apply. One hypothesis is that the inflammatory cytokine environment may play a role. Transgenic mouse models of HBV have shown high levels of IFN-γ within the liver (Guidotti et al. 1996). In situations of chronic bacterial stimulation in mice, IFN-γ has been shown to indirectly induce CD3ζ downregulation within T cells, through induction of reactive oxygen intermediate production (Bronstein-Sitton et al. 2003). Therefore, IFN-γ mediated CD3ζ downregulation may be one potential mechanism.

Similarly, Isomaki et al. showed that chronic exposure to TNF-α suppressed peptide specific IL-2 production and this was associated with TNF mediated downregulation of CD3ζ. Withdrawal of TNF resulted in a reconstitution of both CD3ζ expression and cell function (Isomaki et al. 2001). In this system however, downregulation of CD3ζ was in combination with the entire CD3/TCR complex, and not a selective defect as we and others have observed. Nevertheless, high levels of TNF have been reported in the plasma of patients with CHB (Sheron et al. 1991), and we have shown that global CD8 T cells in patients with high viral load maintain their TNF production, thus by selectively studying CD3ζ downregulation on CD3ε gated CD8 T cells, we may have underestimated the total loss of CD3ζ.

Alternatively, global downregulation of CD3ζ may also be related to depletion of amino acids within the micro-environment such as L-arginine (Fallarino et al. 2006;Rodriguez et al. 2003). Previous reports have shown that L-arginine is required for post-transcriptional mechanisms in stabilisation of CD3ζ mRNA, and depletion of extracellular levels results in CD3ζ downregulation in human T cells (Zea et al. 2004). Depletion of other non-essential amino acids such as L-glutamine did not have this effect (Rodriguez et al. 2002). In our study, we showed that CD8 T cells from chronic HBV infected patients depleted of L-arginine in vitro had reduced CD3ζ expression and proliferated poorly compared to CD8 T cells cultured in the presence of L-arginine. Deficiency of L-arginine was also shown to impair the expansion of HBV-specific CD8 T cell responses from resolved individuals in a dose dependent manner. Notably however, CD8 T cells from CHB patients showed some reconstitution of CD3ζ once they were removed from the source of antigenic stimulation and inflammation, even in the absence of arginine. This suggests that other factors within the inflamed liver may also contribute to this defect including exposure to reactive oxygen intermediates (Otsuji et al. 1996) or inflammatory cytokines or depletion of other amino acids. In particular, depletion of tryptophan and accumulation of tryptophan catabolites has also been shown to inhibit T cell proliferation via CD3ζ down-regulation (Fallarino et al. 2006;Frumento et al. 2002).

We found that circulating concentrations of L-arginine were significantly lower in patients with CHB compared to healthy donors. This deficiency could be either due to reduced supply, or increased demand. Since we are not aware of any change in dietary intake of amino acids used to synthesize L-arginine, or defects in L-arginine synthesis in patients

with CHB, it is likely that lower levels of L-arginine in the periphery are secondary to increased breakdown. L-arginine is catabolised in the body by either arginase or nitric oxide synthase. Arginase-1 is found predominantly in the cytosol of hepatocytes and is therefore a prime candidate for L-arginine depletion in HBV (Bronte & Zanovello 2005). We showed that arginase activity was elevated in liver homogenates from patients with CHB, and longitudinal serum arginase activity correlated temporally with flares of HBV DNA and ALT in 2 patients undergoing chronic flares of liver disease.

Elevated arginase activity during HBV infection may have several potential sources. Although arginase is exclusively expressed in the cytosol of hepatocytes (Morris, Jr. 2002), and these cells have been shown to reduce L-arginine levels by arginase-1 release following orthoptic liver transplantation (Roth et al. 1994), arginase may also be induced in other cell types. Macrophages and myeloid suppressor cells recruited to the liver by IFNγ induced chemokine release may further contribute to arginase release and local L-arginine depletion (Kakimi et al. 2001b). Macrophages exposed to Th2 cytokines IL4 and IL13 (Rodriguez et al. 2003) or IL-10 (Modolell et al. 1995;Munder et al. 1999) or bacterial lipopolysaccharide, with which the liver is enriched (Morris, Jr. 2002), upregulate arginase-1 and can downregulate CD3ζ in T cells indirectly by consumption of extracellular L-arginine. We have shown that serum IL-10 levels, like arginase, correlate temporally with flares of HBV DNA and ALT during HBeAg negative flares of HBV and may therefore be a potential inducer of arginase (see chapter six).

In addition to macrophages, arginase-1 released by myeloid-derived suppressor cells can deplete local L-arginine levels (Rodriguez et al. 2005) and this has been associated with CD3ζ downregulation and impaired T cell function (Ezernitchi et al. 2006). They are enriched in settings of chronic inflammation (Bronte & Zanovello 2005;Haile et al. 2008), however what their role is during chronic HBV infection is not yet clear.

Apart from immune cells, certain parasites or bacteria have been shown to produce their own arginase or manipulate the host's own arginase as a mechanism to suppress T cell function and evade immune control. *Helicobacter pylori* for example has been shown to produce arginase-1 which results in L-arginine depletion dependent CD3ζ downregulation within the gut (Zabaleta et al. 2004). It is not known whether HBV can directly release or

modulate host arginase, and thus potentially contribute to subversion of the immune response through arginase-L-arginine mediated impairment of T cell responses.

Although impairment of proliferation may be partially related to CD3ζ downregulation, there is also evidence that L-arginine depletion can attenuate proliferation of T cells directly. L-arginine deprivation has been linked to growth arrest at the G0-G1 phase of the cell cycle (Rodriguez et al. 2007), compatible with the striking loss of CD8 T cell proliferation we observed. This may partially be due to the fact that one of its breakdown products, L-ornithine, is a precursor for synthesis of polyamines by the ornithine decarboxylase pathway, required for cell growth and differentiation (Bronte & Zanovello 2005).
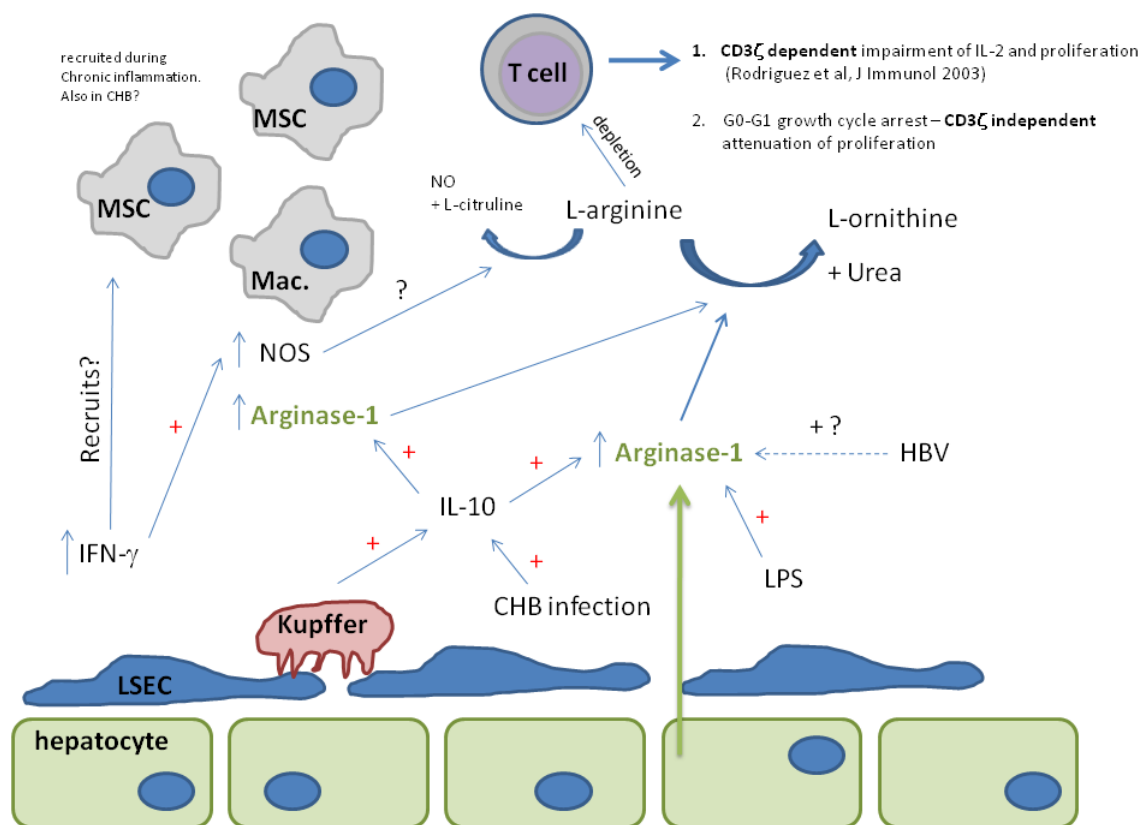

Figure 4b. Schematic diagram to show potential pathways of L-arginine depletion with the HBV inflamed liver. MSC: myeloid suppressor cell, Mac.: macrophage, LSEC: liver sinusoidal endothelial cell.

In addition to CD3ζ downregulation, we found marked CD28 downregulation also in CD8 T cells from patients with high viral load. Loss of CD28 has been related to chronic antigen persistence (Effros et al. 1996), and this could drive HBV-specific CD8 T cells towards a CD28 null phenotype. However, a global loss of CD28 expression would require the influence of bystander stimulation, either through inflammatory cytokines or viral antigens. Notably, both TNF (elevated in the plasma of patients with CHB (Sheron et al. 1991)) and IFN-α (elevated during flares of eAg negative HBV (Dunn et al. 2007)), have been shown to induce CD28 loss (Fletcher et al. 2005;Lewis et al. 2004), and thus could exacerbate signalling dysfunction and T cell hyporesponsiveness in CD8 T cells already dysfunctional through CD3ζ loss.

In summary, we show that non-antigen specific CD8 T cell dysfunction in IL-2 and proliferation may in part be due to aberrant TCR signalling in CHB. Reconstitution of signalling molecule expression is able to partially reverse the IL-2 defect. Furthermore, we show that both downregulation of CD3ζ and impaired proliferative function of CD8 T cells within the inflamed liver, may be exacerbated by local depletion of the nutrient L-arginine, which is lower in the serum of patients with liver inflammation, and may be further depleted in the HBV-infected liver where we have shown arginase-1 to be upregulated. These data provide a novel mechanism for loss of CD8 T cell function and impairment of viral control in CHB.

5 Premature senescence of CD8 T cells in patients with chronic HBV infection.

Background

During chronic viral infection, a high dose of antigen can often persist over many years in the host. In this setting, we hypothesize that chronic activation of CD8 T cells may drive them prematurely towards a terminally differentiated phenotype and replicative senescence: an end stage state associated with functional exhaustion and loss of proliferative capacity. In this chapter, we investigate whether the qualitative and quantitative defects within virus specific and non-antigen specific CD8 T cells described in CHB, could partially be due to accelerated ageing of this compartment associated with chronic activation by the virus itself, or cytokines induced in the surrounding environment.

As a background to this, I will first review the normal differentiation pathway of CD8 T cells during an acute anti-viral response, and then examine how this pathway may be altered if antigen instead is not cleared and persists indefinitely.

5.0.1 CD8 T cell differentiation

There are three phases to an anti-viral CD8 T cell response during acute infection. 1) Antigen specific CD8 T cells undergo clonal expansion to give rise to a population of effector cells. 2) this large number of effector cells is then diminished during the contraction phase, at which point 85-90% of them undergo activation induced cell death. 3) From the remaining cells, a population of memory CD8 T cells are generated which are required for long-term immunity (Zimmerman et al. 1996).

Therefore, antigen encounter can result in the formation of either an 'effector' or 'memory' CD8 T cell. These two populations have phenotypically and functionally distinct characteristics. Hamann et al showed that effector cells were characterised by expression of CD45RA, but not CD27 or CD62L and that these cells contained granzyme B, had immediate cytolytic capacity and could produce IFNγ and TNF upon mitogenic stimulation, which has been associated with control of virus replication. These cells could not produce IL-2 however and had lost their ability for autocrine proliferation. Memory cells on the other hand, defined as CD45RA-CD45RO+, were able to produce a wide range of cytokines including IL-2, IL-4, TNF and IFNγ, however were not cytotoxic without prior in vitro stimulation. Naïve cells, which expressed both CD45RA and CD27 were the main producers of IL-2, however, unlike primed cells, produced relatively low quantities of IFNγ and TNF as determined by intracellular staining and ELISA (Hamann et al. 1997). Therefore the distinct functional characteristics of these cells may hint towards independent roles in the immune response against the virus.

Although the above model suggests a linear differentiation pathway for CD8 T cells from naïve→effector→memory, whether or not the memory subset actually arise from the effector population is still under debate. A number of alternative models have been put forward.

Model 1: This supports the notion of a divergent pathway whereby upon antigenic stimulation of naïve T cells, either a memory or effector cell can be generated. Lanzavecchia et al proposed a model in which they suggested that the duration and strength of TCR stimulation could induce hierarchical levels of CD8 T cell differentiation characterised by distinct survival, homing and functional capacity (Lanzavecchia & Sallusto 2000). In this model, fit cells which had received optimal stimulation were

arrested at intermediate stages of differentiation and survived as effector memory (Tem) or central memory (Tcm) T cells. Too much stimulation initiated activation induced cell death whereas too little stimulation resulted in death by neglect. Therefore, in this model, naive T cells reached hierarchical thresholds of differentiation depending on the 'instructions' provided by dendritic cells and cytokines which they themselves 'decoded' into a cell fate decision. If a sufficient threshold of stimulation was reached, naïve T cells could become memory T cells and bypass the effector stage altogether (Kaech et al. 2002). In support of this instructional hypothesis, Yang et al. showed that different costimulatory pathways could induce differential phenotypes in CD8 T cells. For example, HSA/B7 costimulation was required to induce a memory phenotype, whereas B7 not HSA was required for generation of effector CD8 T cells. Therefore, in this model effector cells were not a mandatory intermediary for memory cell induction (Liu et al. 1997).
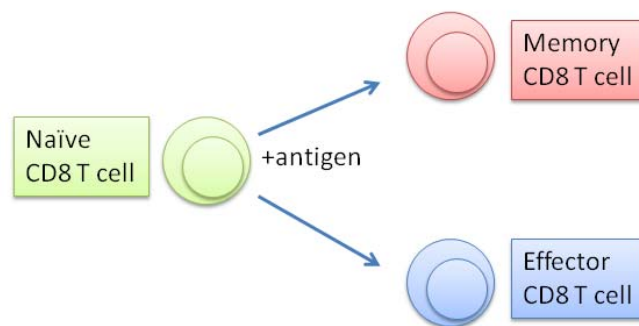
Figure 5a. Divergent model of CD8 T cell differentiation. Adapted from (Kaech et al. 2002)

Model 2: In contrast to model 1, longitudinal studies in mice with LCMV infection favour a linear differentiation pathway (Naïve→effector →memory) whereby memory cells arise from the effector population. Expression of CD127 and responsiveness to homeostatic cytokines are characteristic of memory cells. Sarkar et al showed that, during an acute response to LCMV, all the antigen specific effector CD8 T cells were CD127 low. These cells could be subdivided into killer cell lectin-like receptor subfamily G, member 1 (KLRG-1) intermediate or high expressers, which functionally were similar in their cytotoxic ability, granzyme B expression, and cytokine profiles. However, only the intermediate KLRG-1 expressing cells could produce IL-2 and upon resolution of infection, had the capacity to upregulate CD127 expression. Therefore, they showed with

KLRG-1 expression that memory precursors were present within the effector population (Sarkar et al. 2008).
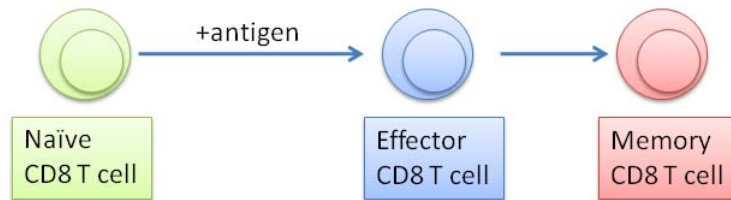
Figure 5b Linear model of CD8 T cell differentiation. Adapted from (Kaech et al. 2002)

In another study, Opferman showed that only B6.2.16 CD8 T cells (transgenic T cells specific for male H-Y antigen) which underwent at least 5 rounds of stimulation (as determined by CFSE) on stimulation with H-Y male antigen were able to give rise to H-Y specific CD8 memory T cells upon adoptive transfer into mice, whereas transfer of undivided pre-effector B6.2.16 CD8 T cells did not. This suggested that memory cells could arise from effector populations, but not the pre-effector, which is against the divergent hypothesis (Opferman et al. 1999).

In acute HBV and HCV infection, Urbani showed that the majority of HBV and HCV tetramer positive CD8 T cells belonged to the effector memory subset (CCR7-CD5RA-). Therefore it is likely that these cells are the precursors for memory cells which are detectable on resolution of these infections (Urbani et al. 2002).

Model 3: This has been termed the 'decreasing potential hypothesis' and suggests that the distinguishing factor between death and survival for an effector T cells is the duration and dose of antigenic stimulation it is exposed to. Therefore, with persistent long-term antigenic stimulation, analogous to a situation of chronic viral infection, effector cells would progressively lose function, and thus be progressively less capable of generating memory populations. Late stage effector cells are unfit both metabolically and functionally to generate memory populations and undergo apoptosis instead. It has been suggested, that an accumulation of these late stage effectors during persistent viral infections may be a potential mechanism for impaired memory cell production (Kaech et al. 2002).
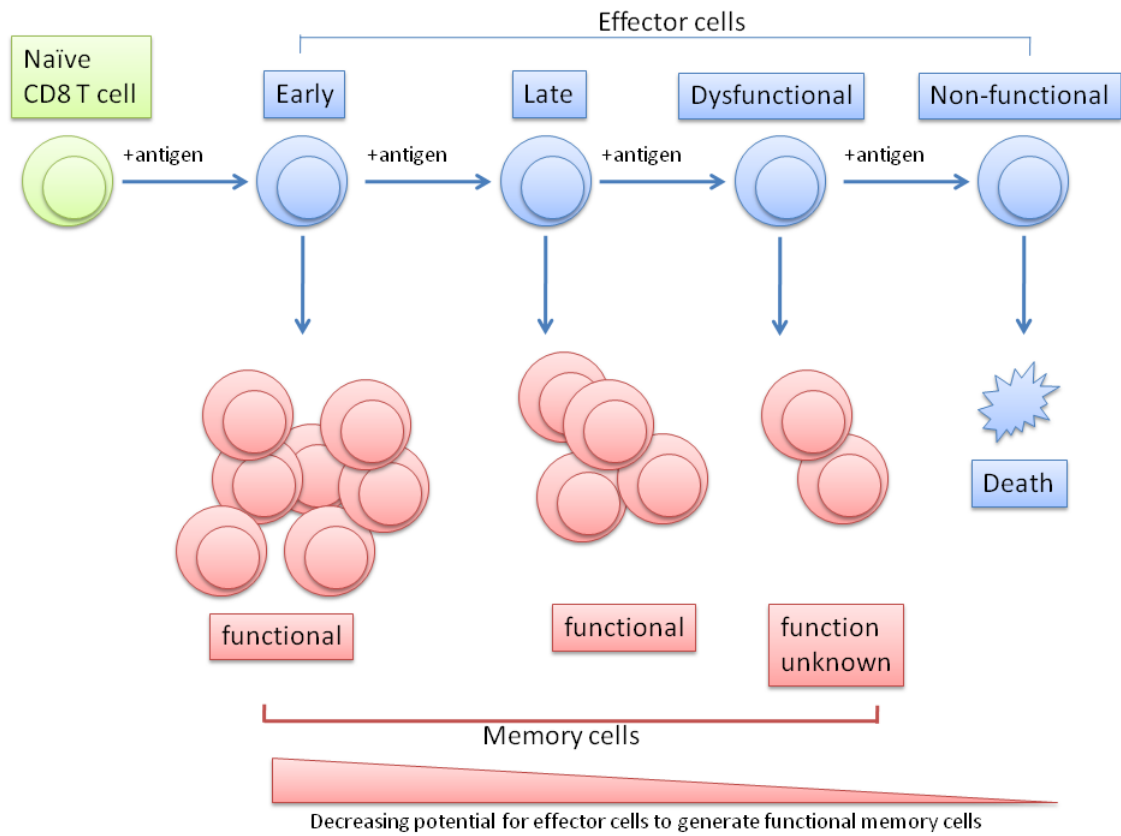
Figure 5c A model of the decreasing potential hypothesis. Adapted from (Kaech et al. 2002)

5.0.2 Subsets of memory CD8 T cells

Memory CD8 T cells facilitate a rapid immune response upon secondary encounter with a specific antigen. In humans, they can be subdivided into either central memory (Tcm) or effector memory T cells (Tem) dependent on expression of molecules, CCR7 and CD62L involved in homing to lymph nodes (Sallusto et al. 1999). CCR7 is a chemokine receptor required for migration through high endothelial venules in lymph nodes into T cell areas (Lanzavecchia & Sallusto 2000).

Central memory CD8 T cells home to lymph nodes and can proliferate and produce IL-2 but have limited cytolytic function. Effector memory CD8 T cells on the other hand, home to tissues and can rapidly produce IFNγ on antigen encounter. They also have cytolytic capacity as they contain prestored perforin, however unlike Tcm, have a low proliferative capacity. Based on their immediate effector capabilities and homing capabilities, it was suggested that Tem were required to rapidly contain pathogens at tissue sites, whereas Tcm were more important for long-term protection by sustaining the effector pool (Lanzavecchia & Sallusto 2005). Tcm may also have additional regulatory functions as these cells have been shown to help B cell proliferation, modulate the function of antigen carrying dendritic cells and IL-10 producing cells are found in this compartment (Wherry & Ahmed 2004).

The relationship between Tcm and Tem is not clear cut. I have discussed briefly the model put forward by Lanzavecchia and colleagues, who suggested that the threshold of activation of naive T cells could determine which cell fate decision was reached (Lanzavecchia & Sallusto 2000). However, other studies suggest a more intimate connection between Tem and Tcm and that CD8 T cells may even interchange between the two compartments. Wherry et al. showed that following antigen clearance in a mouse model of LCMV, effector memory CD8 T cells developed into central memory CD8 T cells over time (Wherry et al. 2003b). Sallusto et al however, have shown that in vitro stimulation of central memory CD8 T cells resulted in loss of CCR7 expression and differentiation to effector memory (Sallusto et al. 1999). The close association between these subsets was analysed further by Romero et al, who characterised effector CCR7-CD45RA-CD8+ T cells by expression of CD27 and CD28, and discovered a population of EM1 cells(CD8+CD27+CD28+CD45RA-CD127^{hi}perforin^{lo}granzymeB^{lo}CCR7-) which were phenotypically and functionally similar to central memory cells bar the expression of

CCR7. These CCR7- EM1 cells were suggested therefore to be a distinct memory population of similar lineage to the central memory, which could potentially mediate the same function within the tissues rather than the lymph nodes (Romero et al. 2007).

A further population of memory cells which re-express CD45RA have also been described. These 'revertant' CD8 T cells have low proliferative capacity and are relatively resistant to apoptosis (Dunne et al. 2002). Additionally, they have been shown to have immediate cytolytic activity and can produce cytokines IFNγ and TNF similar to effector memory CD8 T cells, however are unable to produce IL-2 (Hamann et al. 1997). Faint et al. showed using HLA class I restricted tetramers that a significant fraction of herpes-virus specific CD8 T cells reverted from CD45RO to CD45RA after priming, and were CCR7^{low}LFA1^{high} in phenotype. These cells furthermore had shorter telomere lengths, and potently produced IFN-γ on stimulation suggesting that they were memory and not naïve CD8 T cells (Faint et al. 2001). Recent studies have shown that revertant populations originate from central memory CD8 T cells exposed to IL-15, in an antigen-independent manner (Dunne et al. 2005;Geginat et al. 2003). In support of this, Geginat et al. showed that sorted Tcm cultured with homeostatic cytokines IL-7 and IL-15 could differentiate into revertant cells, however upon antigenic stimulation through the TCR, Tcm instead differentiated almost exclusively into CD45RA-CCR7- effector memory cells (Geginat et al. 2003).
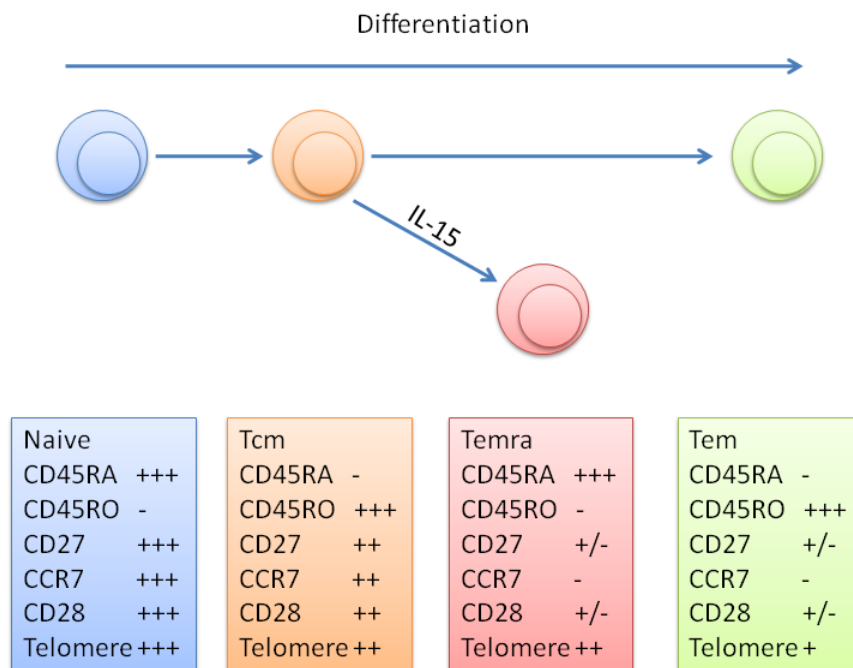

Figure 5d T cell differentiation. Modified from (Akbar & Fletcher 2005).

Although there is still some debate on the lineage of central and effector memory CD8 T cells, it is clear nonetheless that they have distinct phenotypic and functional profiles which may allow them to mount synergistic control of pathogen in the lymph nodes and within tissues.

5.0.3 Homeostatic maintenance of memory populations

The fact that thymic involution occurs at a young age suggests that survival of memory cells over many decades involves proliferation and periodic turnover (Akbar & Vukmanovic-Stejic 2007). This 'homeostatic proliferation' requires cytokines IL-15 and IL-7, which signal through the common gamma chain of the IL-2 receptor, however does not require self/MHC and antigenic stimulation. Cytokine responsiveness and IL15 receptor expression was low in naïve T cells for example, however progressively increased in Tcm, Tem and revertant CD8 T cells (Geginat et al. 2003). Experiments by Tan et al. have shown in cytokine knockout mice that either IL-7 or IL-15 were sufficient for CD8 T cell homeostatic proliferation, however in the absence of both cytokines, this process was arrested. Interestingly, in CD4 T cells, homeostatic proliferation was independent of both IL-7 and IL-15 production (Tan et al. 2002). Additionally, Tough et al. showed that bystander production of type I IFN could induce TCR independent proliferation and could further contribute to maintenance of memory (Tough et al. 1996). Therefore, these cytokines allow for memory populations to be maintained over a lifetime without altering the total size of the memory T cell pool.

Unlike memory cells, naïve CD8 T cells require constant low affinity interaction or 'tickling' with self MHC and antigen as well as IL-7 for their survival (Lanzavecchia & Sallusto 2000;Tan et al. 2002). This is illustrated by a high expression of CD127 (IL-7Rα) on their surface. Interestingly however, Alves et al identified a new population of naïve CD8 T cells which were CD127 low both at the mRNA and protein level and exhibited a low proliferative response to IL-7 but not anti CD3/anti CD28 stimulation. These cells were enriched in circumstances where a relative paucity of classical naïve CD8 T cells was expected, for example in the elderly, early after heamopoeitic stem cell transplantation or in HIV. The authors suggested therefore that these cells may be a population of naïve T cells which had recently received homeostatic signals, which are known to induce short-term rapid downregulation of CD127 expression (Alves et al. 2007).

5.0.4 Altered CD8 T cell differentiation during chronic viral infection

5.0.4a Virus-specific CD8 T cells

If antigen is not cleared and instead persists, the above paradigm of effector and memory cell differentiation may be altered. Wherry et al. showed that during chronic LCMV infection of mice, persistent antigenic stimulation in the absence of CD4 T cell help accelerated differentiation of CD8 T cells towards an end stage phenotype. This progressive 'exhaustion' was associated with a hierarchical loss of effector function; IL-2 production and cytolytic capacity were lost first, followed by ability to produce TNF-α, whereas IFN-γ production was most resistant to loss. Fully exhausted CD8 T cells were further rendered incapable of IFN-γ production and were susceptible to deletion (Wherry et al. 2003a). Therefore, in this model, antigen load itself could drive exhaustion of the CD8 T cell response.

This may not necessarily occur with all chronic viral infections however, as the ability to influence CD8 T cell responses depends on the type of virus, its tissue distribution, differential responses to viral epitopes and availability of CD4 help. For instance, viruses may establish either latent infection with periods of reactivation (e.g. EBV) or chronic infection with life-long low level viral persistence (e.g. CMV) or high level viral persistence (HBV/HIV/HCV). The important difference between the latter two varieties is low level 'smoldering' infections provide intermittent antigen exposure with rest periods, whereas during chronic infection with high levels of viraemia, there is constant barrage of antigen and CD8 T cell activation in the absence of critical rest periods. This may make CD8 T cells in these infections more susceptible to functional exhaustion (Wherry & Ahmed 2004).

In this study, we investigated whether the virus-specific and global CD8 T cell defects in IL-2 production and proliferation could partially be attributable to functional exhaustion of CD8 T cells in CHB. Similar to chronic LCMV infection, CHB is characterised by high level viral persistence over many decades. Additionally, as a unique phenomenon, HBV infection is also associated with production of high levels of sub-viral particles which have no known role in viral replication, however instead have been implicated as having 'immunomodulatory' function (Milich et al. 1990). Furthermore, the virus has a selective tropism to hepatocytes, making the liver environment the site of highest viral replication

and inflammation. At this site, there has also been reported to be a relative lack of CD4 T cells compared to the periphery (Norris et al. 1998). Under these circumstances it is feasible that local chronic activation of CD8 T cells by high viral load in the absence of rest periods and CD4 help, may be conducive to a loss of CD8 T cell function and premature exhaustion of virus-specific CD8 T cells. This could provide a partial mechanism for the failure of viral control in this setting.

5.0.4b Global CD8 T cells

Persistent TCR-mediated antigenic stimulation may drive progressive exhaustion of HBV-specific CD8 T cells, however would not be expected to do the same for the non-antigen specific CD8 T cell population. Based on our observations that these cells are IL-2 and proliferation impaired and CD28 low in patients with high level CHB, we hypothesize that they may also be driven to functional exhaustion. Putative factors that may drive these global CD8 T cells towards dysfunction may include the inflammatory cytokine milieu in the HBV inflamed liver, or exposure to high levels of sub-viral particles, which may be tolerogenic (Milich et al. 1990). As discussed in chapter 4, high levels of TNF and IFNα have been observed in the plasma and serum respectively in patients with CHB (Dunn et al. 2007;Sheron et al. 1991), and have been shown to induce loss of CD28 (Fletcher et al. 2005;Lewis et al. 2004). In addition, homeostatic cytokines such as IL-15 can drive differentiation of central memory CD8 T cells into CD45RA re-expressing memory cells without the requirement for antigen (Dunne et al. 2005;Geginat et al. 2003). Longitudinal fluctuations of IL-15 mRNA have been observed within serial liver biopsies from woodchucks infected with chronic WHV, although they did not correlate with the weak changes in viraemia (Schildgen et al. 2006). One may speculate therefore, that dysregulated production or responsiveness to homeostatic cytokines may alter both the survival capacity of memory cells and possibly the differentiation status of CD8 T cells within the liver. In addition to cytokines, large quantities of viral antigens including the HBeAg of HBV, may have an immunotolerant role (Milich et al. 1990).

5.0.5 Telomeres, Telomerase and Replicative Senescence

Hayflick et al first showed in 1965 that somatic cells had a finite replicative capacity, demonstrated by their observations that fibroblasts cultured in vitro divided only a limited number of times (Hayflick 1965). This 'mitotic clock' is attributable to telomere length in cells.

In mammalian cells, telomeres are made up of hexanucleotide TTAGGG repeats that cap the ends of chromosomes and protect from DNA damage. With each cell division, 50-200 bases of terminal DNA is lost due to the inability of DNA polymerase to replicate the terminal portion (Hodes et al. 2002). In accordance with this, telomere length was found to be shorter in memory compared to naïve CD8 T cells (Weng et al. 1995). Shortening of telomeres continues with divisions until a critical length is reached, (<4kbases) at which point the cell reaches an end stage phenotype known as replicative senescence associated with poor functional capacity and impaired proliferation analogous to functionally exhausted cells described by Wherry et al. Indeed, studies have shown that telomere length is shortest in highly differentiated cells and is associated with an inability of these cells to divide (Akbar & Fletcher 2005;Plunkett et al. 2007).

However, progression to replicative senescence can be postponed by the enzyme telomerase which has the capability to add back telomeric repeats onto the chromosome ends. Telomerase is a ribonucleoprotein enzyme made up of a catalytic reverse transcriptase subunit and an RNA template which it uses to synthesize telomeric repeats (Akbar & Vukmanovic-Stejic 2007). Resting lymphocytes have a low baseline telomerase activity (Satra et al. 2005), however this can be upregulated on stimulation. This capability to upregulate telomerase expression may be crucial during an acute anti-viral response. For instance, during the expansion phase of acute infectious mononucleosis, there are a series of multiple large clonal expansions of antigen specific CD8 T cells (Callan et al. 1996), with up to 28 population doublings. This rapid number of divisions over a condensed timeframe would normally be expected to result in telomere shortening and predispose to replicative senescence. However, in this setting, Maini et al. showed that despite exhibiting a CD45RO+CD28- memory phenotype, there was a preservation of telomere length (TRF length) associated with a compensatory increase in telomerase activity in CD8 T cells but not CD4 T cells. Preservation of telomere length in this case may be required to generate a population of long lived memory cells. Interestingly however, in five controls and five

patients with resolved infection, there was no significant difference in TRF length between CD8 and CD4 T cells. Furthermore on follow up 1-3 months post AIM infection, there was rapid telomere shortening within CD8 T cells to the extent that no difference in TRF length between CD8 and CD4 T cells were observed (Maini et al. 1999b). These findings collectively suggested attenuation of telomerase activity over time in chronic infection. This was supported by in vitro studies by Valenzuela et al. who demonstrated significant upregulation of telomerase activity in CD4 and CD8 T cells upon primary stimulation with antigen, however on repetitive stimulation, the ability of CD8 T cells to upregulate telomerase was lost. These data have also been shown in vivo in X-linked lymphoproliferative disease (XLP) and during HIV infection. Young children with XLP displayed short CD8 T cell telomere lengths comparable to those observed in the elderly population. This premature shortening of telomere length was driven by excessive stimulation of these cells (Plunkett et al. 2005). In HIV infection, in which there is persistent infection of high viraemia, Effros et al. showed that there was an expanded CD28-CD8+ population associated with reduced proliferative capacity which had TRF lengths 5-7kb shorter than CD28+CD8+ T cells from the same patient. These TRF lengths were analogous to those in PBMC from uninfected centenarians (Effros et al. 1996). Similarly Palmer et al. showed that TRF length was shorter in CD8 T cells from HIV infected patients compared to uninfected individuals (Palmer et al. 1997) and Franzese et al showed reduced telomerase activity in CD4 T cells infected with HIV-1 (Franzese et al. 2007). Upon transduction of HIV-specific CD8 T cells with hTERT, the catalytic subunit of telomerase, there was prolonged secretion of IFNγ and TNF by these cells, a delay in the loss of CD28, delayed accumulation of cell cycle inhibitors and increased longevity of these cells (Dagarag et al. 2004). In addition, Herpesviruses EBV and CMV, which develop latent and smoldering infections respectively, have also been shown to drive immunosenescence, and in elderly individuals, a large accumulation of effector CMV specific cells have been associated with an immune risk phenotype (Akbar & Fletcher 2005).

Figure 5e. Progressive loss of telomeres leads to end stage replicative senescence. The enzyme telomerase can 'rescue' telomeres by addition of telomeric repeats onto chromosome ends.

These data collectively suggest that upon acute infection, short term exposure to antigen allows for clonal expansion of antigen-specific cells, associated with telomerase upregulation and maintenance of telomere lengths (in the case of AIM infection), and formation of a memory population upon disease resolution. However, on persistent stimulation, CD8 T cells may be driven prematurely towards a terminally differentiated phenotype associated with shortened telomeres and inability to upregulate telomerase. This 'end stage' phenotype associated with replicative exhaustion and functional impairment could hinder the virus-specific response.

5.0.6 Aims

In this study we investigate whether chronic infection with HBV could result in premature immunosenescence of CD8 T cells, and partially account for the functional defects observed within this compartment.

Results

5.1 Enrichment of CD27 negative effector memory and revertant CD8 T cells in patients with CHB

To determine whether functional impairment of CD8 T cells could partially be attributable to premature ageing within this compartment, PBMC were isolated from patients and healthy donors and CD8 T cells were phenotyped ex vivo. In confirmation of previous work, we observed that CD8 T cells could be subdivided into four phenotypically distinct subsets dependent on expression of CD27 and CD45RA (Hamann et al. 1997), figure 5.1.1. The proportions of these CD8 T cell subsets were then compared between 20 healthy donors, 19 patients with low viral load and 17 patients with high viral load (table 5.1) and representative dot plots are shown for each group in figure 5.1.2a. On cross-sectional analysis, there was a trend towards a progressive decline in naïve CD8 T cells with increasing viral load. Patients with high viral load had the lowest proportion of this subset (35±20.1) compared to those with low viral load (51±14.5) ($p<0.05$) and healthy donors (60.8±19.5) ($p<0.001$). No significant difference in the proportion of central memory CD8 T cells were observed between any group. Conversely, effector memory and revertant CD8 T cells were both enriched in patients with high viral load compared to controls ($p<0.01$, $p<0.001$) (fig. 5.1.2b). Interestingly, revertant but not effector memory CD8 T cells were enriched in patients with low viral load compared to controls. Based on this observation one could speculate that at a lower threshold of viral load, naïve CD8 T cells may preferentially be induced to become revertant over effector cells. Indeed, previous studies have shown that central memory CD8 T cells in the presence of IL-15 could differentiate directly into revertant CD8 T cells in the absence of antigen (Geginat et al. 2003). However, an alternative scenario is that this discrepancy may partially be attributable to other variables known to influence the phenotypic repertoire of CD8 T cells, such as age and CMV/EBV seropositivity of the patients, or viral factors such as HBeAg status and liver inflammation.

Subject	HBV DNA (IU/ml)	ALT (IU/L)	HBeAg	AGE	SEX	CMV	Zeta MFI done
HD 1	N/A	Normal	N/A	34	M	neg	
HD 2	N/A	Normal	N/A	34	M	neg	yes
HD 3	N/A	Normal	N/A	34	F	neg	yes
HD 4	N/A	Normal	N/A	23	M	pos	yes
HD 5	N/A	Normal	N/A	24	F	pos	
HD 6	N/A	Normal	N/A	34	M	pos	yes
HD 7	N/A	Normal	N/A	34	F	pos	
HD 8	N/A	Normal	N/A	22	F	not tested	yes
HD 9	N/A	Normal	N/A	22	F	not tested	
HD 10	N/A	Normal	N/A	22	M	not tested	
HD 11	N/A	normal	N/A	23	M	not tested	
HD 12	N/A	normal	N/A	24	M	not tested	yes
HD 13	N/A	normal	N/A	24	M	not tested	
HD 14	N/A	normal	N/A	24	M	not tested	yes
HD 15	N/A	normal	N/A	24	F	not tested	
HD 16	N/A	normal	N/A	32	F	not tested	yes
HD 17	N/A	normal	N/A	32	F	not tested	
HD 18	N/A	normal	N/A	33	F	not tested	yes
HD 19	N/A	normal	N/A	33	?	not tested	yes
HD 20	N/A	normal	N/A	36	?	not tested	yes
CHB 7	1400	30	neg	21	M	not tested	
CHB 9	4100	42	neg	45	?	not tested	
CHB 13	72172	405	neg	46	M	not tested	
CHB 61	?	?	?	54	?	not tested	yes
CHB 62	?	?	?	64	?	not tested	yes
CHB 63	420000	270	pos	25	M	pos	yes
CHB 4 '	440	23	meg	29	M	pos	
CHB 59	blq	32	neg	33	F	pos	
CHB 2	blq	57	neg	38	M	pos	yes
CHB 64	blq	25	neg	43	M	pos	
CHB 14	170000	56	neg	48	F	pos	yes
CHB 65	570	23	neg	49	M	pos	
CHB 5	850	22	neg	25	F	not tested	
CHB 66	140000	31	pos	27	M	not tested	
CHB 12	40000	24	Neg	31	F	not tested	
CHB 8	1400	56	Neg	32	M	not tested	
CHB 53	blq	18	Neg	35	M	not tested	
CHB 67	510	17	Neg	40	F	not tested	
CHB 68	1300	29	Neg	50	F	not tested	
CHB 69	15000000	88	Pos	25	M	not tested	
CHB 70	high *		Pos	25	F	not tested	yes
CHB 71	60000000	35	Pos	34	M	not tested	

CHB 57	330000000	39	Pos	34	M	not tested	
CHB 20	1127317	189	Neg	42	F	not tested	
CHB 72	high *	high*	Pos	45	F	not tested	yes
CHB 30 '	260000000	196	Neg	51	F	not tested	yes
CHB 21	1400000	58	Pos	62	M	not tested	
CHB 33 '	11000000	62	Pos	24	F	pos	yes
CHB 26 '	23000000	322	Pos	26	F	pos	
CHB 28	110000000	24	Pos	31	F	pos	
CHB 34 '	290000000	418	Pos	36	M	pos	yes
CHB 32 '	120000000	76	Pos	40	M	pos	yes
CHB 27	67000000	96	Pos	40	M	pos	yes
CHB 19	1100000	151	Neg	47	F	pos	
CHB 22	2000000	69	Pos	60	M	pos	yes
CHB 73	1548076	112	Pos	62	F	pos	

Table 5.1 Characteristics of patients and controls used for study of CD8 T cell differentiation. Blq: below quantification. (*) denotes that HBV DNA/ALT levels were not available at the time of sampling, and instead values from the previous clinic visit are shown. A dash (') next to the patient code denotes that this patient has been used in more than one assay, and the sampling timepoint studied, may vary between different experiments. HD = healthy donor.
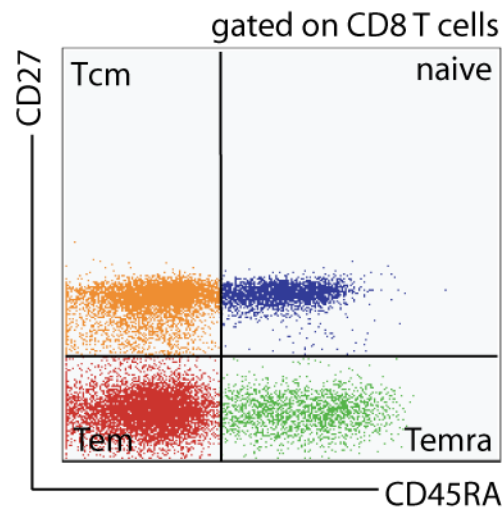
Figure 5.1.1: CD8 T cells can be subgrouped by expression of CD27 and CD45RA. PBMC were isolated from patients and healthy donors and stained for surface markers CD8, CD27 and CD45RA ex vivo. Representative dot plot from a patient with high viral load shows the distribution of CD8 T cells into four distinct groups; naïve (CD27+CD45RA+), central memory (Tcm, CD27+CD45RA-), effector memory (Tem, CD27-CD45RA-) and revertant (Temra, CD27-CD45RA+).
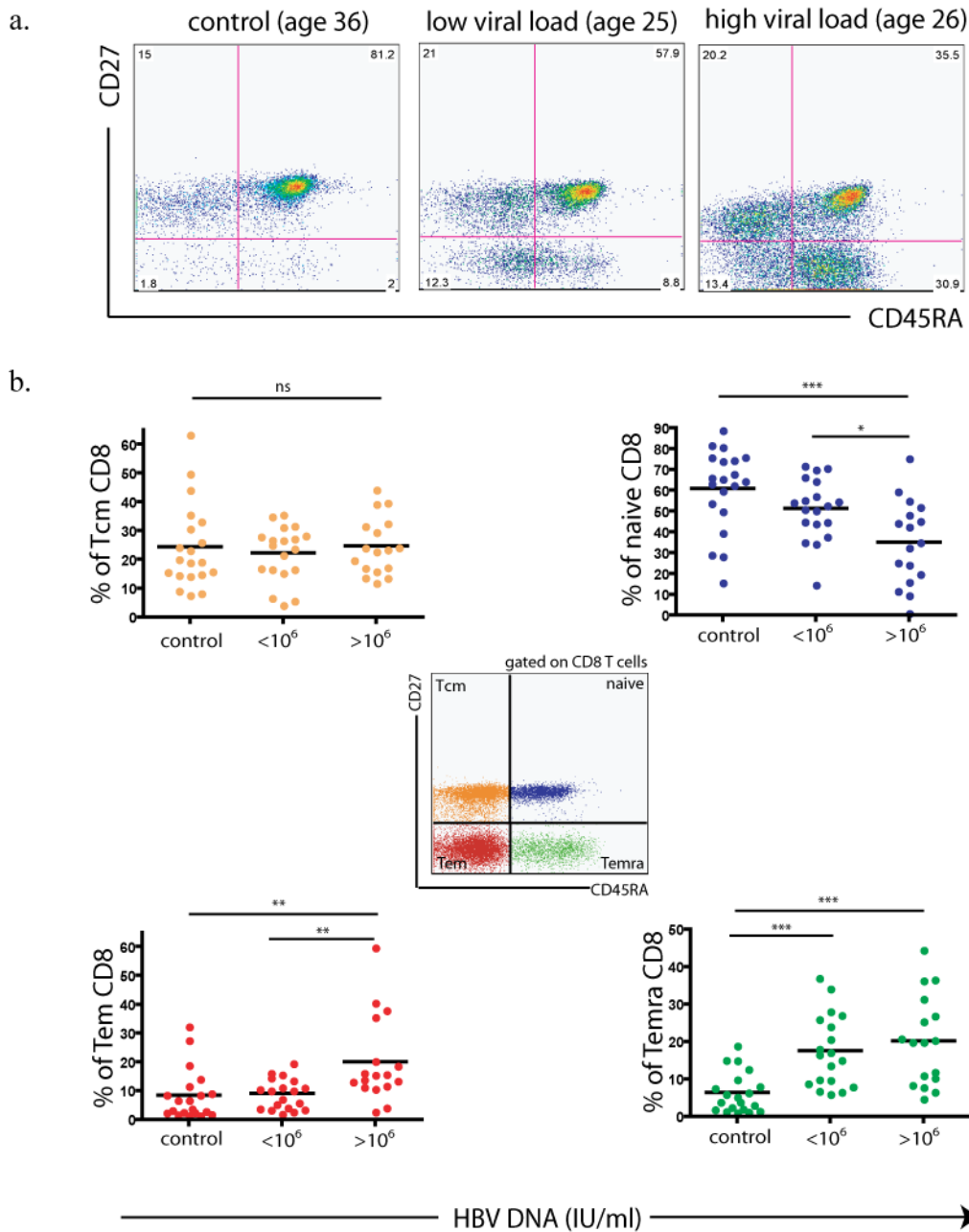
Figure 5.1.2: Effector memory and revertant CD8 T cells are enriched in patients with CHB. a) Representative dot plots from an age matched control, patient with low viral load ($<10^6$ IU/ml) and patient with high viral load ($>10^6$IU/ml) depict the subdivision of CD8 T cells into subsets dependent on expression of surface markers CD27 and CD45RA. Cross sectional data in (b) compares the proportions of each subset against viral load in 20 healthy donors, 19 patients with low viral load and 17 patients with high viral load. (*=p<0.05, **=p<0.01, ***=p<0.001, non-parametric Mann-Whitney U test).

5.2 Enrichment of CD27 negative CD8 T cells in age and CMV matched individuals

Increasing age and CMV status are known to drive CD8 T cell differentiation towards a terminally differentiated phenotype. To confirm that the accumulation of highly differentiated CD8 T cells in patients with high viral load was a consequence of HBV infection and not simply due to differences in age/CMV status between different patient groups, data were re-analysed in either young age-matched individuals (figure 5.2a) or only CMV seropositive individuals under the age of 50 years (figure 5.2b). From these data, we showed that even within young individuals, patients with highest viral load had the lowest proportion of naïve CD8 T cells, and an accumulation of both effector and revertant CD8 T cells. Similarly, in CMV positive individuals, there was an incremental increase in the proportion of revertant CD8 T cells with viral load and those individuals with highest viral load conversely had the lowest proportion of naïve CD8 T cells.
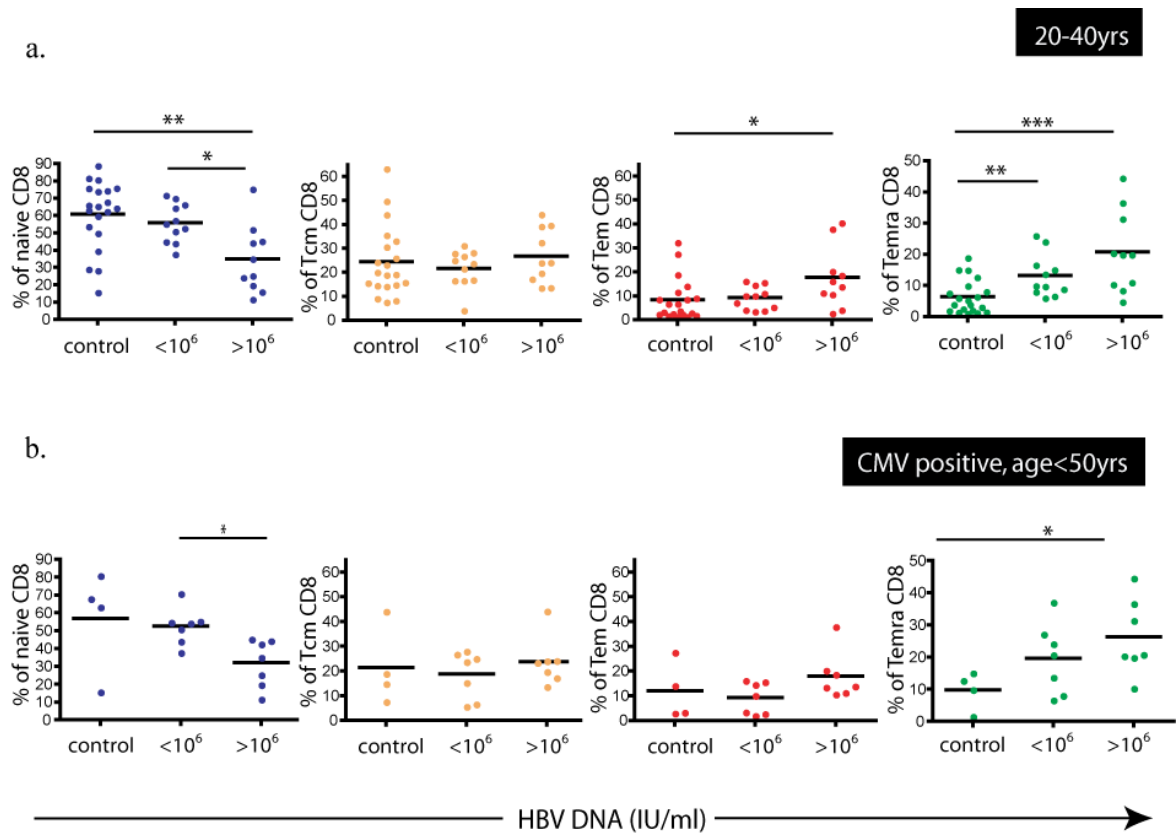
Figure 5.2: Enrichment of CD27- negative CD8 in age and CMV matched individuals. a) Graphs show the proportion of each CD8 T cell subset against viral load in age matched individuals (20-40years) and only CMV seropositive individuals under the age of 50 in (b). (*=p<0.05, **=p<0.01, ***=p<0.001, non-parametric Mann-Whitney U test).

5.3 Progressive downregulation of CD3ζ with differentiation

In the previous chapter we had observed that CD3ζ downregulation was greatest in patients with high viral load, in whom we have now shown to have an accumulation of highly differentiated CD8 T cells. Based on this correlative finding, we hypothesized that downregulation of CD3ζ could be a contributory mechanism for the functional exhaustion seen in these terminally differentiated cells. To test this hypothesis, PBMC were isolated from healthy donors and patients, surface stained for markers CD8, CD27 and CD45RA, then intracellularly stained for CD3ζ. As shown in figure 5.3a, following separation of CD8 T cell subsets by CD27 and CD45RA expression, the mean fluorescence intensity of CD3ζ expression was then compared between the 4 subsets. We observed in almost all patients, that MFI of CD3ζ expression was reduced in CD27 negative subsets, compared to CD27 positive CD8 T cell subsets from the same individual. In some cases, there was a progressive reduction in CD3ζ expression as cell progressed from naïve→central memory→effector memory→revertant, especially in patients with low viral load (fig 5.3b). Progressive loss of CD3ζ MFI was not observed in healthy donor CD8 T cells, although a subtle decrease in CD3ζ MFI was observed in the Tem subset compared to naïve CD8 T cells in some patients. These data suggest therefore that highly differentiated CD27 negative CD8 T cells from patients with CHB have lower CD3ζ. This may reduce their ability to respond to stimuli through the T cell receptor complex and partially explain why they are characteristically less able to produce IL-2 and proliferate than CD27 positive CD8 T cells. Furthermore, only revertant CD8 T cells from patients with CHB, but not healthy donors, downregulated CD3ζ, suggesting that accumulation of differentiated subsets is not in itself enough to account for the discrepancy in global CD8 T cell CD3ζ expression between patients and controls.
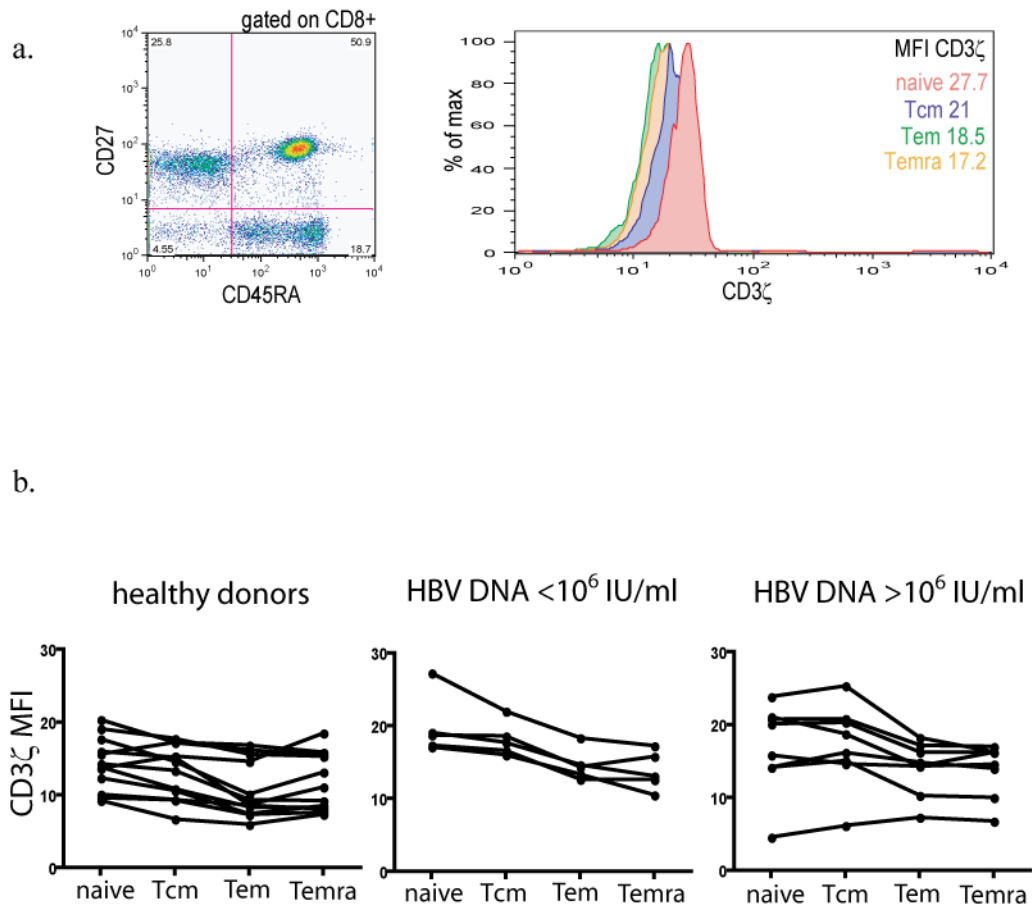
Figure 5.3: Progressive downregulation of CD3ζ with differentiation in CD8 T cells from patients with CHB. PBMC were surface stained for CD8, CD27 and CD45RA, and intracellularly stained for CD3ζ. a) Representative dot plot shows the distribution of CD8 T cell subsets in a typical low level carrier (left) and the mean fluorescence intensity of CD3ζ expression for each subset is shown on a histogram (right). (red=CD45RA+CD27+, blue=CD45RA-CD27+, green=CD45RA-CD27-, orange=CD45RA+CD27-). b) Graphs depict the difference in CD3ζ MFI between CD8 T cell subsets in healthy donors, patients with low viral load (HBV DNA $<10^6$IU/ml) and patients with high viral load (HBV DNA $>10^6$IU/ml).

5.4 Global CD8 T cells from patients with chronic HBV have shorter telomeres

Telomere length is shortened in highly differentiated CD8 T cells, and is associated with reduced replicative ability. To determine whether global CD8 T cells from patients with CHB had shorter telomeres, PBMC were surface stained for CD27, and telomere length was determined by a flow based fluorescence in situ hybridisation assay (flow FISH). Briefly, telomere specific FITC-labelled PNA probe was added to the cell suspension, and heated to $82^{\circ}C$ in order to allow denaturation of DNA and subsequent hybridisation of the fluorescent probe onto the telomeric ends. The level of fluorescence detected on the flow cytometer could therefore be used as a quantitative measure of the telomere length on a per cell basis. As shown in figure 5.4a, telomere length was determined both within global CD8 T cells, as well as separately within CD27 negative and CD27 positive fractions. From the cross-sectional analysis, we observed a trend towards a decrease in telomere length in the global CD8 T cells with increasing viral load, with shortest telomere length in CD8 T cells from patients with high viral load ($p<0.05$) (patient details; table 5.2). Previous data from the group of Professor Akbar have determined telomere length by flow fish in CD8 T cells obtained from young, middle aged and elderly individuals (unpublished data). In their study, the range of telomere MFI ranged from 176 in cord blood CD8 T cells, to 82 in CD8 T cells from an elderly individual (age 63). We observed a mean drop in telomere MFI from 150 (controls) to 135 (high viral load), corresponding to shortening of TRF lengths from 9.1 to 8 kilobases (TRF length was previously determined by southern blotting and plotted on a standard curve against MFI values obtained by flow FISH). This was a biologically significant reduction and roughly equated to premature ageing of the immune system by up to 10 years. Furthermore, in the one individual who showed a telomere MFI less than 100 (98.25), HBV DNA was in excess of 1×10^{8} IU/ml at this timepoint. This trend was maintained in the CD27 negative fraction, however, there was no significant alteration of telomere length within the CD27 positive fraction. For all individuals, telomere length was shorter by at least an MFI of 20 in the CD27 negative fraction compared to the CD27 positive fraction with a mean difference of 31.27. Therefore, not only was there an accumulation of CD27 negative CD8 T cells in chronic HBV infection, but these CD8 T cells also had shorter telomeres than the equivalent subset in healthy donors.

These initial data suggest that premature senescence of CD8 T cells may be secondary to chronic HBV infection, however are still preliminary in nature due to the limited sample sizes. In order to confirm these preliminary findings and also dissect out the relationship between telomere length and viral load/liver inflammation, it would be important in future experiments to:

1. Increase the sample size within the healthy donor and patient cohorts
2. Determine telomere length longitudinally in patients in whom viral load and liver inflammation are fluctuating temporally.

Name	HBVDNA (IU/ml)	HBeAG	ALT (IU/L)	age	Sex
HD 12	N/A	N/A	Normal	23	M
HD 6	N/A	N/A	Normal	32	M
HD 2	N/A	N/A	Normal	28	F
HD 1	N/A	N/A	Normal	32	M
HD 3	N/A	N/A	Normal	32	F
HD 16	N/A	N/A	Normal	32	F
HD 21	N/A	N/A	Normal	28	M
HD 7	N/A	N/A	Normal	32	F
HD 18	N/A	N/A	Normal	33	F
CHB 59	blq	Neg	32	27	F
CHB 58	160	Neg	39	36	M
CHB 74	380	Neg	29	34	F
CHB 75	460	Neg	58	33	M
CHB 5	850	Neg	22	25	F
CHB 7	1400	Neg	30	23	M
CHB 8	1400	Neg	56	32	M
CHB 12	40000	Neg	24	30	F
CHB 66	140000	Pos	31	27	M
CHB 63	420000	Pos	270	24	M
CHB 19 '	1400000	Neg	62	47	F
CHB 31 '	20000000	Pos	83	23	M
CHB 71	60000000	Pos	35	34	M
CHB 33 '	78000000	Pos	103	24	F
CHB 28	110000000	Pos	24	30	F
CHB 27 '	150000000	Pos	63	40	M
CHB 57	330000000	Pos	39	35	M
CHB 32 '	Not done	Pos	73	40	M

Table 5.2 Clinical characteristics of patients and healthy donors used for determination of telomere length by Flow FISH. HD = healthy donor.
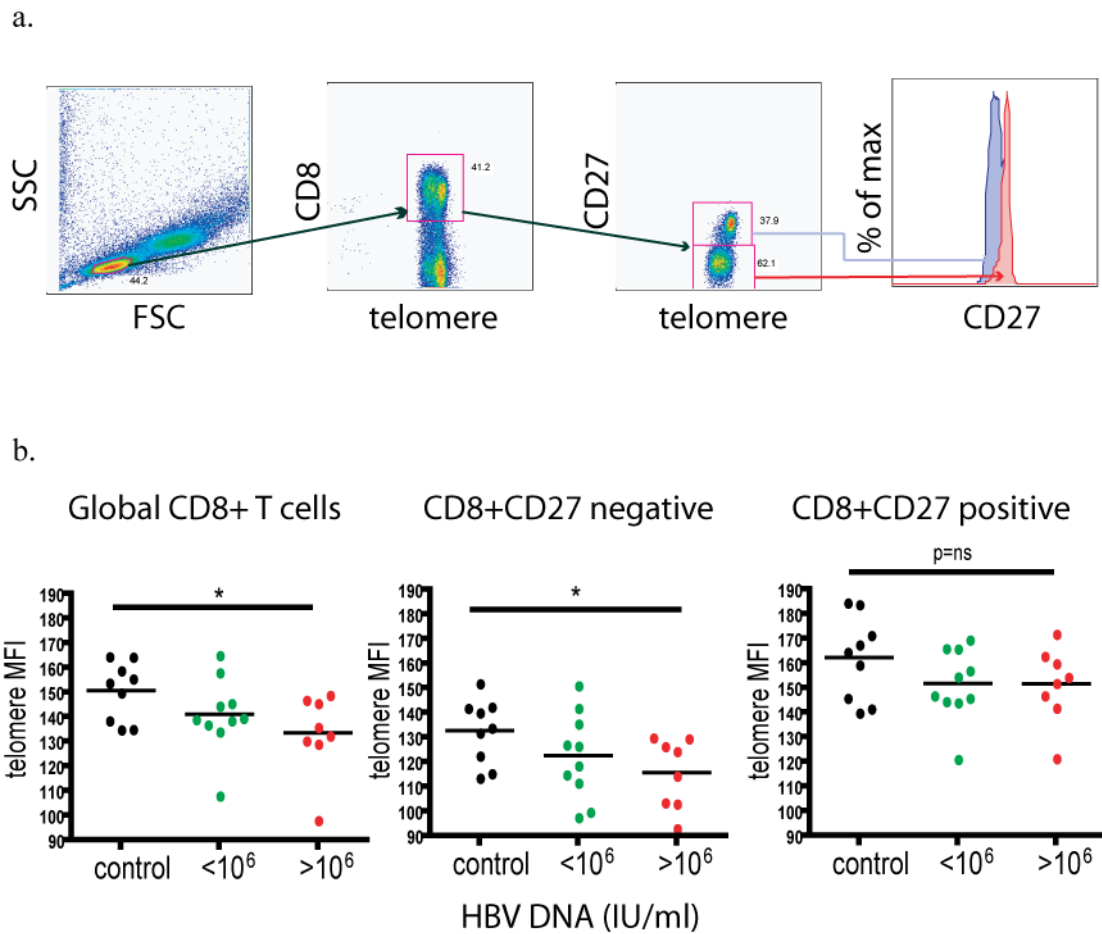
Figure 5.4 Global CD8 T cells from patients with CHB have shorter telomeres. Telomere length in PBMC was determined ex vivo by flow FISH. a) flow cytometric plots depict the gates used to analyse telomere MFI on either global CD8 T cells, or within either CD8+CD27+ or CD8+CD27- T cells. b) Cross-sectional data shows telomere MFI vs. viral load for global CD8 T cells, and CD8+CD27+ and CD8+CD27- cells. (*=p<0.05, non-parametric Mann-Whitney U test).

5.5 HBV-specific CD8 T cells have the shortest telomeres

To determine whether HBV-specific CD8 T cells were more susceptible to telomere loss than CD8 T cells of other viral specificities, PBMC from HLA-A2 positive donors were stimulated with viral peptides representing immunodominant epitopes of CMV, EBV and HBV for 10 days, following which telomere length was determined by flow FISH. Dot plots in figure 5.5.1 illustrate the magnitude of virus-specific CD8 T cell responses as determined by IFN-γ production in two patients with low viral load. After gating on the virus specific populations, the telomere MFI was then compared between different CD8 T cell specificities in the same individual. As shown in the right hand histograms, telomere MFI was significantly lower in HBV-specific CD8 T cells compared to EBV-specific CD8 T cells in patient 1 and CMV-specific CD8 T cells in patient 2. In both cases, telomere length in HBV-specific CD8 T cells was subtly also shorter compared to the global CD8 T cell population (Patient 1 global=154, C18=149), (Patient 2 global=158, C18=153). One caveat to this finding, however, was that MFI of telomere length in these experiments was at a higher range than that seen ex vivo. For example, CMV specific CD8 T cells in patient 1 had an MFI of 175, which is similar to that seen in cord blood T cells.

Therefore, to rule out artefacts associated with tissue culture, these experiments were repeated ex vivo in a patient with high viral load. As shown in figure 5.5.2, we observed that the telomere MFI was significantly shorter in HBV-specific CD8 T cells compared to EBV or CMV-specific CD8 T cells from the same individual (5.5.2 middle panel). Since there was slight variation in the global CD8 MFI between individual tubes, samples were run in triplicate to reduce variability and a mean of the triplicates was taken (figure 5.5.2 lower panel). On this analysis, we observed that the telomere MFI in global CD8 T cells was highly consistent between samples. The MFI of EBV and CMV-specific cells was similar to the global population or slightly elevated respectively. Only HBV-specific cells consistently had a lower telomere MFI compared to the global CD8 T cells, with a mean reduction of 13 (global MFI 115, HBV specific MFI 102). This again would equate to premature ageing of this compartment by 10 years and may partially explain the marked quantitative and qualitative defects observed in this compartment. These preliminary data suggest that HBV-specific CD8 T cells may have even shorter telomeres than global CD8 T cells and those of other viral specificities, however further ex vivo experiments are required to explore this finding.

One finding of note in this study is that it is extremely unusual to detect HBV-specific CD8 T cells ex vivo, especially from patients with high viral load in whom there are barely detectable circulating frequencies. Despite this, we were able to detect an ex vivo response towards the HBV c18-27 epitope in one high level carrier (patient CHB 34). In support of this data, Boni et al have shown that following stimulation with a pool of overlapping peptides covering the whole HBV genome, mainly responses to core and polymerase but not to envelope were detected (Boni et al. 2007). This suggested that CD8 T cells specific to different epitopes may have differential susceptibility to a hierarchical loss of T cell effector function and exhaustion. Envelope specific CD8 T cells may have undergone deeper exhaustion due to exposure to large frequencies of circulating HBeAg. It is feasible therefore, that comparison of telomere length between CD8 T cells specific for different HBV epitopes may further reveal differential telomere erosion; this is a subject of future study.
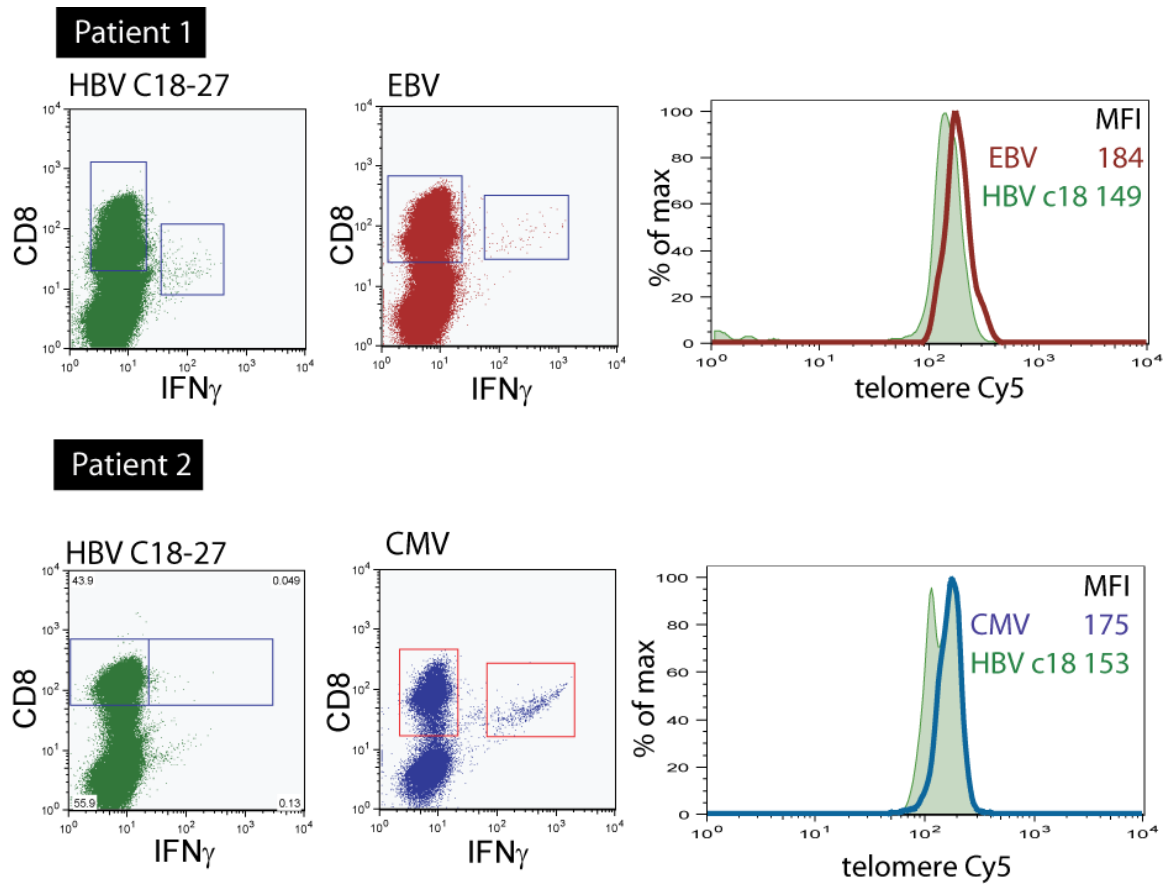
Figure 5.5.1 HBV-specific CD8 T cells have the shortest telomeres. PBMC from two patients with CHB were stimulated with HLA-A2 restricted viral peptides representing immunodominant epitopes on HBV, CMV and EBV and virus-specific responses were allowed to expand for 10 days. Telomere length within virus-specific CD8 T cells was subsequently determined by flow FISH.

	Sample 1 Median	Sample 2 Median	Sample 3 Median	Mean of triplicate
Global CD8	(120)	(113)	(109)	(114)
EBV+ CD8	117	111	108	112
Global CD8	(119)	(114)	(112)	(115)
HBV+ CD8	104	102	99.8	102
Global CD8	(119)	(117)	(115)	(117)
CMV+ CD8	131	124	124	126.3
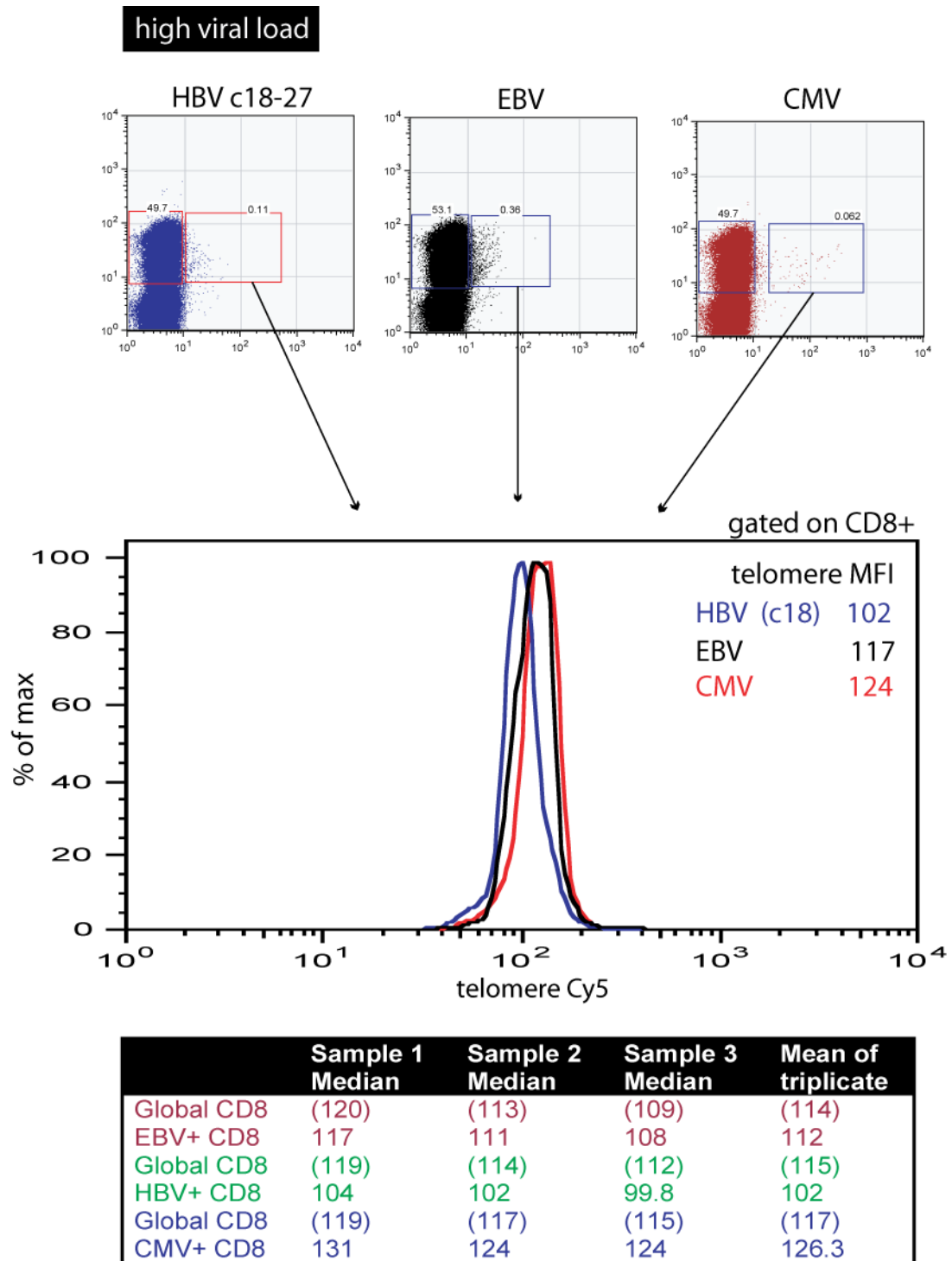
Figure 5.5.2 HBV-specific CD8 T cells ex vivo display shortest telomere length.

5.6 Determination of telomerase activity in CD8 T cells in patients and controls

Telomerase is a ribonucleoprotein enzyme that can compensate for progressive telomere loss, and can delay progression to replicative senescence. CD8 T cells can upregulate telomerase activity upon activation, and a compensatory increase has been associated with preservation of telomere length in EBV-specific CD8 T cells during acute infectious mononucleosis (Maini et al. 1999b). To determine whether loss of telomere length during CHB is secondary to aberrations in telomerase activity, CD8 T cells were negatively selected, pelleted and lysed, and cell extract was obtained to determine telomerase activity.

This was done by a gel based TRAPeze system, a modification of the PCR based TRAP (telomeric repeat amplification protocol) assay, and involved a two step process in which telomerase added telomeric repeats onto the 3' end of a substrate oligonucleotide (TS), following which the TS-telomerase product was amplified by PCR. This generated a ladder of products with 6 base increments as shown in 5.6b. Total product generated (TPG) was then used as a quantitative measure of telomerase activity. A representative dot plot in 5.6a shows the purity of CD8 T cells upon negative selection (91%) in a patient with high viral load. Standardisation of this assay required the absolute number of CD8 T cells, which was enumerated using Trucount beads, and information on the stage of cell cycle for each cell, which was determined by Ki67 staining (which identifies cells in all phases of the cell cycle). Telomerase activity could be corrected per 500 cycling cells- and thus direct comparison of telomerase activity within cycling cells could be done between CD8 T cell extracts isolated from patients and healthy donors (Plunkett et al. 2007;Reed et al. 2004). As we detected virtually no telomerase activity in our ex vivo PBMC samples (fig 5.6), in this assay, CD8 T cells were additionally stimulated with anti-CD3 and irradiated APC for 3 days to initiate proliferation and induce telomerase upregulation. Two separate gels depicting telomerase activity before and after stimulation in six patients with CHB, and one healthy donor are shown in 5.6b. Comparison of data between gels was difficult due to much higher background values in one gel compared to the other. However, comparison of TPG content between ex vivo (day 0) and stimulated (day 3) was possible, and revealed paradoxically that there was a downregulation of telomerase activity upon stimulation in all cases 5.6c. This may potentially have been an artefact of the assay however. The number of Ki67+ cells was used to calculate how much each sample should be diluted in CHAPS lysis buffer. This meant that stimulated samples, which had a large number of

cycling cells, were much more diluted than unstimulated samples which had <1% Ki67+ cells (data not shown). However, because the volume of CHAPS lysis buffer was much lower for the unstimulated sample, the concentration of cells was higher. Although resting cells have been reported to have undetectable telomerase, in reality small baseline levels may be present (Dr. Franzese, personal communication). Therefore, the higher concentration of cells used for the ex vivo samples could partially explain the bias towards higher telomerase activity in this sample. In future experiments, direct comparison of telomerase activity between stimulated and unstimulated samples without correction for cycling may provide a more accurate comparison.

Subject	HBV DNA (IU/ml)	ALT (IU/L)	HBeAg	AGE	SEX	CMV
CHB 24 '	12000000	73	pos	38	M	Pos
HD 11	N/A	Normal	N/A	23	M	not tested
CHB 30 '	260000000	196	neg	51	F	not tested
CHB 34 '	290000000	418	pos	36	M	Pos
CHB 27	67000000	96	pos	40	M	Pos
CHB 28	110000000	24	pos	31	F	Pos
CHB 54 '	Blq	46	neg	55	M	not tested

Table 5.3. Characteristics of patients used for detection of telomerase by gel based TRAPeze assay. Blq: below level of quantification.

SAMPLE	R1 Lymphs	R2 Beads	R4 CD3	Bead #	TC Vol	Tot Vol frozen	CD3 #	Lymph #	% ki67 CD3	#ki67 CD3	VOL CHAPS	per 500
hd	6208	3782	5560	47923	100	100	70452.63882	78663.66579	25	17613.1597	352.2631941	176.131597
CHB 54	1305	2363	1201	47923	100	100	24356.97122	26466.15108	24	5845.673094	116.9134619	58.45673094
CHB 28	5497	4893	4787	47923	100	100	46884.81525	53838.69426	36	16878.53349	337.5706698	168.7853349
CHB 27	5788	4607	4725	47923	100	100	49150.46125	60208.01476	53	26049.74446	520.9948893	260.4974446
CHB 34	4226	3083	3854	47923	100	100	59907.63607	65690.10639	40.1	24022.96206	480.4592413	240.2296206
CHB 30	2206	7094	1676	47923	100	100	11322.09586	14902.47223	37	4189.175467	83.78350933	41.89175467
CHB 24	7069	3427	6531	47923	100	100	91329.18383	98852.54946	29.8	27216.09678	544.3219357	272.1609678

Figure 5.6: Telomerase activity in patients and controls. PBMC were snap frozen ex vivo, or following stimulation with anti-CD3 and irradiated APC for 3 days. Extracts were then collected and telomerase activity was determined by the gel based TRAPeze assay. a) representative dot plots depict how the number of Trucount beads, total lymphocytes and the percentage of Ki67+ CD8 T cells was determined by flow cytometry. These values were then used to calculate volume of CHAPS lysis buffer (see table above). b) Gels illustrate ladders of telomeric products and summary data is shown in (c).

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5.7 Determination of telomerase activity by PCR ELISA plus kit

A variety of assays are now available for the determination of telomerase activity and each have been reported to have different sensitivities. In addition to the Chemicon TRAPeze assay, we also tested a modified protocol by which the endpoint of telomerase activity could be determined by ELISA rather than with a gel. This assay was less labour intensive, allowed more samples to be run at once reducing inter-assay variability, and did not use radioactivity. Similar to the TRAPeze assay, 0.1 million negatively selected CD8 T cells were snap frozen, and upon thawing, were diluted in different amounts of CHAPS lysis buffer to correct for the number of Ki67+ cycling cells. Lysate was then collected and subjected to a similar PCR based amplification step after which product was detected by ELISA and expressed as relative telomerase activity. Preliminary data shown in fig. 5.7 shows no significant difference between telomerase activity in 4 patients and 5 controls following 5 day in vitro stimulation with anti-CD3 and irradiated antigen presenting cells. Notably, we did not observe an increase in telomerase activity during CHB infection, which we might expect as a compensatory mechanism to counteract the accelerated telomere loss in CD8 T cells from these individuals.

Subject	HBV DNA (IU/ml)	ALT (IU/L)	HBeAg	AGE	SEX	CMV
HD 15	N/A	normal	N/A	24	F	not tested
HD 9	N/A	normal	N/A	22	F	not tested
HD 6	N/A	normal	N/A	34	M	pos
HD 5	N/A	normal	N/A	24	F	pos
CHB 2	blq	57	neg	38	M	pos
CHB 53	blq	18	neg	35	M	not tested
CHB 68	1300	29	neg	50	F	not tested
CHB 60	360	not done	neg	31	M	not tested
CHB 21	1400000	58	pos	62	M	not tested

Table 5.4 Patients used for detection of telomerase by Roche PCR ELISA plus telomerase kit.
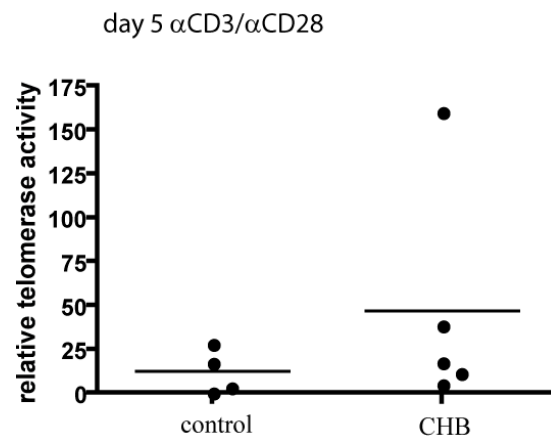
Figure 5.7: Telomerase activity determined by Roche PCR ELISA plus kit.

Discussion

In chapter one, we showed that global CD8 T cells in patients with CHB were impaired in their ability to produce IL-2 and to proliferate, but retained their capacity to produce proinflammatory cytokines. This skewed functional profile is reminiscent of that found in terminally differentiated CD8 T cells (upon PMA/ionomycin stimulation of healthy donor purified CD8 T cells) (Hamann et al. 1997), and CD8 T cells which had been driven to functional 'exhaustion' by persistent high level virus (Wherry et al. 2003a). In this chapter, we investigated the phenotypic properties of virus-specific and global CD8 T cells in patients with CHB in order to determine whether replicative senescence of these cells could partially account for their loss of effector function and proliferation.

We found that in patients with high viral load, there was enrichment in the proportion of effector and revertant CD8 T cells, and a reduction in the proportion of naïve CD8 T cells compared to age-matched healthy donors. Global CD8 T cells from these patients also had the shortest telomeres, most pronounced within the CD27 negative fraction which was enriched in CHB, indicative of their highly differentiated status and susceptibility to end stage replicative senescence. Preliminary experiments showed no difference in the activity of telomerase between healthy donor and patient CD8 T cells.

Chronic HBV infection is characterised by long-term high dose virus persistence. In this setting, persistent activation of HBV-specific CD8 T cells could drive them prematurely towards a terminally differentiated state associated with functional exhaustion. Urbani et al. showed that during acute HBV infection, the majority of virus specific CD8 T cells were of an effector memory phenotype (CCR7-CD45RA-) (Urbani et al. 2002). Whether this phenotype is altered during chronic HBV infection is as yet unclear. The phenotype of virus-specific CD8 T cells is known to vary depending on the virus in question. Appay et al showed that during chronic infection, EBV and HCV-specific CD8 T cells were mainly CD28+CD27+ double positive, whereas CMV-specific CD8 T cells were conversely mainly CD28-CD27- double negative. HIV-specific CD8 T cells had an intermediate phenotype (CD28-CD27+). Thus, depending on the viral infection, there were enrichments of distinct memory populations arrested at different stages along the differentiation pathway (Appay et al. 2002). Similarly, Fletcher et al. showed that after stimulation of CD4 T cells with different viral antigens, only CMV-specific CD4 T cells from young individuals had a significant enrichment of CD27-CD28- cells, however this was not the

case for Varicella zoster virus (VZV), EBV or Herpes simplex virus (HSV) antigen-specific CD4 T cells for which, in most cases, less than 25% of these cells occupied this compartment (Fletcher et al. 2005). These data suggested that the type of viral infection did not necessarily correlate with the differentiation status of the virus-specific T cells. For example, one might expect that virus-specific CD8 T cells would be more differentiated if there was chronic infection with high level viraemia (HCV/HIV), and less differentiated during either latent (EBV) or low level 'smoldering' infection (CMV). Since this is not the case, we cannot use the type of chronic infection established to accurately predict what the phenotype of the majority of HBV-specific CD8 T cells is likely to be, and we can so far only speculate based on the trends observed in the global CD8 T cell population.

The altered memory characteristics of virus-specific CD8 T cells in different infections suggested that factors other than virus load must additionally contribute to driving CD8 T cells towards a terminally differentiated phenotype. In CHB infection, the circulating frequencies of HBV-specific CD8 T cells are barely detectable ex vivo (after screening with overlapping peptides covering the HBV genome), thus the majority of these cells are non-HBV specific (Boni et al. 2007). Since we also see a shift in the phenotype of these global CD8 T cells in those patients with high virus load and liver inflammation, this supports the notion that other factors may be influencing CD8 T cell phenotype and function.

One possibility is that CD8 T cells specific for other viruses, occupy a large amount of the 'immunological space' and may skew the phenotypic profile of the global CD8 T cells (Akbar & Fletcher 2005). For example, large oligoclonal expansions of CMV-specific CD8 T cells accumulate in individuals with age, and are highly differentiated compared to EBV, HCV or HIV-specific CD8 T cells from the same subjects (Appay et al. 2002). We found that, in a sample of 25 patients in whom we tested CMV serology, over 80% of patients were CMV positive (16/19 CMV+), however the proportion of CMV positive healthy donors was much lower (1/6 CMV+). Although it is possible that this variable could skew global CD8 T cell phenotype, after re-analysing the data excluding CMV negative individuals, there was still a significant accumulation of both effector memory and revertant CD8 T cells in patients compared to healthy donors.

As well as competing for immunological space, viruses may exert a pervasive influence on the phenotype of global CD8 T cells. After acute infectious mononucleosis, Sauce et al.

showed that both EBV-specific and global CD8 T cells (as well as CD4 T and NK cells) lost expression of IL-15Rα, and this correlated with impaired responsiveness to IL-15 in vitro. Furthermore, loss of IL-15Rα was maintained for up to 14 years after AIM, however was not observed in healthy EBV carriers or EBV naïve individuals (Sauce et al. 2006). Whether or not HBV infection induces similar changes in global T cell IL-15Rα expression has not been tested. However, co-infection with EBV (in those who have recovered from AIM) could potentially skew the IL-15 responsiveness of global and HBV-specific CD8 T cells. IL-15 is a homeostatic cytokine required for the proliferation and maintenance of memory CD8 T cells. Altered responsiveness to IL-15 could therefore impair the ability of global and HBV-specific CD8 T cells to survive and expand which may further exacerbate quantitative and qualitative defects observed. The dependence of HBV-specific CD8 T cells on IL-15 in vivo remains unclear however, and requires further attention.

Hepatitis C virus has been described to have a pervasive influence on the phenotype of circulating CD8 T cells, as CMV-specific CD8 T cells from these individuals lost markers associated with maturity, and had increased expression of CCR7 (Lucas et al. 2004). Although, in this study, HCV infection paradoxically conferred a less differentiated phenotype onto CMV-specific CD8 T cells, in a study by Golden Mason et al., CMV specific CD8 T cells from patients with chronic HCV had upregulated expression of the inhibitory receptor PD-1, signalling through which is known to suppress CD8 T cell responses (Golden-Mason et al. 2007).

In another study by Fletcher et al., it was shown that in elderly subjects, CD4 T cells of different virus specificities were more differentiated in CMV sero-positive individuals compared to CMV sero-negative individuals. This bystander effect on non-CMV specific CD4 T cells was mediated partially through IFN-α production by CMV stimulated plasmacytoid dendritic cells (pDC). PBMC stimulated with CMV lysate in which pDC had been depleted did not produce IFNα. Additionally, PPD specific CD4 T cells were cultured in vitro in the presence or absence of IFN-α to determine whether it could modulate the phenotype of these cells. By day 21, the proportion of cells with a highly differentiated CD28-CD27- phenotype was 25% compared to 3% in the absence of IFNα- suggesting that IFNα could induce loss of costimulatory receptors on non CMV positive cells (Fletcher et al. 2005).

In patients with chronic HBV infection with high liver inflammation, there is elevated serum IFNα compared to those with low ALT or healthy controls. Furthermore, levels of IFNα correlated temporally with HBeAg negative flares of HBV (Dunn et al. 2007). Loss of CD28 and CD27 in global CD8 T cells observed in those patients with highest viral load and liver inflammation could therefore partially be mediated by IFNα. Another cytokine which has been associated with CD28 loss is TNF-α. In JTag cells (a subline of Jurkat cells which upregulate CD28 upon PMA stimulation), addition of exogenous TNF was able to inhibit PMA mediated upregulation of CD28 by inhibiting the formation of INR specific transcription complexes, and thus silenced CD28 transcription (Lewis et al. 2004). We showed that global CD8 T cells from patients maintained their production of TNF, and this cytokine was also shown to be elevated in the plasma of patients with HBeAg positive chronic HBV compared to healthy donors (Sheron et al. 1991). In addition, IL-15 has been shown to induce generation of CD28 null cells from freshly sorted CD8+CD28+ T cells, partially through induction of TNF. In these experiments, IL-15 induced proliferation resulted in stable downregulation of CD28 over a month, and this was partially reconstituted upon addition of neutralising anti-TNF antibody to block TNF in the culture medium (Chiu et al. 2006). These data may hint as to a possible role in dysregulation of homestatic cytokines in the altered phenotypic profile in CHB infection, however whether IL-15 levels are significantly altered in the intrahepatic cytokine milieu or circulation in these patients is as yet unclear. They do suggest however that TNFα and IFNα, which are both elevated in patients with high viral load and liver inflammation, may play a bystander role in driving antigen independent CD8 T cell differentiation towards a terminally differentiated phenotype in CHB.

Another characteristic of exhausted CD8 T cells is the expression of molecules such as PD-1; an inhibitory receptor of the CD28 family induced on T cells, B cells and myeloid cells upon activation (Greenwald et al. 2005). Its ligand PD-L1, is constitutively expressed on liver antigen presenting cells including LSEC's, Kuppfer cells (Iwai et al. 2003) and Stellate Cells (Chen et al. 2006), and can be upregulated on hepatocytes stimulated with IFNα or IFNγ (Muhlbauer et al. 2006). Barber et al., in a gene profiling study, found PD-1 to be selectively upregulated on functionally impaired-virus specific CD8 T cells from mice infected with chronic LCMV infection compared to functional memory CD8 T cells. Injection of mice with anti PD-1 or anti PD-L1 restored proliferation and cytotoxic

function in exhausted LCMV specific CD8 T cells which were then able to kill infected cells and reduce viral load. This was not seen however when the CTLA-4 signalling pathway was blocked (Barber et al. 2006).

In humans, PD-1 has been shown to be selectively upregulated on virus-specific CD8 T cells during CHB (Boni et al. 2007), HCV (Radziewicz et al. 2007;Urbani et al. 2006) and HIV infection (Day et al. 2006). PD-1 has also been reported to be upregulated on the global CD8 T cell subset in both chronic HCV (Golden-Mason et al. 2007) and HBV infections (Peng et al. 2008b), and thus could potentially mediate a pan CD8 T cell suppression in exhausted CD8 T cells. In order to investigate expression of PD-1 on HBV specific CD8 T cells, Boni et al. used pools of overlapping peptides covering the overall protein sequence of HBV genotype D, to detect HBV-specific CD8 T cell responses ex vivo over the course of chronic flares of HBeAg negative flares of HBV. The responses detected were mainly of core and polymerase specificity, and these cells hyperexpressed PD-1 compared to CMV/FLU virus specific CD8 T cells from the same individuals. Blockade of PD-1/PDL-1 signalling rescued HBV-specific CD8 T cell responses as detected by tetramers (Boni et al. 2007). These data suggest that hyperexpression of PD-1 serves as a molecular signature for exhaustion of HBV-specific CD8 T cells at least, and blockade of PD-1 signalling is partially able to restore functional exhaustion.

The fact that HBV-specific CD8 T cells are under the added pressure of high dose antigenic stimulation through the T cell receptor, which would induce repetitive mitotic cycling, could explain their further propensity to have shorter telomeres and telomerase loss compared to other virus specificities. We measured telomere length in both global and virus-specific CD8 T cells. Our initial preliminary studies showed that with increasing viral load, there was a trend towards a progressive reduction in global CD8 T cell telomere length. A similar global CD8, but not CD4 T cell, telomere erosion had been observed previously in HIV discordant monozygotic twins. In these twin pairs, CD8 T cells from HIV positive individuals consistently had shorter TRF lengths compared to their non HIV infected twins (Palmer et al. 1997). Secondly, we further observed that HBV-specific CD8 T cells (ex vivo (n=1), and after short term culture (n=2)) had shorter telomeres compared to CD8 T cells of other virus specifities from the same individuals. Although these findings are preliminary, one could speculate that further telomere attrition in HBV-specific CD8 T cells could be compatible with their added stimulation by antigen through the TCR.

Telomeric shortening is counteracted by the enzyme telomerase, which can add back telomeric repeats onto chromosome ends and delay progression to replicative senescence. Inability to upregulate telomerase would therefore hasten telomere attrition. Telomerase activity may be influenced by a number of factors. Firstly, Reed et al showed that following purified protein derivative (PPD) injection into the skin, specific CD4 T cells isolated from the site of antigenic challenge at day 19 had significant telomere erosion (400bp-equivalent to 8 years ageing) compared to the CD4 T cells in the blood, and this was partially due to high quantities of IFNα in the tissue fluid which inhibited telomerase activity of PPD specific CD4 T cells both in vivo and in vitro (Reed et al. 2004). Therefore in patients with CHB infection, IFNα induced during HBeAg negative flares of HBV, could account for a non-antigen specific mechanism of telomerase inhibition, and accelerated telomere shortening. Other cytokines such as IL-15 are known to induce stable telomerase expression over long periods of time in memory cells, in this case by signalling through JAK3 and PI3K pathways (Li et al. 2005b), however whether this cytokine is altered during chronic HBV is unclear.

Previous studies have shown that the ability to induce telomerase in CD8 T cells may require a signal from the costimulatory molecule CD28. Consistent with this is the finding that telomere length was shorter in CD28-CD8+ compared to CD28+CD8+ T cells (Monteiro et al. 1996). Plunkett et al. showed that telomere erosion in highly differentiated CD8+CD27-CD28- CD8 T cells was associated with defective AKT phosphorylation (Plunkett et al. 2007). hTERT, the catalytic subunit of telomerase, is a substrate for the AKT kinase, and itself requires phosphorylation at two sites for its activity. Highly differentiated CD8 T cells had a specific defect in phosphorylation of AKT at the Ser 473 site, and were thus unable to upregulate telomerase. Since in patients with high viral load, we have shown that there is an accumulation of CD27 and CD28 negative CD8 T cell fractions in patients with high viral load, this mechanism could contribute to impaired telomerase upregulation in highly differentiated subsets. In support of this, we found that telomere length was shortest within CD27 negative CD8 T cells. Interestingly, PD-1 is known to limit AKT activation by inhibiting CD28 mediated activation of PI3K (Greenwald et al. 2005). Therefore elevated PD-1 on CD8 T cells in HBV may partially contribute also to inhibition of telomerase activity.

Loss of telomere length may not always follow a linear progression, and at some points during the lifespan of a cell, it may occur sporadically as well. For example, telomeres are G-rich sequences, which are highly sensitive to oxidative damage. DNA damage, replication errors and failure to repair properly could provide additional stochastic events which could accelerate the linear loss of telomere length with ageing (Goronzy et al. 2006). Telomere loss has also been observed in other chronic inflammatory disorders including Rheumatoid Arthritis (Koetz et al. 2000), SLE (Honda et al. 2001), Psoriasis (Wu et al. 2000) and Scleroderma. Although this telomere loss may be secondary to chronic antigenic stimulation, Artlett et al. showed that telomere erosion was found in patients with scleroderma, but also in their family members suggesting that telomere loss could be due to genetically imposed chromosome instability (Artlett et al. 1996). In patients with rheumatoid arthritis, telomere shortening was found both in naïve and memory T cells, indicating that chronic antigenic stimulation was not the only mechanism for telomere loss. Finally, Epel et al have provided evidence that chronic emotional stresses can accelerate telomere shortening (Epel et al. 2004). These data provide alternative mechanisms for global T cell telomere attrition, which could add to the heterogeneity of telomere loss within patients and controls.

In this study we have shown that CD8 T cells from patients with chronic HBV infection and high viral load are more differentiated and may have significantly shortened telomeres. Although preliminary experiments have not shown any difference in telomerase activity between patients and controls, sample sizes were small and it will be worth undertaking future studies to determine telomerase activity over the course of HBeAg negative flares of HBV, during which we know factors such as IFNα fluctuate and may influence its activity. In conclusion, these data imply that CD8 T cells from patients with HBV may have a shorter residual lifespan and a greater propensity to replicative senescence, and this could partially contribute to their proliferative defects.

6 The role of IL-10 producing regulatory B cells in the pathogenesis of CHB

Background

In this chapter, we investigate the role of IL-10 in chronic HBV infection in the context of viral control and pathogenesis of disease. This cytokine is well known to be immuno-suppressive and has been shown to modulate T cell responses during bacterial, viral and parasitic infection and autoimmune disease. In the setting of CHB, it is feasible that such a cytokine mediated suppression could contribute to both the virus specific and pan-CD8+ T cell defects we have seen. Thus we investigate the potential impact of IL-10 on the host virus interaction in HBV infection.

6.0.1 Biology of IL-10

IL-10 was first described as CSIF; a cytokine synthesis inhibitory factor produced by Th2 clones upon conA/antigen stimulation, which was able to suppress cytokine production (IL-2, IL-3, TNF, IFN-γ, GM-CSF) by activated Th1 clones but not Th2 clones (Fiorentino et al. 1989). Thus, its reputation as an inhibitory cytokine was established. The mechanism by which it exerts its influence on T cells is thought to be twofold; either a) *directly* through binding to IL-10 receptor on T cells (de Waal et al. 1993) or b) *indirectly* through downmodulation of MHC II (de Waal et al. 1991), B7-1/B7-2 expression (Ding et al. 1993) and chemokine and cytokine production (Berkman et al. 1995;Fiorentino et al. 1991) by monocytes, macrophages and dendritic cells leading to impaired antigen presentation to CD4 T cells. Either way, this leads to a profound suppression of primarily Th1 cytokine production. It should be noted however that IL-10 is pleiotropic in nature, and also serves other functions such as induction of proliferation and survival of B cells (Levy & Brouet 1994;Rousset et al. 1992).

The molecule itself is 178 amino acids long and binds to its receptor, which is a tetrameric structure composed of two IL-10R1 (alpha) chains and two IL-10R2 (beta) chains (Moore et al. 2001). IL-10R1 is expressed at very low levels on lymphocytes and is downregulated upon activation (Liu et al. 1994). It serves as the docking site for IL-10. IL-10R2 is not required for binding but is rather an accessory subunit for signalling. It is expressed constitutively on most cell/tissue types (Moore et al. 2001). On binding of IL-10 to its receptor, kinases JAK-1 and Tyk2 are activated and tyrosine residues on the intra-cytoplasmic domain of Il-10R1 chain become phosphorylated. This serves as a docking

site for STAT-3 which along with STAT-1 and STAT-5 are phosphorylated by the aforementioned JAK kinases. These STAT proteins subsequently form homo/heterodimer structures which translocate to the nucleus and drive transcription of STAT-3 responsive genes like SOCS-1 and SOCS-3 (Pestka et al. 2004).
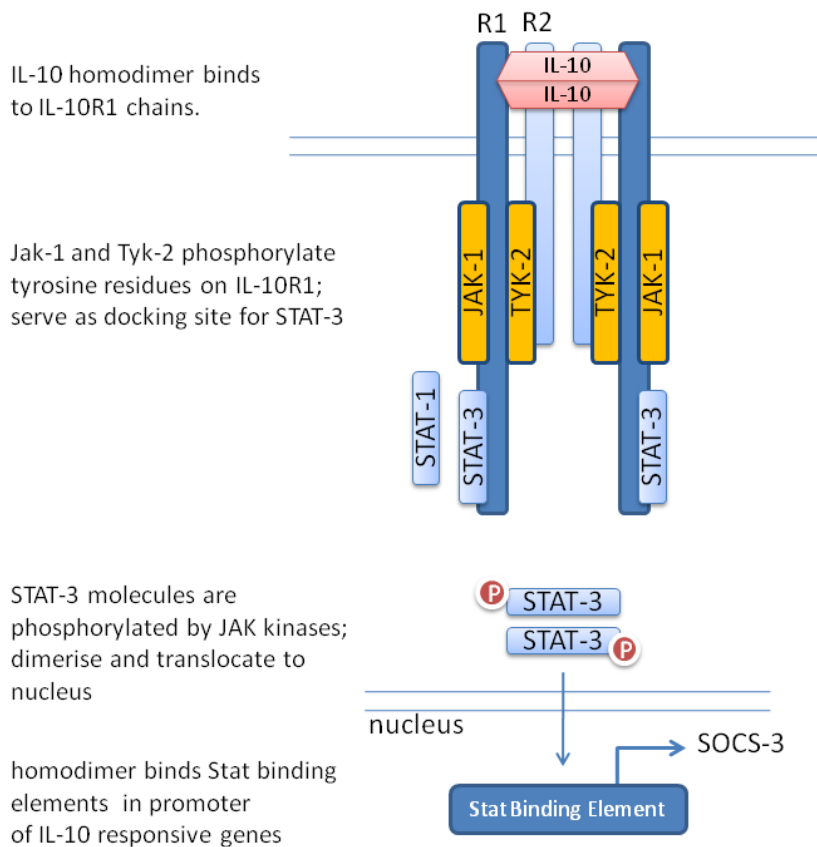
Figure 6a: IL-10 signalling pathway. STAT-3, signal transducer and activator of transcription-3. SOCS-3, suppressor of cytokine signaling-3. Adapted from (Donnelly et al. 1999).

6.0.2 IL-10 in disease

IL-10 has a dual role in chronic infection. On the one hand, during certain bacterial (Kullberg et al. 1998), fungal, viral (Suvas et al. 2004) and parasitic infections (Couper et al. 2008;Gazzinelli et al. 1996;Hunter et al. 1997), IL-10 is required to suppress an over-reactive Th1 response primarily mediated by IFNγ and TNFα. Ablation of IL-10 signaling in this setting results in severe immunopathology. On the other hand, mistimed or excessive IL-10 production has been shown to dampen both innate and adaptive host immune responses against pathogens, and thus promote persistence (Couper et al. 2008). Which of these effects is dominant in any particular chronic infection likely depends on the source and kinetics of IL-10 production in addition to the pathogen involved.

To determine indeed whether the kinetics of IL-10 are important in manipulating the course of chronic infection, previous studies have tried to address whether IL-10 is the cause or consequence of high pathogen burden. However, the data which have been published provide convincing evidence for both sides of the argument, further eluding to the complex pleiotropic actions of IL-10. On the one hand, there is evidence that viruses such as CMV and EBV produce their own viral homologues of IL-10 which have up to 84% sequence homology (for EBV) and can bind the human IL-10 receptor (Kotenko et al. 2000;Moore et al. 1990). In this case, IL-10 may be a viral escape mechanism to dampen the host immune response and promote long-term persistence. On the other hand, IL-10 may be induced secondary to a pathogen insult, as a homeostatic mechanism designed to control an excessive inflammatory response against that pathogen. During infection with *Leishmania major*, high pathogenic load drives an excessive Th1 response characterised by production of IFNγ. This in turn has been shown to induce the production of IL-10 producing adaptive T cells at the site, which act in a positive feedback loop to dampen Th1 responses. Overactivation of IL-10 in this setting, can render Th1 responses ineffective and persistently dampen anti-microbial immune responses, resulting in maintenance of pathogen burden and prevention of lesion healing (Anderson et al. 2005).

These studies therefore have attempted to dissect the functions of IL-10 production at various stages of disease, and have shown that IL-10 cannot only be assigned one function in any particular chronic infection, but rather is involved in striking a fine balance between ameliorating immunopathology and impeding pathogen clearance at the same time.

6.0.3 IL-10 in chronic viral infection

The most compelling evidence to show a role for IL-10 in chronic viral infection has been provided recently by two independent studies in LCMV infection, which have both shown that blockade of the IL-10 receptor is associated with resolution of infection. The mouse model of LCMV is a useful tool in the investigation of chronic infection, primarily because it is possible to reliably induce either chronic or acute infection depending on the strain of virus used, its dose and route of infection. Ejrnaes et al showed that mice infected with the Armstrong Clone strain 13 who developed chronic infection produced drastically higher amounts of IL-10 than those with acute infection. The source of this IL-10 was primarily from CD4 T cells which were thought to be IL-10 primed by CD8alpha negative dendritic cells. Blockade of IL-10R in vivo enhanced antiviral IFN-γ responses, reduced IL-10 secretion and led to resolution of persistent infection with clearance of LCMV in the liver and kidneys by approximately 26 weeks (Ejrnaes et al. 2006). In similar experiments, Brooks et al. showed that therapeutic administration of an IL-10R blocking antibody restored T cell function and resolved established infection. However, as a slight contradiction, they suggested that the antigen presenting cells were the direct source of the elevated IL-10 seen (Brooks et al. 2006). Furthermore, these findings have been mirrored in human studies of HIV (Clerici et al. 1994) and HCV infection in which Rigopoulou et al. showed that blockade of IL-10 receptor in vitro enhanced CD4 proliferative responses to HCV antigens NS3, NS4 and Core (Rigopoulou et al. 2005).

In support of these findings, genetic studies have also provided an insight into the importance of IL-10. In chronic HBV infection, Miyazoe et al. and Cheong et al. have independently shown in Japanese and Korean patient cohorts that polymorphisms of the IL-10 promoter associated with elevated IL-10 production increase disease severity and progression and are associated with viral persistence (Cheong et al. 2006;Miyazoe et al. 2002). This association is not unique to HBV, and such polymorphism resulting in increased IL-10 have also been associated with a negative outcome of HCV infection (Knapp et al. 2003).

The impact of these data provide mixed messages for the development of new therapeutic strategies against IL-10. On the one hand blockade of IL-10 in vivo in mice has shown a very promising effect on disease resolution. However, translation of this finding into the

clinic may prove more difficult. Firstly, as we have learned, IL-10 likely mediates the critical balance between ameriolating immunopathology and impeding pathogen clearance. Blockade of IL-10 therefore may boost anti-viral immune responses, however tip the balance towards a pro-inflammatory state in an otherwise benign infection. Studies by Nelson et al. in chronic HCV infection have shown, for example, that long-term IL-10 therapy reduced ALT and ultimately the incidence of fibrosis, however actually elevated the HCV RNA levels in the patients studied (Nelson et al. 2000;Nelson et al. 2003). Therefore for the success of IL-10 targeted therapies, it will be critical to choose the correct timepoint of drug administration as well as to identify the particular group of patients who would be most responsive to it.

6.0.4 Source of IL-10 in chronic HBV infection

IL-10 is produced by mainly cellular sources including dendritic cells, monocytes, macrophages, T cells, B cells and NK cells (Couper et al. 2008). In this section, we discuss the potential sources of IL-10 in the HBV infected liver which could impact on the course of disease.

6.0.4a Liver resident macrophages and endothelial cells

The liver is an immunotolerant environment in which there is thought to be a constitutive suppression of immune response against gut derived bacterial antigens. In a recent study by Tu et al., it was shown that this suppression may be mediated by IL-10 produced by liver resident macrophages (Kupffer cells) in response to toll like receptor signals (Tu et al. 2008a). In support of this theory, it has previously been shown that IL-10 deficient mice develop a spontaneous colitis in the absence of IL-10, suggesting that this may mediate a similar constitutive suppression against commensal gut bacteria (Kuhn et al. 1993). Furthermore, in patients undergoing orthoptic liver transplantation, le Moine et al suggested that IL-10 released by the graft could promote immune tolerance (Le Moine et al. 1994). Therefore, it is feasible that a single cytokine could alone maintain a strong tolerogenic influence on the liver.

In the study by Tu et al, they showed that Kupffer cells isolated from liver mononuclear cells with magnetic beads produced IL-10 in a dose dependent manner in response to TLR ligands poly I:C, LTA and LPS. Interestingly, most of the IL-10 was produced following stimulation with bacterial products which signal through the MyD88 dependent TLR2 and TLR4 pathways, and less IL-10 was produced following signalling through the TRIF dependent TLR-3 pathway which is used more by viruses. These findings confirmed those previously supported by Knolle et al, who showed that Kupffer cells secreted IL-10 in response to LPS most optimally 12-24hours after challenge (Knolle et al. 1995).

In a later study by Knolle et al, it was further shown that in a transgenic mouse model, Liver sinusoidal endothelial cells, but not macrovascular endothelial cells from the aorta, could prime naïve CD4+ T cells (with a transgenic receptor specific for influenza virus hemagglutinin) towards an IL10+IL4+ Th0 but not Th1 phenotype, which could further contribute to IL-10 production (Knolle et al. 1999).

6.0.4b Virus specific/regulatory T cells

Regulatory T cells (Tregs) have been implicated in the control of immune responses. They may be subdivided into natural CD4+CD25+FoxP3+ regulatory T cells which develop in the thymus, and adaptive regulatory T cells which are generated from naïve T cells in the periphery after encounter with antigen presented on dendritic cells with a distinct activation status and IL-10 (Mills 2004). Inducible regulatory subsets include CD4+ T regulatory 1 (Tr1) cells which predominantly mediate their suppressive function through secretion of IL-10 (Groux et al. 1997), and CD4+ T helper 3 (Th3) cells which secrete TGF-β (Miller et al. 1992). Another subset of CD8+ regulatory cells which have the capacity to release IL-10 and TGF-β have also been identified (Mills 2004). Natural and adaptive regulatory T cells differ by the way they mediate suppression of T cell responses. Natural regulatory T cells have the capacity to suppress via production of cytokines IL-10, soluble and surface bound TGF-β and IL-35 (Vignali et al. 2008), although there is debate as to the relative contribution of cytokine secretion in their suppressive action. This is highlighted by the fact that allergic inflammation and airway hypersensitivity can still be suppressed by transfer of IL-10 deficient natural regulatory T cells (Kearley et al. 2005), however in a different study, ablation of regulatory T cell IL-10 exacerbated lung allergic inflammation and hyper-reactivity (Rubtsov et al. 2008). The main mode of action for these cells is thought to be through contact dependent mechanisms. These include 1) engagement of cytotoxic T-lymphocyte antigen 4 (CTLA-4) on regulatory T cells with CD80/CD86 on dendritic cells which induces indoleamine 2,3-dioxygenase (IDO), an enzyme which catabolises tryptophan and has an immunosuppressive role (Fallarino et al. 2003) 2) granzyme A/granzyme B/perforin dependent killing (Vignali et al. 2008) 3) direct transfer of suppressive cyclic AMP to effector T cells through membrane gap junctions (Bopp et al. 2007) 4) consumption of IL-2 by CD25 (high affinity IL-2Rα), resulting in IL-2 dependent apoptosis (de la et al. 2004) and 5) elaboration of adenosine generated by ectoenzymes CD39 and CD73 (Deaglio et al. 2007). Tr1 cells secrete high amounts of IL-10, no IL-4 and low IL-2 and IFN-γ (Roncarolo et al. 2006). IL-10 released by these cells inhibits cytokine production (TNF and IL-12) by dendritic cells and macrophages (Mills 2004), and also downregulates expression of costimulatory molecules CD80 and CD86 (Ding et al. 1993). IL-10 can also directly act on effector T cells by binding to IL-10R and suppress proliferation and IL-2 production. In the setting of the HBV inflamed liver, Tr1

cells may be the more relevant source of IL-10, although natural and adaptive regulatory T cells may work together to amplify regulatory function.

In chronic HBV infection, the frequency and functional characteristics of CD4+CD25+ regulatory T cells has been studied in both the periphery and liver of patients. In one report, Franzese et al. found no increase in the circulating frequencies of CD4+CD25+ regulatory T cells in HBeAg+ immunotolerant patients, as well as no fluctuations with treatment induced flares of chronic hepatitis, although they stress that this was a small exploratory study in which treatment induced flares may not necessarily represent those during disease flares. They did find however that on depletion of Treg populations, there was an increase in frequency and functional capability of HBV-specific CD8+ T cell responses (Franzese et al. 2005). More recently, Xu et al. performed a larger study in which they did find significantly elevated frequencies of CD4+CD25+ regulatory T cells in patients with severe HBV infection ($>10^7$ copies/ml HBV DNA) compared to healthy donors. Furthermore, immunohistochemical staining of paraffin embedded tissue showed enrichment of CD4 and Foxp3 (transcription marker expressed on regulatory T cells) on liver sections from patients with severe HBV infection suggesting that these cells were also enriched within the liver. The frequency of circulating T regs correlated with serum HBV DNA levels and were able to suppress proliferation of PBMC following stimulation with HBV antigens (Xu et al. 2006). There are now a number of studies which have further reproduced the findings of Xu et al in patients with chronic HBV (Peng et al. 2008a;Stoop et al. 2005).

Antigen-induced regulatory T cells can also be generated in the liver and produce IL-10 in chronic infection. In chronic HBV infection, Chang et al. showed that following stimulation with overlapping peptides spanning the whole HBV genome, there was a higher magnitude of intrahepatic CD8 T cells producing IL-10, especially in HBeAg+ patients, compared to peripheral CD8 T cells, which were instead skewed towards production of Th1 cytokines (Chang et al. 2007). Similarly, an enrichment of virus specific IL-10 producing CD8 T cells within the liver has also been observed in chronic HCV infection. Accapezzato showed, for instance, that an enriched HCV specific CCR7-CD8+ T cell population in the liver suppressed T cell responses via IL-10 production (Accapezzato et al. 2004). Indeed, HCV-specific CD8 T cells within the liver have been shown to have poor cytolytic ability and are unable to mediate viral control. Instead they

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may have a protective anti-fibrotic role, as they have been shown to cluster in areas of low liver fibrosis and low hepatcellular apoptosis where they might block inflammation by bystander T cells via IL-10 (Abel et al. 2006).

Finally, antigen-induced CD4 T cells may also be induced in a similar way to the CD8 T cells. Indeed in both chronic HBV and HCV infections, stimulation of CD4 T cells, in HBV by HBcAg (Hyodo et al. 2004), and, in HCV by HCV core protein (MacDonald et al. 2002), could induce IL-10 production. HBcAg is analogous to HBeAg, the secreted form of core protein, which has a short leader sequence cleaved from it. One feature unique to CHB is the production of vast amounts of circulating HBeAg, which has no known role in viral replication, but may instead have a tolerogenic effect on immune responses (Milich et al. 1990). Induction of immunosuppressive IL-10 production by CD4 T cells and other mononuclear cells could perhaps contribute to this tolerogenic effect.

6.0.4c Regulatory B cells

The antibody-independent function of B cells in regulating immune responses has become a recent topic of interest. In 2000, Harris et al. were the first to identify two subsets of B cells, B effector 1 (Be1) and B effector 2 (Be2), which produced different panels of cytokines, dependent on their initial interaction with antigen and T cells. Be1 cells primarily produced IFN-γ, whereas Be2 primarily produced IL-4. Both subsets were able to produce IL-10. Importantly, they showed that these cytokine-producing effector B cells could regulate the differentiation of naïve CD4 into either Th1 or Th2 helper cells, suggesting an important antibody-independent role of B cells in modulating the immune system (Harris et al. 2000). Indeed, it is thought that B cells are required for the Th1—Th2 shift which is required to ameriolate disease pathology following an inflammatory cytokine response against *Schistosoma mansoni* infection. In B cell deficient uMT mice, there was a sustained Th1 response against the helminth egg compared to wildtype mice, which accelerated granuloma development (Hernandez et al. 1997).

In addition to influencing the Th1-Th2 shift however, there is now strong evidence mainly in murine studies that a population of IL-10 producing B cells, termed 'regulatory B cells' by Mizoguchi, may regulate immune responses in chronic inflammatory autoimmune, infectious and tumor models (Mauri & Ehrenstein 2008;Mizoguchi & Bhan 2006).

The most compelling evidence for the existence of regulatory B cells comes from three separate murine studies of collagen induced arthritis, EAE and a model of chronic intestinal inflammation in the Gut Associated Lymphoid Tissue (GALT).

Immunisation of transgenic mice with type II collagen in Complete Freund's adjuvant leads to a chronic collagen-induced arthritis, associated with a pathogenic Th1 response. In this model, Mauri et al showed that stimulation of B cells with antigen and agonistic CD40 generated IL-10 producing B cells, which upon transfer into transgenic mice prevented arthritis development or ameriolated established disease. B cells isolated from IL-10 knockout mice, however, had no protective function against arthritis, showing that this effect was IL-10 dependent (Mauri et al. 2003). In a followup study, these IL-10 producing regulatory B cells were shown to be phenotypically analogous to transitional 2 marginal zone precursor B cells (T2-MZP) found in the spleen of naïve mice. Transfer of T2-MZP, but not follicular or marginal zone B cells, into immunized DBA/1 mice, suppressed antigen-specific T cell responses and significantly prevented new disease and ameriolated established disease. T2-MZP from IL-10 knockout mice however could not mediate a protective effect (Evans et al. 2007).

Akin to collagen induced arthritis, a similar protective role has been ascribed to regulatory B cells in Experimental Autoimmune Encephalomyelitis (EAE), a paralysing demyelinating model of multiple sclerosis. In this setting, antigen-specific IL-10-producing regulatory B cells were required for recovery of disease, and the absence of B cells (in uMT mice) resulted in severe type 1 autoimmunity. Furthermore, in a bone marrow chimeric system in which only B cells but not T cells were deficient in IL-10 production, the inflammatory response persisted and impeded recovery. Only transfer of IL-10-producing regulatory B cells from B6 mice who had recovered EAE could result in resolution. Thus regulatory B cells control autoimmunity in this case by dampening pro-inflammatory responses via IL-10 (Fillatreau et al. 2002).

Finally, Mizoguchi et al. showed in a model of chronic intestinal inflammation that a subset of B cells expressing high CD1d could be induced in the inflammatory environment of the GALT and suppressed IL-1 mediated inflammation through IL-10 (Mizoguchi et al. 2002). In confirmation of this, Yanaba et al have shown that a unique subset of CD1d^{high}CD5+ B cells, which were the main subset producing IL-10 in their CD19

overexpressing transgenic mouse model, normalised T cell mediated inflammation upon adoptive transfer into CD19 knockout mice (Yanaba et al. 2008).

These studies therefore prove at least that B cells can mediate potent regulation of immune responses via IL-10 in different murine disease models. Less work has been done in human studies and this field is newly developing. However, studies by Duddy et al. have shown that IL-10 producing B cells exist in humans, and that they are phenotypically naïve (CD27 negative), whereas those polarised towards TNF and IFNγ production have a memory phenotype (CD27+). This IL-10 production is context dependent though, and can be induced in vitro with CD40L stimulation alone, whereas CD40L and BCR dual ligation polarised B cells more towards lymphotoxin production (Duddy et al. 2004). Interestingly, they showed that in multiple sclerosis, stimulation of B cells with either stimulus produces less IL-10 in patients compared to healthy donors. IL-10 production could be recovered on mitoxontrone therapy (Duddy et al. 2007). These findings are analogous to the protective role attributed to B cells by Fillatreau et al. in EAE (Fillatreau et al. 2002).

Whether regulatory B cells are protective as suggested by the above studies, or pathogenic in the context of chronic viral infection, is yet to be investigated. Studies in patients with cancer have shown that B cell depletion therapeutically enhanced anti-tumour immunity due to lack of B cell IL-10 (Inoue et al. 2006). In such cases, IL-10-producing regulatory B cells may be deleterious to the immune response against the tumour and hence be construed as pathogenic.

6.0.5 Aims

In this chapter we investigate the role of IL-10 in chronic HBV infection, including its ability to modulate anti-viral CD8 T cell responses. Furthermore, we study whether regulatory B cells could be a potential source of this cytokine, and thus play a role in regulating immune responses in chronic HBV infection.

Results

6.1 IL-10 is raised in the serum of patients with chronic HBV infection

IL-10 is a pleiotropic cytokine which both protects from immunopathology while at the same time can impede pathogen clearance in a number of chronic infectious diseases. To determine whether IL-10 plays a role in chronic HBV infection, we first investigated the circulating levels of this cytokine in the serum of patients and healthy donors by ELISA or with a cytometric bead array (CBA) inflammation kit. As shown in figure 6.1a, IL-10 levels were significantly raised in patients compared to healthy donors by either assay. Furthermore, patients with high viral load and elevated ALT (surrogate marker of liver inflammation) had the highest circulating IL-10 levels compared to patients with low viral load (vs. DNA $p<0.05$, vs. ALT $p<0.001$) and healthy donors (vs. DNA $p<0.001$, vs. ALT $p<0.001$). IL-10 levels were also determined in 15 patients on anti-viral therapy (for varying periods of time), in whom viral load had been suppressed to below quantifiable levels. These patients had a significant reduction in serum IL-10 levels compared to those with high viral load not on treatment ($p<0.01$), down to levels seen in controls (fig 6.1b).

Interestingly, we observed that the mean serum IL-10 level was higher in patients when measured by CBA (18.08pg/ml, n=16) compared to ELISA (3.32p/ml, n=34). Although this could be due to the CBA being a more sensitive assay, it is more likely this disparity is a result of different patient characteristics in the two cohorts. In the CBA cohort for instance, 10/16 patients had HBeAg negative chronic hepatitis with raised ALT (>60 IU/l) and/or intermediate to high viral load (HBVDNA $>10^5$ IU/ml) (table 6.1). In clinical terms, patients infected with viral variants incapable of expressing HBeAg have a unique course of disease, as they are more prone to spontaneous recurrent flares of hepatic inflammation associated with fluctuations in viral load (which is not typical of patients with HBeAg positive hepatitis). In these patients it is possible to monitor rapid changes in disease activity over a condensed timeframe. Out of the six patients with the highest IL-10 in the ELISA cohort, 4 had HBeAg negative hepatitis and their levels of IL-10 were comparable with those in patients in the CBA cohort. Therefore, the skewing towards higher IL-10 levels in the CBA cohort may be a true effect dependent on patient characteristics, rather than an artefact of assay variability.

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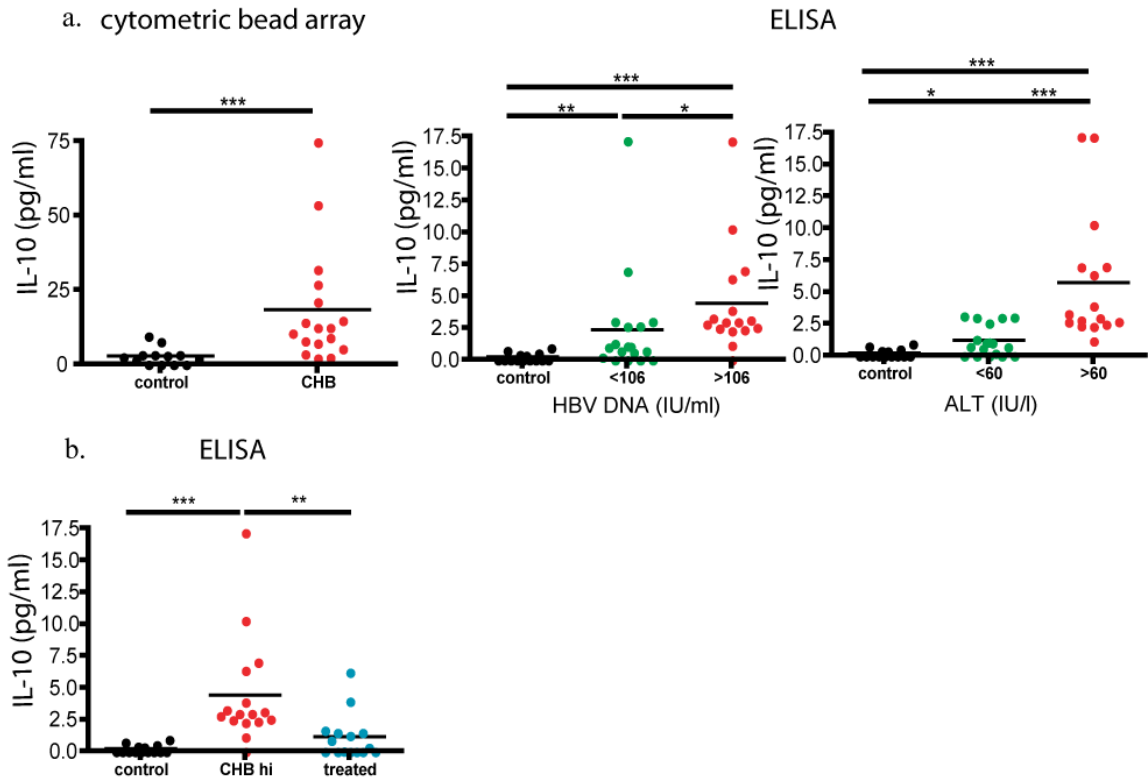
Figure 6.1: IL-10 is raised in the serum of patients with chronic HBV infection. Serum IL-10 levels were determined by cytometric bead array (left) or by ELISA (right), and values for both healthy donors and patients are shown in (a). (b) Graph to compare serum IL-10 levels in controls, patients with high viral load ($>10^6$ IU/ml), and patients with chronic HBV currently on treatment. In most cases, treated patients had suppression of virus to below quantifiable levels and had normalised their ALT. (*=p<0.05, **=p<0.01, ***=p<0.001, non-parametric Mann-Whitney U test).

Patient	DNA (IU/ml)	ALT (IU/L)	HBeAg	IL-10 (pg/ml)
CHB 1	Blq	34	Neg	1.30
CHB 2	Blq	57	Neg	0.00
CHB 3	160	39	Neg	0.00
CHB 4	440	23	Neg	3.01
CHB 5	850	22	Neg	0.00
CHB 6	870	28	Neg	1.06
CHB 7	1400	30	Neg	3.02
CHB 8	1400	56	Neg	0.73
CHB 9	4100	42	Neg	0.20
CHB 10	7100	50	Neg	1.01
CHB 11	7800	73	Neg	17.19
CHB 12	40000	24	Neg	0.70
CHB 13	72172	405	Neg	2.65
CHB 14	170000	56	Neg	0.58
CHB 15	250000	48	Neg	1.08
CHB 16	418571	116	Neg	6.96
CHB 17	730000	102	Pos	2.68
CHB 18	770000	33	Neg	0.00
CHB 19 *	1100000	151	Neg	6.36
CHB 20	1127317	189	Neg	2.81
CHB 21	1400000	58	Pos	0.00
CHB 22	2000000	69	Pos	3.29
CHB 23 *	3800000	158	Neg	3.89
CHB 24	12000000	73	Pos	1.16
CHB 25	30000000	54	Pos	2.57
CHB 26	35000000	89	Pos	2.29
CHB 27	67000000	96	Pos	2.36
CHB 28	110000000	24	Pos	3.00
CHB 29	160000000	156	Pos	3.13
CHB 30 *	160000000	125	neg	10.29
CHB 31	160000000	54	pos	7.01
CHB 32	166000000	64	pos	2.98
CHB 33 *	320000000	77	pos	2.49
CHB 34 *	510000000	206	pos	17.15
T1	19	26	neg	0.88
T2	37	50	pos	0.33
T3	43	51	pos	0.03
T4	1615	38	neg	1.67
T5	2000	17	neg	0.00
T6	26621	47	pos	1.50
T7	Blq	16	neg	0.00
T8	Blq	17	neg	0.00
T9	Blq	42	neg	1.28
T10	Blq	30	neg	0.00
T11	Blq	25	neg	3.96
T12	Blq	30	neg	6.23
T13	Blq	37	neg	0.00
T14	Blq	17	pos	0.00
T15	Blq	24	pos	1.51

Serum IL-10 was determined in these patients by ELISA.

Subject	HBV DNA (IU/ml)	ALT (IU/L)	HBeAg	IL-10 (pg/ml)
CHB 35 *	13250	81	neg	14.8
CHB 36 *	175000	403	neg	74.8
CHB 37	397500	42	neg	10.5
CHB 38 *	510000	472	neg	7.9
CHB 39 *	552500	75	neg	5.3
CHB 40	2105000	368	neg	12.4
CHB 41	12875000	314	neg	53.7
CHB 42 *	33750000	880	neg	26.9
CHB 43 *	35250000	432	neg	9.1
CHB 44	450000000	207	neg	7.2
CHB 45	-	-	-	14.2
CHB 46	-	-	-	31.9
CHB 47	-	-	-	3.6
CHB 48	-	-	-	2.4
CHB 49	-	-	-	2.2
CHB 50	-	-	-	12.4

Serum IL-10 was determined in these patients by CBA.

Table 6.1 Clinical characteristics of patients in whom IL-10 levels were measured in serum by ELISA (above table) and by cytometric bead array (bottom table). * = serum samples from these 11 individuals were collected at multiple timepoints, and longitudinal fluctuations in IL-10 are shown in fig 6.2. CHB = chronic HBV infection. T = on treatment.

6.2 Temporal correlation between serum IL-10 and flares of chronic liver disease

In order to dissect further the relationship between IL-10 and viral load/ALT, serum samples were collected at multiple timepoints before, during and after chronic flares of HBeAg negative HBV in 9 patients and in 2 patients with HBeAg positive hepatitis (patients CHB 34 and CHB 33) and IL-10 was measured. Graphs in figure 6.2 show that in most cases, IL-10 peaked at a time point when both viral load and ALT were elevated; sampling was not frequent enough to distinguish with which it was more closely temporally correlated. However, in at least some cases, peaks in IL-10 clearly coincided with viral load and preceded liver inflammation (patients CHB 33 + CHB 43) or alternatively peaked with liver inflammation alone (patient CHB 36). Therefore these data imply that both ALT and viral load may be intimately associated with IL-10 levels. Only a study with more frequent sampling timepoints over the progression of a flare could perhaps detail any further subtle temporal correlations.

In addition to these findings, we have also observed that in some cases, IL-10 was induced in response to increased inflammation (patients CHB 19, CHB35 and CHB 38) suggesting that in the context of HBV, it may be required as a physiological brake to attenuate an overactive immune response. However, this was not demonstrated in all individuals, as in other cases, IL-10 levels peaked before, and coincided with, the rise in ALT.
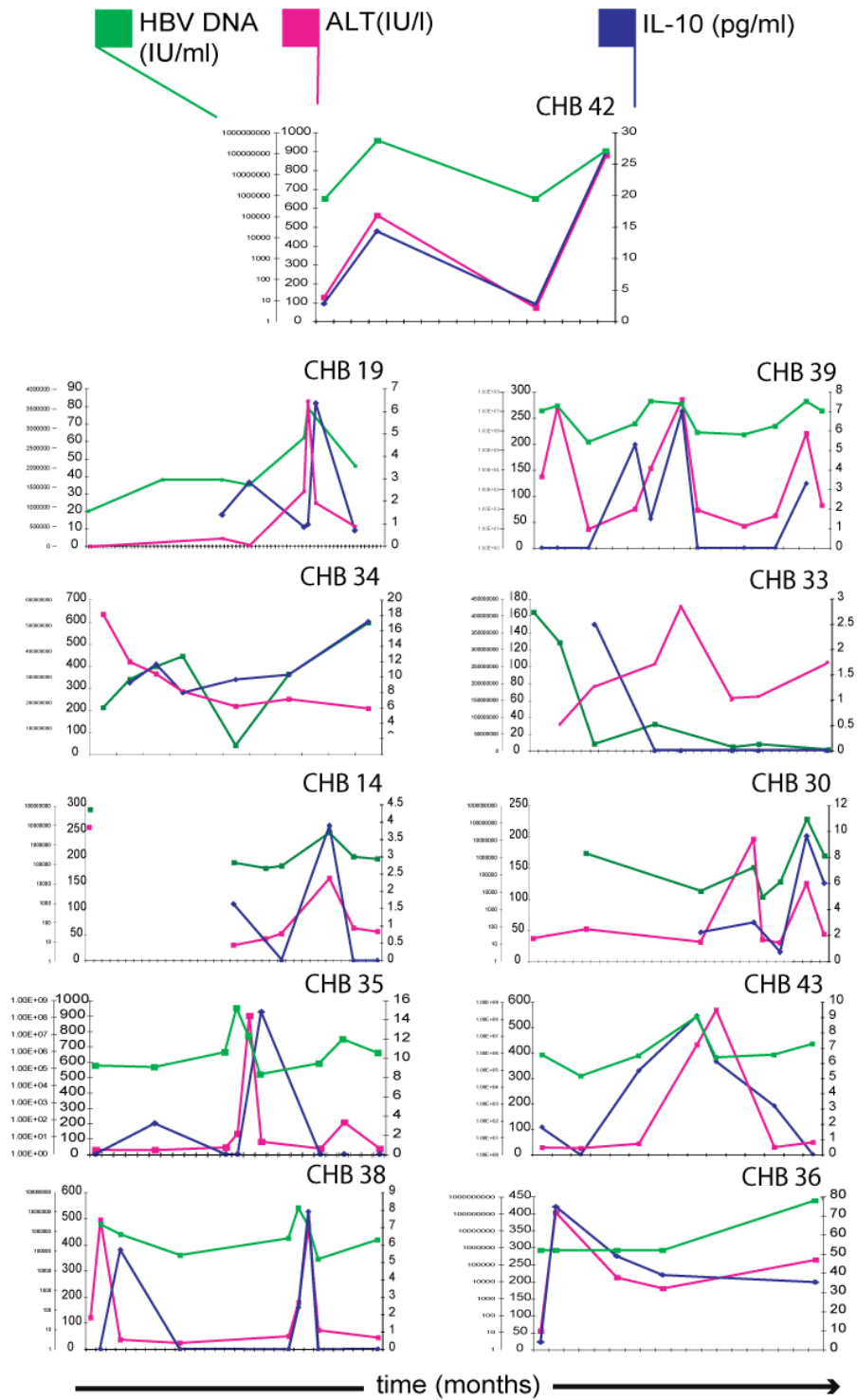
Figure 6.2: Temporal correlation between serum IL-10 and chronic flares of liver disease. IL-10 levels were measured in serum samples collected before, during and after chronic flares of liver disease in 11 patients with chronic HBV. A graph for each patient shows the temporal longitudinal fluctuations in IL-10 (blue), in association with viral load (green) and ALT of the patient (pink).

6.3 Elevated supernatant IL-10 from PBMC in patients with high viral load

In order to investigate the capacity of peripheral blood mononuclear cells to produce IL-10 in chronic HBV, PBMC were stimulated with mitogens PMA and ionomycin for 5 hours and supernatants were harvested for ELISA. As shown in figure 6.3, there was a trend towards a progressive increase in IL-10 production by PBMC taken from patients with increasing viral load. PBMC from patients with high viral load ($>10^6$IU/ml) produced significantly more IL-10 than those from healthy donors ($p<0.05$).

Subject	HBV DNA (IU/ml)	ALT (IU/L)	HBeAg
CHB 52	Blq	24	Neg
CHB 53	Blq	18	Neg
CHB 54	Blq	34	Neg
CHB 1	Blq	34	Neg
CHB 6	870	28	Neg
CHB 12	40000	24	Neg
CHB 14	170000	56	Neg
CHB 11′	320000	96	Neg
CHB 55	1000000	474	Neg
CHB 33′	19000000	65	Pos
CHB 56	20000000	83	Pos
CHB 25	30000000	54	Pos
CHB 26	35000000	89	Pos
CHB 27	67000000	96	Pos
CHB 28	110000000	24	Pos
CHB 17	130000000	193	Pos
CHB 30	160000000	125	Neg
CHB 34′	310000000	249	Pos
CHB 57	330000000	39	Pos

Table 6.2 Clinical characteristics of patients used in graph 6.3. An (') beside a patient code denotes that a different sampling timepoint has been studied, compared to a previous assay.
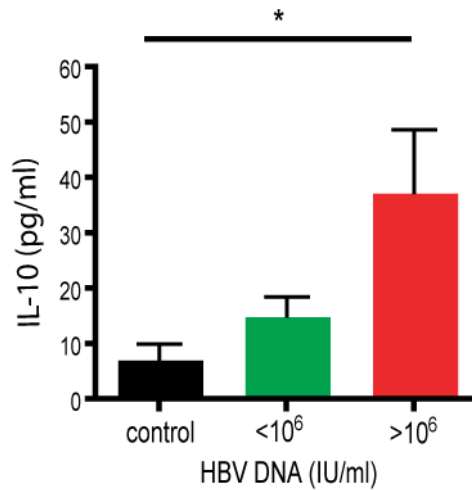
Figure 6.3: Elevated supernatant IL-10 from PBMC in patients with high viral load. PBMC from patients and controls were stimulated for 5 hours with PMA and ionomycin, and supernatants were analysed for IL-10 content by ELISA. Graphs show the mean IL-10 in supernatant in controls (n=9), patients with low viral load ($<10^6$ IU/ml) (n=8) and patients with high viral load ($>10^6$ IU/ml) (n=11). (*=p$<$0.05, non-parametric Mann-Whitney U test).

6.4 IL-10 producing B cells are enriched in patients with CHB

Recent studies have shown that IL-10 producing B cells can regulate immune responses in chronic inflammatory disorders. In order to identify whether such a population may exist in chronic HBV infection, PBMC were stimulated with either CpG type B or HBV surface and core antigens for 4 days. IL-10 producing B cells were then detected by intracellular cytokine staining after a further short incubation with PMA/ionomycin. A representative dot plot in figure 6.4a shows the frequency of IL-10 producing B cells following CpG stimulation in a typical HBV carrier. Upon this particular stimulation, the majority of IL-10 produced was from CD19+ B cells. On cross-sectional analysis, there was a mean 5 fold increase in the frequency of IL-10 producing B cells in patients with low or high viral load compared to controls (p<0.001). No difference was observed however between patients with low and high viral load, in contrast to serum IL-10 levels and PBMC IL-10 production which were both elevated in patients with high viral load. These data suggest therefore that the enriched fraction of IL-10 producing B cells may partially contribute to the regulatory framework in HBV infection, however it is likely that other cellular sources of IL-10 also contribute.

During HBV infection, large quantities of HBsAg and HBeAg (the secreted form of HBV core antigen) are secreted, and are present in milligram concentrations in the serum. HBeAg has no discernible role in virus replication or binding, but instead, has been linked with tolerance of T cell responses (Milich et al. 1990), and has been shown to bind to lymphocytes including B cells (Cao et al. 2001). To determine whether antigen specific-B cells were a source of IL-10, PBMC were also stimulated with HBV core and surface antigens with a similar protocol. No IL-10 production was observed with surface antigen either by intracellular staining or by ELISA following positive magnetic bead separation of CD19+ B cells (data not shown). However, stimulation with core antigen alone or in combination with a co-stimulus CD40L was able to induce single positive IL-10 producing or TNF-α producing B cells (fig 6.4c). These data highlight the potential therefore for B cells in vivo to respond to circulating HBV antigens which could induce IL-10 production.

Subject	HBV DNA (IU/ml)	ALT (IU/L)	HBeAg	B cell IL-10
CHB 1	Blq	34	Neg	2.81
CHB 54	Blq	34	Neg	1.19
CHB 59	Blq	32	Neg	1.04
CHB 58	160	39	Neg	2.39
CHB 60	360	not done	Neg	5.87
CHB 5	850	22	Neg	2.75
CHB 6	870	28	Neg	2.97
CHB 8	1400	56	Neg	0.94
CHB 9	4100	42	Neg	2.27
CHB 10	7100	50	Neg	3.95
CHB 11	7800	73	Neg	3.45
CHB 12	40000	24	Neg	3.97
CHB 15	250000	48	Neg	3.11
CHB 55	1000000	474	Neg	1.13
CHB 22	2000000	69	Pos	3.26
CHB 33*	19000000	65	Pos	0.91
CHB 27*	24000000	61	Pos	7.282
CHB 25	30000000	54	Pos	5.18
CHB 28	110000000	24	Pos	2.46
CHB 32*	120000000	76	Pos	3.67
CHB 17*	130000000	193	Pos	1.96
CHB 30	160000000	125	Neg	2.05
CHB 34*	310000000	249	Pos	1.64
CHB 24*	not done	76	Pos	1.49

Table 6.3 Clinical characteristics of patients studied in figures 6.4 – 6.10.
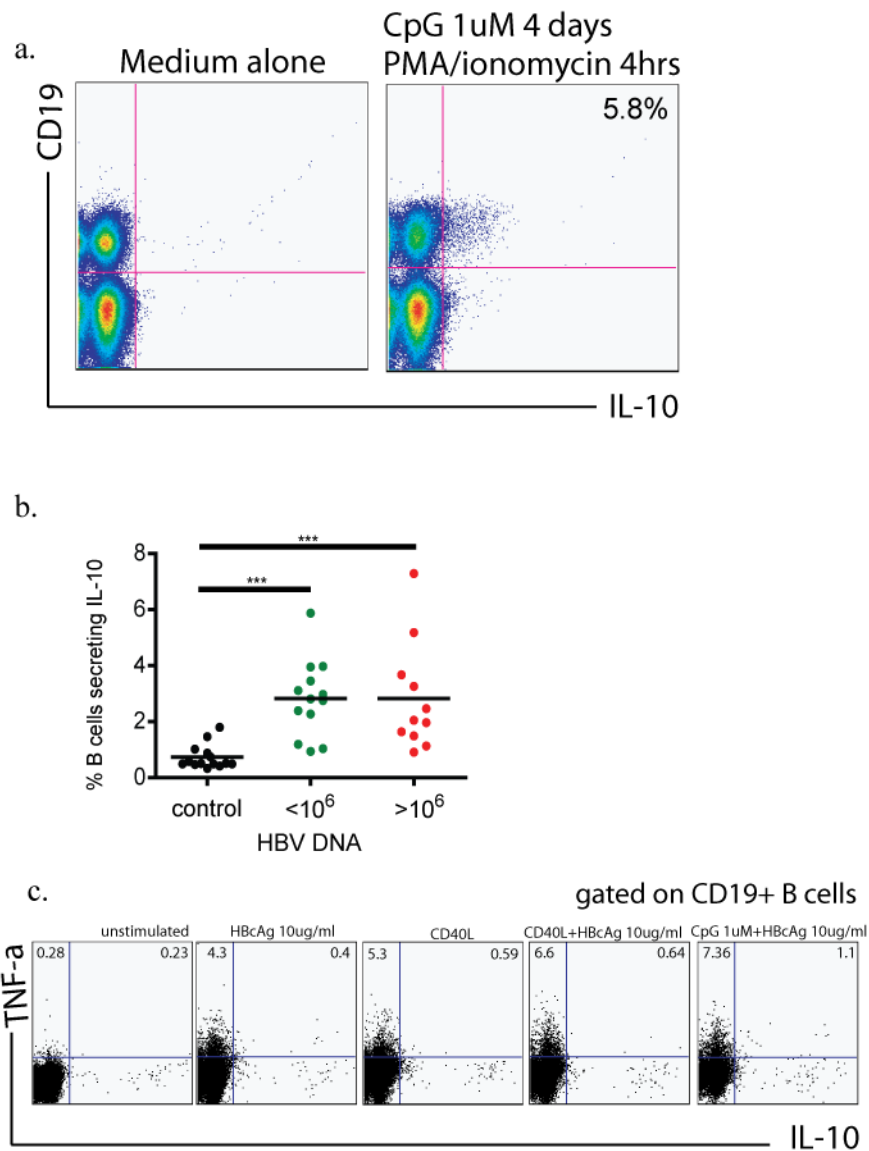
Figure 6.4: IL-10 producing B cells are enriched in patients with CHB. In order to analyse the frequency of IL-10 producing B cells, PBMC were stimulated for 4 days with CpG (PMA/ionomycin was added for the last 4 hours) and IL-10 production in CD19+ B cells was determined by flow cytometry a) representative dot plot to show the frequency of CD19+IL-10+ cells in a low level carrier (HBV DNA<10^6IU/ml). Cross-sectional data is summarised in (b) (***=p<0.001, non-parametric Mann-Whitney U test). c) Dot plots show IL-10 and TNF-α production in CD19+ gated B cells from a typical low level carrier, upon stimulation with HBV core antigen (HBcAg) alone or in combination with CD40L or CpG.

6.5 IL-10 producing B cells are phenotypically naïve

Dependent on expression of surface markers CD24 and CD38, peripheral B cells can be further subdivided into antigen naïve B cells (transitional and mature) and memory B cells (Carsetti et al. 2004). To investigate the phenotypic characteristics of IL-10 producing B cells, we next surface stained PBMC with the above markers prior to intracellular staining. As shown in figure 6.5, the majority of IL-10 producing CD19+ cells were transitional or mature, however only a small proportion were memory. This is consistent with observations by Duddy et al. who showed only CD27- human B cells produced IL-10, whereas CD27+ memory cells were skewed toward production of Th1 cytokines (Duddy et al. 2007). Furthermore, this data is supported by murine studies which have shown that transitional 2 marginal zone precursor B cells (phenotypically similar to human transitional B cells) but not follicular B cells were able to suppress collagen induced arthritis dependent on IL-10 production (Evans et al. 2007).

As an alternative to markers CD24 and CD38, human transitional B cells also express CD21, CD23 and L-selectin and are CD10 negative (Carsetti et al. 2004). These additional phenotypic markers may further help to differentiate this population from the other B cell subsets.
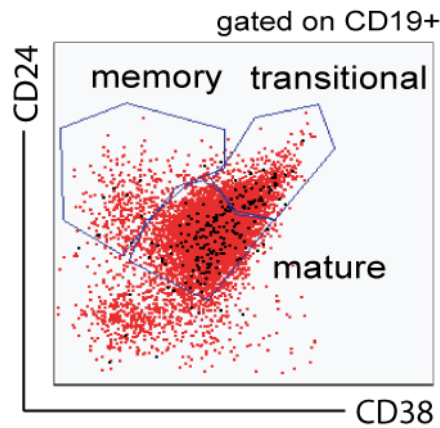
Figure 6.5: IL-10 producing B cells are phenotypically naive (mature and transitional). CD19+ B cells can be subdivided into three phenotypic groups dependent on their expression of surface markers CD24 and CD38; transitional (CD24^{high}, CD38^{high}), mature (CD24^{dull}, CD38^{dull}) and memory (CD24^{high}, CD38^{dull}). Transitional and mature populations are the naïve subsets. A representative dot plot for a high level carrier gated on CD19+ B cells shows the phenotypic distribution of IL-10 positive B cells (black dots) overlayed over IL-10 negative B cells (red dots) upon overnight stimulation (16hours) with CpG.

6.6 Transitional B cells are enriched in the periphery of patients with CHB and correlate with disease flares

Having characterised IL-10 producing B cells as naïve, we next determined whether the proportions of naïve and memory subsets were altered in patients with HBV (n=22) compared to healthy donors (n=14). As shown in figure 6.6a, on average twice as many transitional cells, but not mature or memory, were significantly enriched in patients compared to controls (p<0.001), and a similar trend was observed ex vivo (data not shown). This is despite the fact that CpG has previously been shown to drive transitional cells towards a memory phenotype (Capolunghi et al. 2008). To confirm that enrichment of transitional cells was specific to chronic HBV infection, we analysed the ex vivo frequency of these cells making use of serially stored PBMC samples collected over the course of flares of chronic HBV (fig. 6.6b). In two out of three patients, the proportion of transitional B cells correlated temporally with liver inflammation (patient CHB 33) and both liver inflammation and viral load (patient CHB 30). No fluctuation was observed in patient CHB 34, an HBeAg positive patient with persistently high viral load and ALT sustained at levels greater than 4 times the upper limit of normal (ie.>200IU/l). However, in this patient, the proportion of transitional B cells consistently accounted for 5-8% of the total B cells, which was almost double the normal range reported in human studies (3-4%) and found in patients CHB 33 and CHB 30. Therefore, this maintained elevated transitional cell proportion could also be a reflection of disease. No temporal correlations with viral load or liver inflammation were observed with memory, mature or total B cell populations.
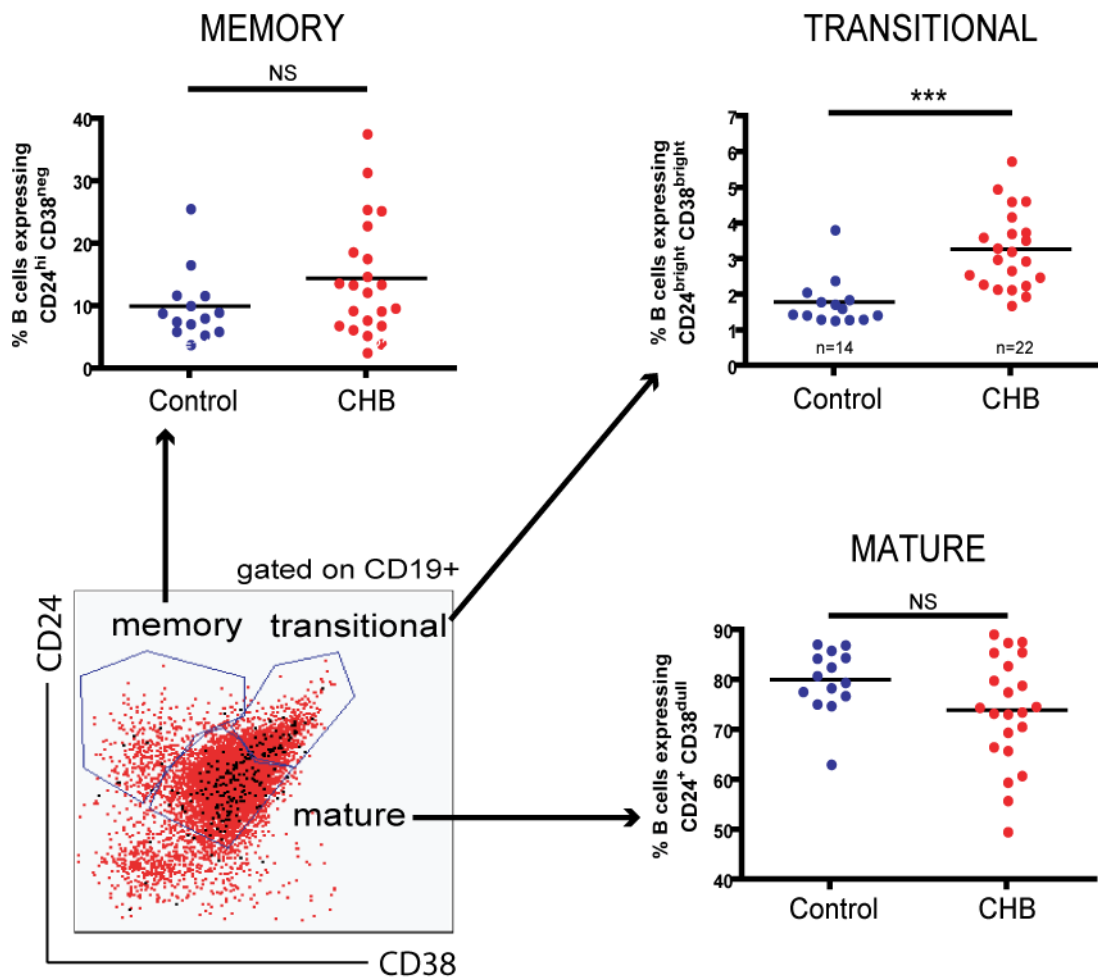
Figure 6.6a: Transitional B cells are enriched in the periphery of patients with CHB. Graphs show the proportions of the different peripheral B cell subsets in patients with CHB vs. healthy donors. A representative example dot plot from a patient illustrates the distribution of CD19+ cells into 3 phenotypic groups dependent on surface expression of markers CD24 and CD38. (***=p<0.001, non-parametric Mann-Whitney U test).
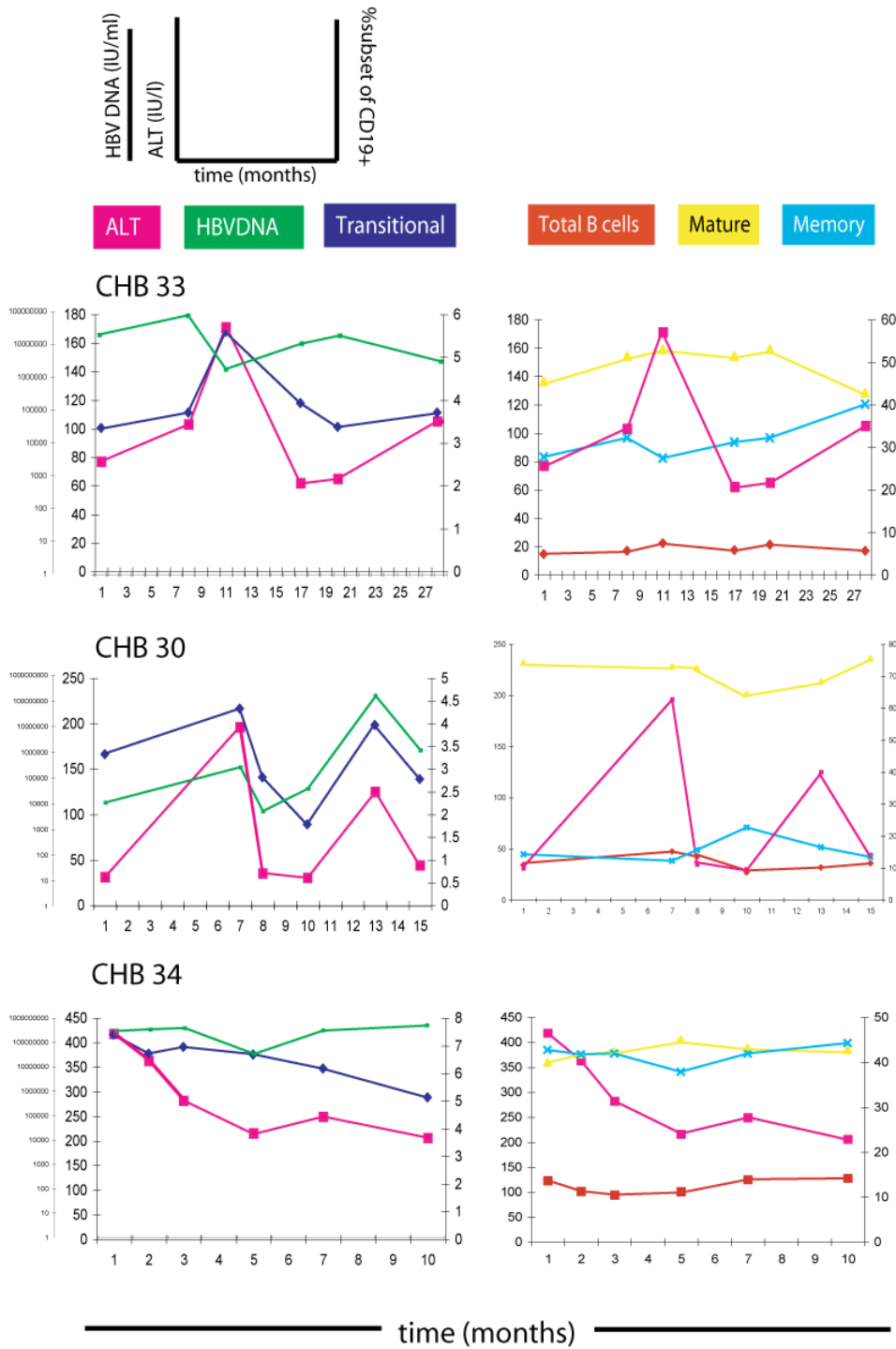
Figure 6.6b: Ex vivo frequencies of transitional cells correlate with disease flares. Graphs show the longitudinal fluctuations in the proportions of the different B cell subsets over the course of HBeAg negative flares in patients CHB 33 and CHB 30, and over the timecourse of disease in a patient with HBeAg positive disease (patient CHB 34).

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6.7 IL-10 producing B cells display a CD24^{high}CD38^{high} transitional phenotype during peak of disease flares

We observed that transitional cells are enriched during chronic flares of disease. Whether these new transitional cells are able to produce IL-10 or not however was unclear. To confirm this, we analysed the frequency of IL-10+ transitional cells longitudinally in flaring patients both ex vivo and following overnight stimulation with CpG. In fig 6.7a, representative dot plots illustrate the phenotype of IL-10 producing cells (black dots) overlayed over IL-10 negative B cells (grey dots) at various timepoints over the course of a flare in 1 patient with HBeAg negative chronic hepatitis. In this patient, during the peak of viral load, all IL-10 producing B cells clustered together in the transitional cell gate or at the margin between mature and transitional cells. At all other timepoints, this population was heterogenous in terms of its phenotype. Furthermore on stimulation with CpG, not only was there an enrichment of transitional cells during the peak of viral load, but there was also an enrichment of IL-10 production within these cells. Indeed, although the transitional cells represented only 12.4% of the total B cells, they accounted for 21.8% of the total IL-10 producing cells at this timepoint (fig 6.7b). Interestingly, no correlation in mature B cell IL-10 production was observed during disease, and indeed during the peak of viral load the production of mature cell IL-10 was lowest. These data suggest that the transitional subset which fluctuates with disease may be the putative regulatory subset in chronic HBV infection, although it is important to note that not all transitional cells produce IL-10, therefore other as yet unknown markers may be required to further differentiate the true regulatory B cell subset.
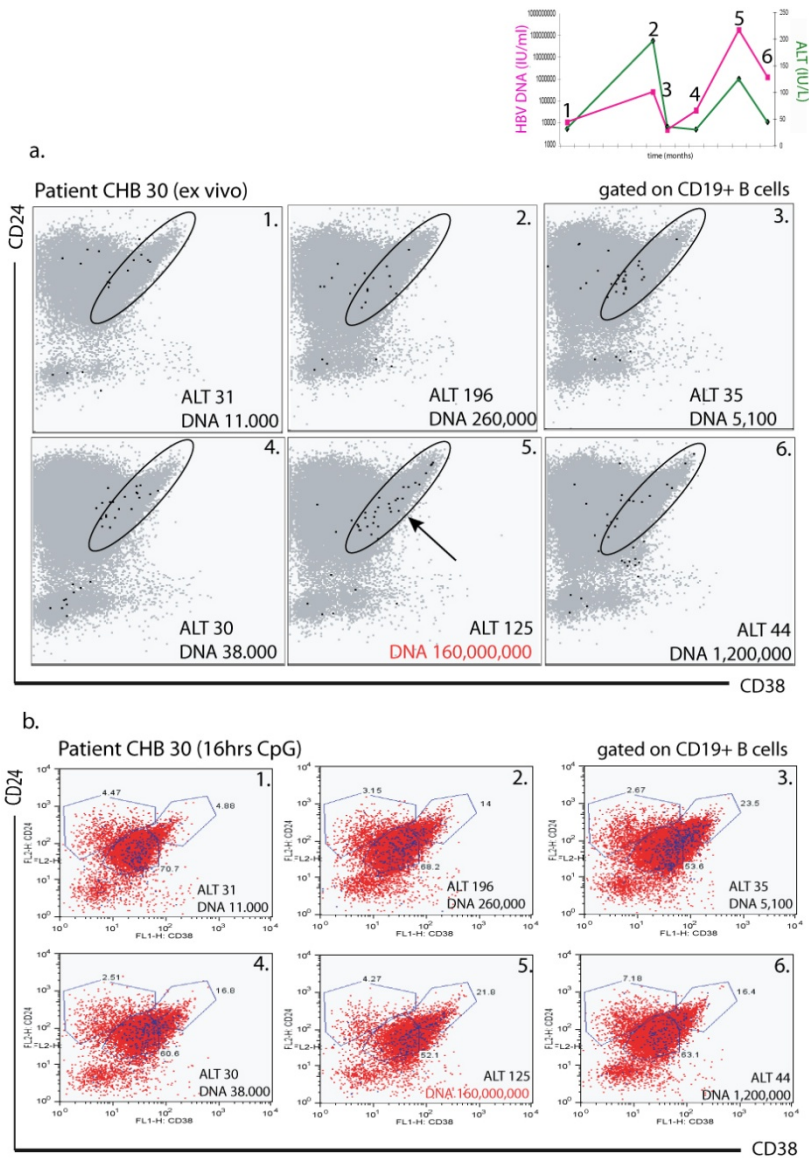
Figure 6.7: IL-10 producing B cells display a CD24^{high}CD38^{high} phenotype during disease flares. Dot plots show the phenotypic characteristics of IL-10 producing B cells over multiple timepoints during an HBeAg negative flare of disease in a representative patient either a) ex vivo or b) following overnight (16hour) stimulation with CpG. The gate applied in (a) encompasses both the CD24^{high}CD38^{high} transitional cells, as well as a subset of B cells at the mature/transitional border. IL-10 producing B cells (black dots) are overlayed on top of IL-10 negative B cells (grey dots). In (b), gates have been drawn to show the different phenotypic B cell subsets subdivided by CD24 and CD38 expression. Numeric values corresponding to each gate represent the proportion of CD19+IL-10+ cells which are of that subset. Red dots = IL-10 negative B cells; Blue dots = IL-10 positive B cells.

6.8 Transitional B cells are present in an HBV liver

Impairment in CD8+ T cell responses are most pronounced within the liver environment, the site of HBV replication. To investigate the potential of B cells to regulate immune responses in this setting, paired samples of peripheral blood and intrahepatic lymphocytes were obtained from two patients with CHB and the proportion of total and transitional B cells was quantified. As shown in a representative patient in 6.8, the proportion of transitional B cells was similar in the blood (6.37% of B cells) and liver (5.23% of B cells), although the total B cell proportion was lower in the liver (8.76% of lymphocytes) compared to blood (14.7% of lymphocytes). This is in concordance with data by Norris et al, who showed that the mean percentage of B lymphocytes in 10 healthy livers studied was 6.1% (Norris et al. 1998). These data therefore show that transitional B cells are present in the liver and have the potential to exert regulatory function. As a limitation to this study, we have only so far analysed the intrahepatic B cell compartment in livers obtained from patients with low viral load and no liver inflammation. As we have shown previously, serum IL-10 levels as well as ex vivo circulating transitional B cell frequencies are often at baseline levels between disease flares. Therefore, to get a true representation of the role of B cells in the liver, further study is required to study liver samples collected at timepoints of high viral load and liver inflammation, at which point we know that there is a non-specific cellular infiltrate into the liver and, in the periphery at least, an enrichment of ex vivo transitional cell frequencies and serum IL-10 production.
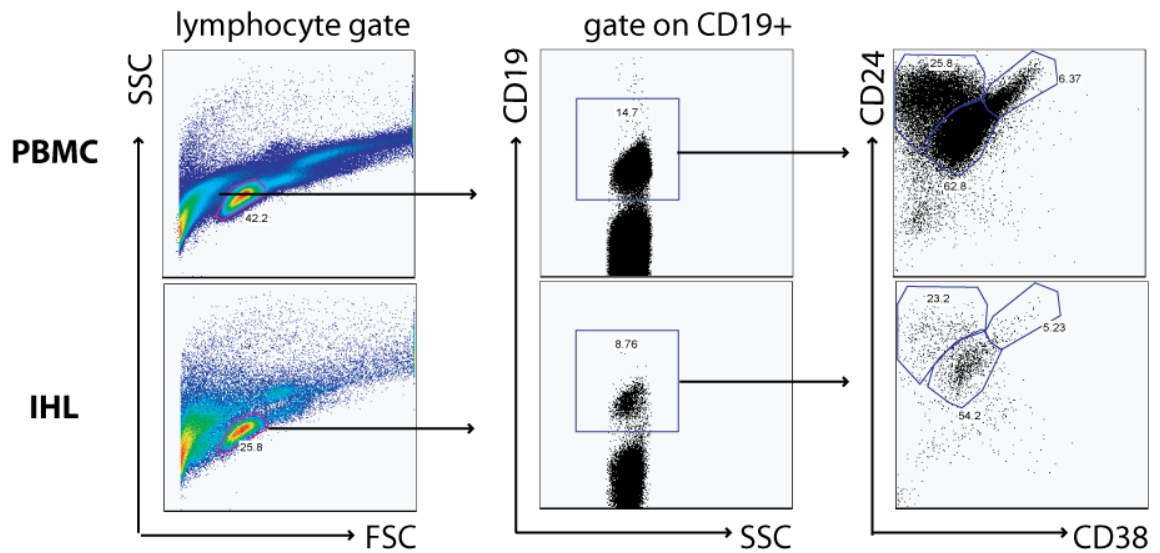
Figure 6.8: Transitional B cells are present in an HBV liver. Dot plots are shown to illustrate the proportions of total and mature/memory/transitional B cells in the periphery and liver of a representative patient with low viral load (right panels) following dual gating on lymphocyte and CD19+ populations.

6.9 Depletion of B cells enhances virus specific CD8 T cell responses

To determine whether B cells regulate CD8 T cell function, we initially studied the impact of depletion of the total B cell population on the magnitude of CD8 T cell responses against a variety of HLA-A2 restricted viral peptides. Shown in figure 6.9.1a are representative examples of CMV, EBV, FLU and HBV pool responses from a healthy donor, patient with low viral load and patient with high viral load. Upon depletion of B cells, there was a marked recovery of CD8 T cell responses to flu peptide in all 3 subjects, however in individual cases CMV, EBV and HBV pool responses were also significantly recovered. These data may suggest therefore that B cells maintain a constitutive low level suppression of virus specific T cell responses. Indeed, as shown in figure 6.9.1b, when B cells were added back to CD19 depleted PBMC, there was a dose dependent suppression of the CD8 T cell response against CMV peptide in a healthy donor.

An impairment of the CD8 T cell response to peptide stimulation due to addition of B cells could be due two potential mechanisms. Either 1) addition of extra lymphocytes during culture increases competition for space and nutrients, therefore limits the expansive capacity of virus specific CD8 T cell responses or 2) a population of B cells actively suppresses virus specific responses. The putative regulatory transitional B cells could potentially mediate this latter effect.

To determine more specifically whether transitional regulatory B cells could dampen virus specific CD8 T cell responses, the above experiments were repeated with the exception that only transitional B cells were depleted. Since we have observed previously that during disease flares, there is a cluster of IL-10 producing B cells at the mature-transitional border (fig 6.7a), we extended our gate slightly during cell sorting to further deplete this intermediate population also. To do this, PBMC from three HLA-A2 positive patients were stained for surface markers CD24, CD38 and CD19 and cells were then passed through a cell sorter. By manually drawing a gate encompassing both the $CD24^{high}CD38^{high}$ and intermediate populations, these cells could be selectively depleted from the whole PBMC population. Dot plots in figure 6.9.2a show the gating strategies used to subdivide the B cell populations. The purity of the transitional cell depleted PBMC fraction is shown in 6.9.2a (right panel). Upon cell sorting, only a small fraction of transitional cells remained (1.67% of total B cells) compared to whole PBMC (30.9% of total B cells) from the same individual passed through the cell sorter as a control.

As shown in 6.9.2b, depletion of transitional cells resulted in nearly a 4 fold increase in the magnitude of the CD8 T cell response to a pool of HBV peptides in one patient (from 0.06% to 0.23%). Interestingly, there was no significant recovery of CMV response in the same individual. When transitional cells were added back (unstimulated or following short term activation with PMA/ionomycin for 5 hours) to depleted PBMC in a 1 (Transitional):4 (PBMC) ratio, in all three patients, there was a notable suppression of the HBV-specific response. Furthermore, in patient 3, transitional cells were added back to PBMC without prior stimulation and still suppressed HBV-specific CD8 T cell responses. On simultaneous blockade of IL-10 and its receptor, this suppression was partially relieved (fig 6.9.2c). Although further experiments are required to increase the sample sizes and confirm these findings, these preliminary data at least suggest that transitional cells may have the capacity to suppress HBV-specific CD8 T cell responses by IL-10.
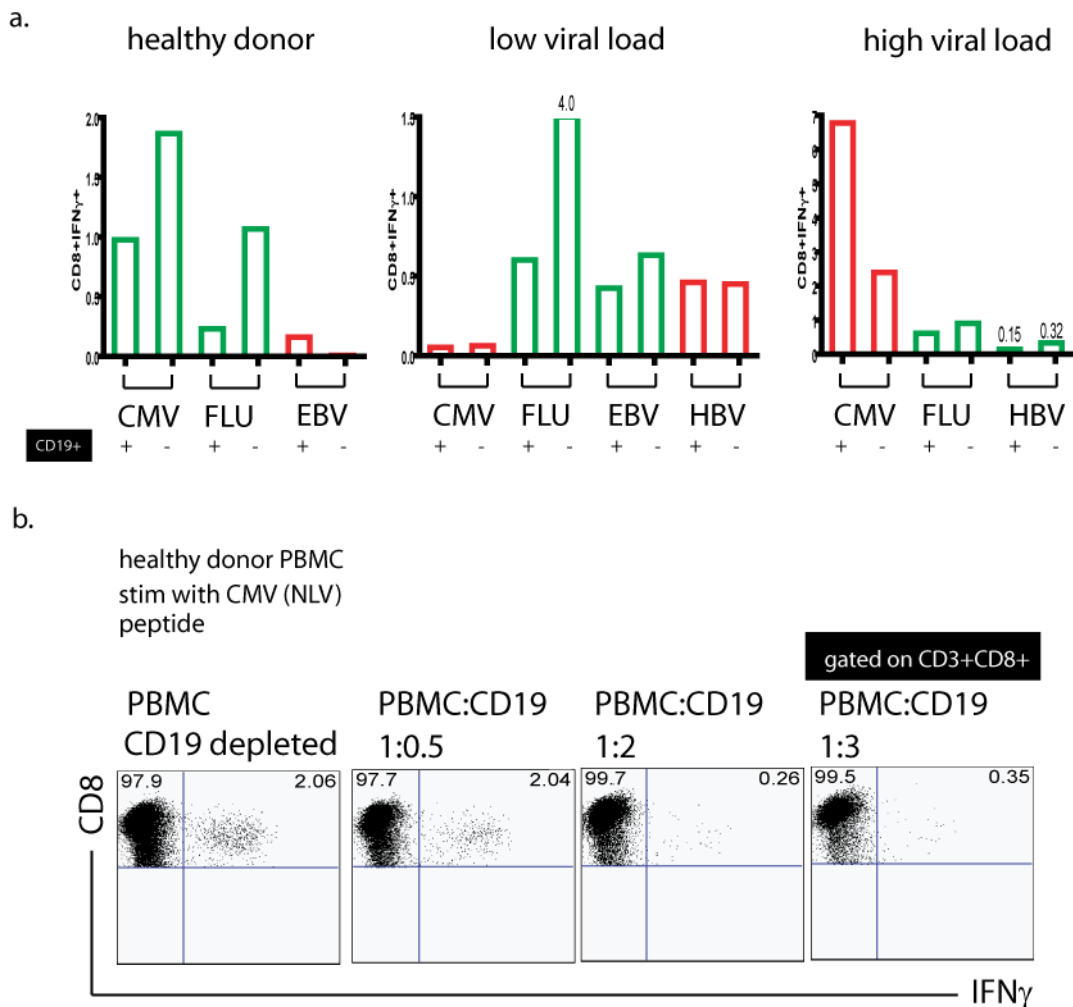
Figure 6.9.1: Depletion of B cells enhances virus specific CD8 T cell responses. PBMC from a healthy donor, patient with low viral load and patient with high viral load were depleted of CD19+ B cells via positive magnetic bead selection, and CD8 T cell responses to different viral peptides were determined by IFN-γ production with or without CD19+ B cells present. a) Graphs show the change in frequency of virus specific CD8 T cell responses upon depletion of B cells following stimulation with either a pool of HBV peptides, or those towards CMV, EBV or FLU. Green bars represent an increase in response upon CD19+ depletion. Red bars represent a decrease or no change in the response. b) CD19+ B cells were then titrated back at increasing concentrations onto PBMC and the effect on the CD8 T cell response to CMV in a healthy donor was measured.
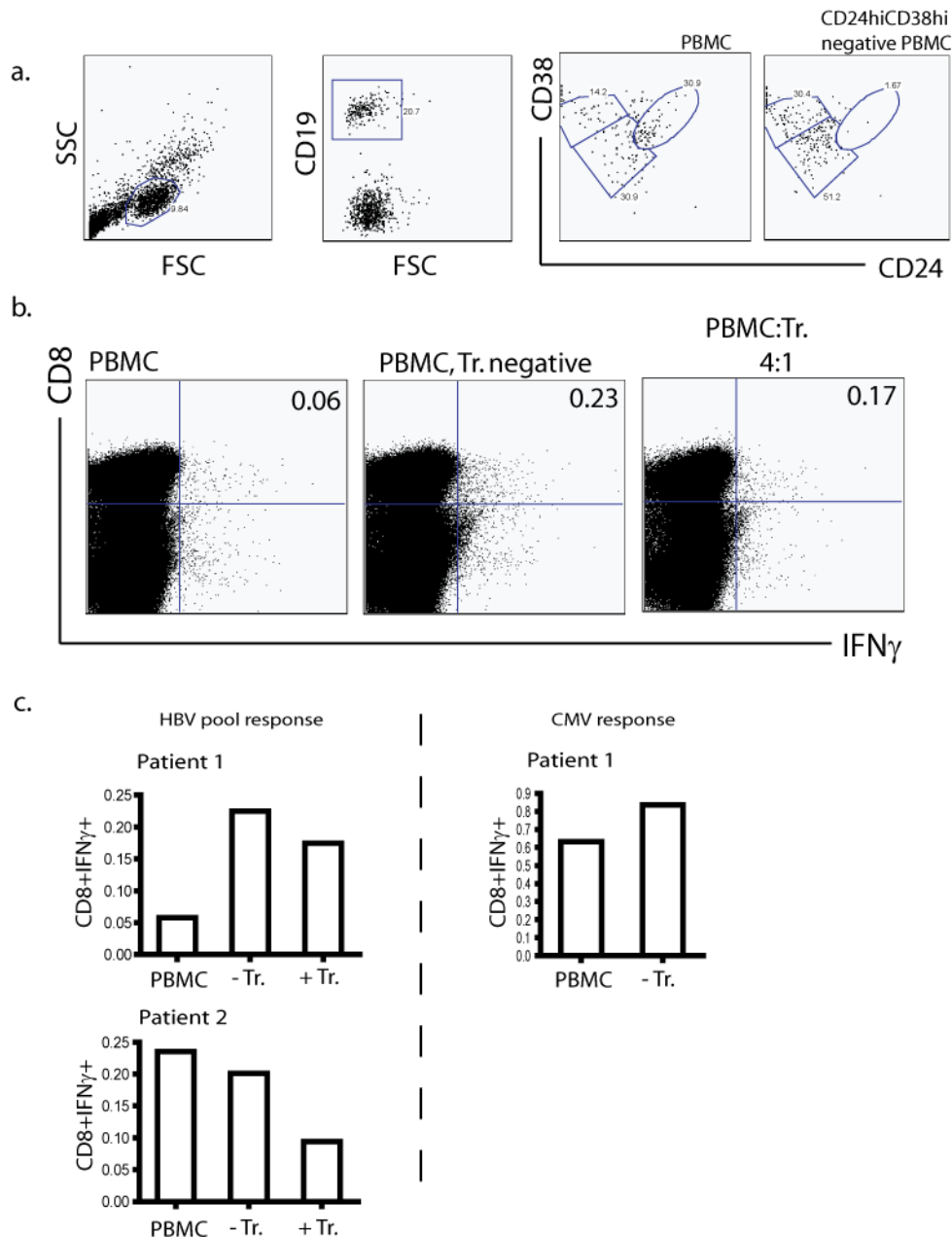
Figure 6.9.2: Suppression of HBV-specific CD8 T cell responses by transitional B cells. PBMC from HLA-A2 positive donors were depleted of CD24^{high}CD38^{high} transitional B cells using cell sorting. a) A representative dot plot shows the gates used to separate the different subsets and the purity of the cell sort in a representative patient with low viral load. b) PBMC were stimulated with a pool of HBV peptides and the frequency of IFNγ producing cells was determined after 10 days of culture with or without the presence of transitional cells; (left- PBMC alone, middle-PBMC depleted of transitional B cells,right-PBMC:transitional at a 4:1 ratio). Background values derived from the unstimulated control samples were subtracted. c) Summary data shows the effect of transitional cell depletion/addition on the HBV-specific CD8 T cell responses in two patients with CHB (left side). An example of the effect of transitional cell depletion on CMV-specific

responses is shown on the right side for patient 1. d) flow cytometric dot plots show the effect of adding back in unstimulated transitional cells (Tr.) on HBV-specific CD8 T cell responses, with or without IL-10 blockade.

d.
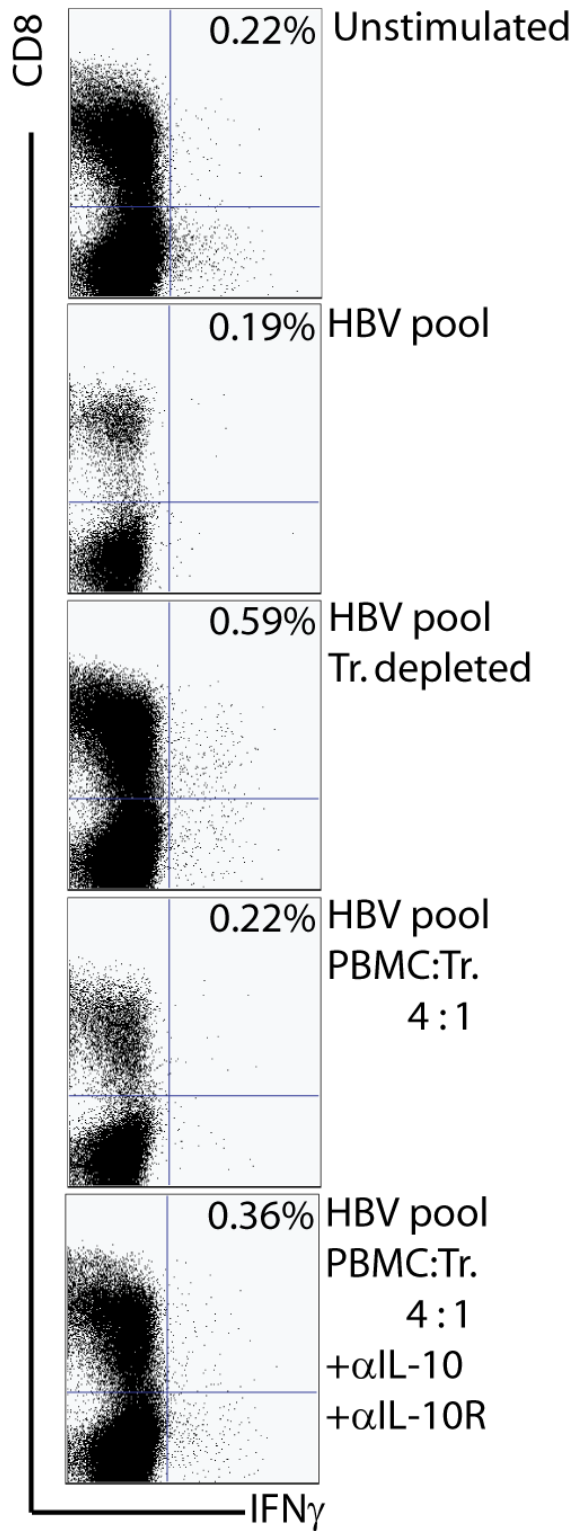
6.10 IL-10 blockade preferentially rescues HBV specific T cell responses

Regulatory B cells modulate immune responses primarily through production of immunosuppressive IL-10. To investigate what impact IL-10 may have on virus specific CD8 T cell responses, PBMC from HLA-A2+ individuals were isolated and stimulated with viral peptides (CMV, FLU or HBV peptide pool) in the presence or absence of anti-IL10 or anti-IL-10 receptor. Following short term in vitro culture, the magnitude of virus-specific responses was then quantified as the number of IFN-γ-producing CD8 T cells. Upon stimulation with a pool of HBV peptides, there was little response above background in patients with high viral load (range 0-0.27%) (representative example of high level carrier shown in 6.10a, summary data; 6.10b). This is fitting with previous data which have characterised profound qualitative and quantitative defects within HBV-specific CD8 T cells (Boni et al. 2007;Maini et al. 2000). Upon addition of anti-IL-10 alone, HBV specific responses were rescued in 2/4 low level carriers and 4/7 high level carriers. In four individuals, blockade of IL-10 revealed new HBV-specific CD8 T cell responses which were previously undetectable. This data implies that certain populations of HBV-specific CD8 T cells, which were previously thought to be deleted in patients with high viral load, may instead have been present in the circulation under a state of constitutive tolerance induced by IL-10. IL-10 blockade was also able to rescue responses to other viral peptides (CMV and FLU) in patients with high viral load.

Interestingly, in resolved individuals there was a preferential rescue of HBV-specific CD8 T cell responses, whereas no CMV or FLU responses from the same individuals were rescued. This may suggest that HBV-specific CD8 T cell responses were preferentially sensitive to the effects of IL-10. This may be because of differential expression of IL-10R on their surface or because the HBV-specific responses tended to much lower than both CMV and FLU responses, and thus lower concentrations of IL-10 were sufficient for their suppression. From our data, we have found that IL-10 levels in resolved individuals resembled that of controls (data not shown), therefore the fact that IL-10 blockade can still rescue HBV-specific responses suggests a greater propensity of these cells to respond to this cytokine. To determine whether certain specificities of HBV-specific CD8 T cells were more susceptible than others to the effects of IL-10, IL-10 blocking experiments were carried out following stimulation of PBMC with overlapping peptides spanning the whole HBV genome. Shown in figure 6.10c are data from two patients with high viral load. In

both these individuals, there was preferential rescue of responses against X and P1 peptide pools, and not against other pools (e.g. Core and envelope) encompassing immunodominant HBV epitopes. A further cross-sectional study is required to elaborate on these preliminary suggestive findings.

To induce a more complete blockade of IL-10, PBMC were also treated with anti-IL-10Receptor in addition to anti-IL-10. The effect on HBV responses in both resolved and chronic individuals is summarized in 6.10d. Upon blockade of IL-10 and its receptor together, in 4/7 CHB patients, there was a profound synergistic effect on HBV-specific responses. Therefore, these data confirm that IL-10 can potently suppress HBV specific CD8 T cell responses and may thus impede virus control.
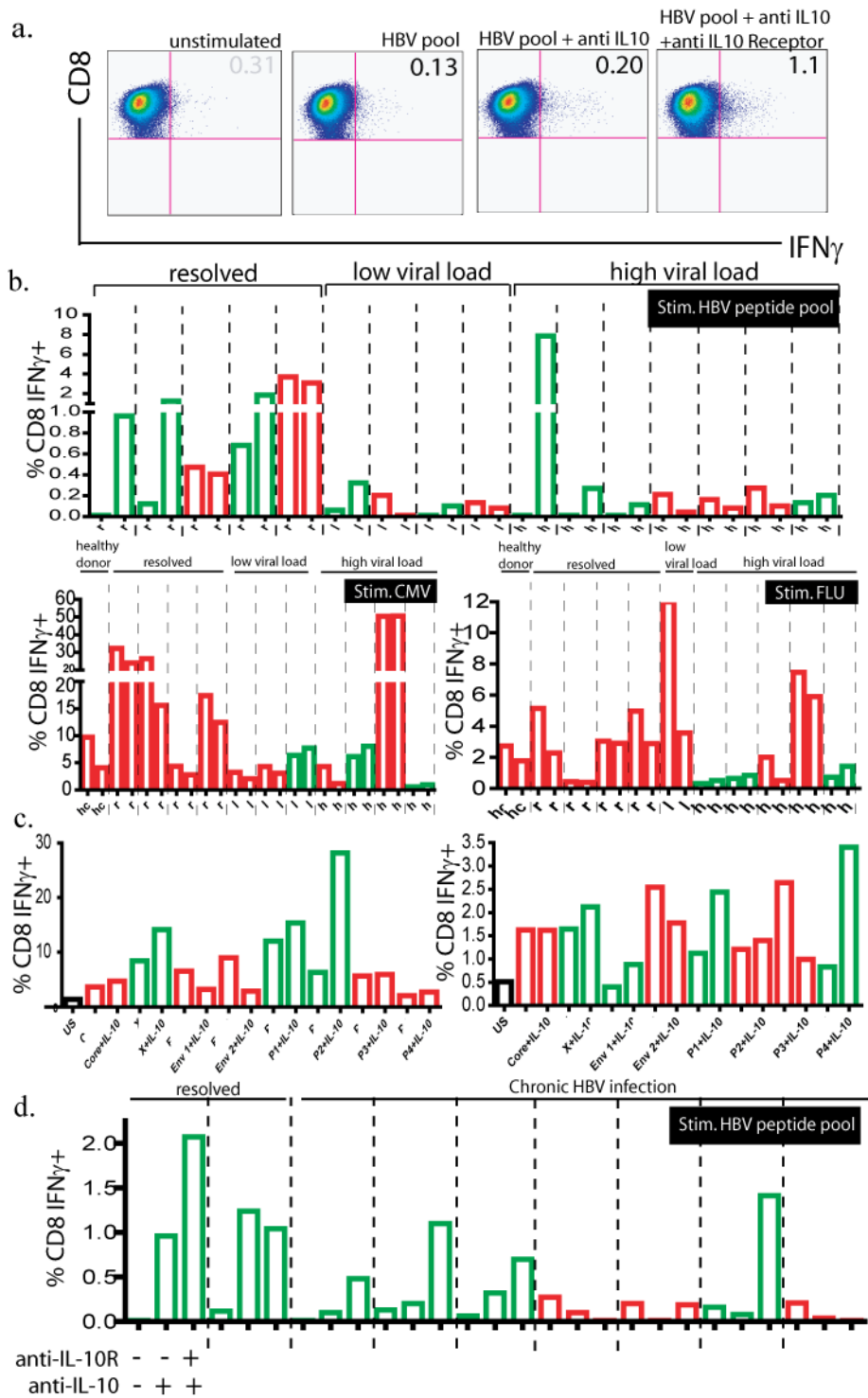
Figure 6.10 Blockade of IL-10 rescues virus specific CD8 T cell responses. PBMC from HLA-A2 individuals were stimulated with viral peptides to CMV, FLU and a pool of HBV peptides with or without blockade of IL-10 and its receptor. a) Representative example of CD8+IFN-γ responses to HBV pool are shown for a high level carrier. b) cross-sectional

data summarises the change in frequency of CD8+IFN-γ cells upon IL-10 blockade alone. (hc=healthy control, r=resolved, l=low viral load, h=high viral load. In all cases green bars represent rescue of response and red bars represent no change or decline of responses upon IL-10 blockade. c) PBMC from two patients were stimulated with pools of overlapping peptides spanning the HBV genome and the effect of IL-10 blockade is shown for each response d) PBMC were additionally treated with anti-IL-10R along with anti-IL-10 in 2 resolved and 7 patients with CHB. The effects of dual blockade is shown.

Discussion

In this study we found that IL-10 was raised in the circulation of patients with CHB, and its levels correlated temporally with flares of disease in patients with HBeAg negative disease. Blockade of IL-10 in vitro was able to rescue virus-specific CD8 T cell responses to different viral peptides (CMV, FLU, HBV pool) in patients with high viral load suggesting a functional suppressive role for this cytokine on the immune response. Furthermore, we showed that regulatory B cells were a potential source of this IL-10, as they were enriched in patients with CHB compared to controls upon CpG stimulation. Upon phenotypic characterisation, these B cells were dispersed between the mature and transitional B cell subsets, however only the transitional B cells were shown to correlate temporally with chronic flares of disease in patients with HBeAg negative hepatitis, suggesting that these were the putative regulatory subset in CHB. Indeed, preliminary data showed that selective depletion of these cells rescued CD8 T cell responses to HBV peptides confirming that these transitional IL-10 producing B cells could suppress and regulate CD8 T cell responses, at least in vitro. These data suggest that IL-10 from regulatory B cells may impede virus control in chronic HBV, and in this setting be pathogenic.

IL-10 is a pleiotropic cytokine which regulates immune responses during bacterial, parasitic and viral infections (Couper et al. 2008). The overall effect it has on the outcome of disease depends on the timing of secretion and the pathogen involved. In *Toxoplasma gondii* infection, blockade of IL-10 signalling results in severe immunopathology due to an overzealous Th1 pro-inflammatory response mediated by IFN-γ and TNF-α (Gazzinelli et al. 1996). Conversely, during infection with LCMV in mice, blockade of IL-10R is able to ameriolate disease and leads to resolution of infection (Brooks et al. 2006;Ejrnaes et al. 2006). Therefore by maintaining a fine balance between protection from immunopathology and impedance of viral control, IL-10 can have a potent effect on the ultimate outcome of disease.

To investigate the role of IL-10 in chronic HBV infection, we first compared the circulating serum levels in patients and healthy donors in a cross-sectional study. From these data, we found that IL-10 was raised in patients, and furthermore its levels correlated with viral load and liver inflammation. Further possibilities for differences in IL-10 could be related to genetic factors such as polymorphisms associated with elevated IL-10

production (Cheong et al. 2006;Miyazoe et al. 2002), however, the dynamic temporal changes in serum IL-10 we have seen over condensed periods of disease activity, are against this interpretation.

To dissect the kinetics of this IL-10 production, we next studied the longitudinal fluctuations of this cytokine over the course of hepatic flares in patients with HBeAg negative hepatitis. These individuals are susceptible to recurrent spontaneous flares of hepatic inflammation, which provides a window of opportunity to study rapid fluctuations of disease over a condensed timeframe. This analysis revealed a strong temporal correlation between IL-10 and both viral load and ALT in the majority of patients studied. Interestingly, as ALT and viral load peaked appeared to peak simultaneously during flares of disease in most individuals, it was hard to determine which variable the IL-10 was most closely linked to. On the one hand, at least in some cases, IL-10 levels correlated better with liver inflammation whilst viral load was relatively static. In this setting, one could hypothesize that induction of IL-10 could be a protective strategy by the immune response to protect from immunopathology. During infection with *Trypanosoma cruzi* for example, inoculation of IL-10 knockout mice with the virulent Tulahuen strain resulted in early mortality associated with CD4+ T cells and IL-12-mediated immunopathology. This effect could be reversed by administration of recombinant IL-10, showing its protective role in this case. (Hunter et al. 1997). On the other hand, in two cases, IL-10 levels peaked with viral load preceding the flare in ALT. This suggested a closer correlation with viral factors. In support of this, we showed that upon anti-viral therapy and suppression of viral load to low/below quantifiable levels in all 15 of our treated subjects, there was a return of IL-10 levels to baseline. Based on this potential association between IL-10 levels and viral load, it is tempting to suggest that IL-10 may be a viral escape mechanism in this case. In support of this theory, CMV and EBV viruses have been shown to produce their own IL-10, with up to 84% sequence homology to that in humans (Kotenko et al. 2000;Moore et al. 1990) that can bind the human IL-10 receptor and may therefore directly subvert anti-viral immune responses. Furthermore, studies in HBV and HCV infection have suggested that HBV core and HCV NS4 antigens themselves induce IL-10 production from peripheral blood leucocytes, which could help to suppress immune responses (Brady et al. 2003;Hyodo et al. 2003;Hyodo et al. 2004). Finally, preliminary data from our group (Dunn et al. unpublished) have shown that in early acute pre-symptomatic HBV infection, there is a peak in IL-10 production associated with the rise in viraemia preceding onset of

liver inflammation. Although the cellular source of this IL-10 is still to be investigated, it is tempting to hypothesize that perhaps an active IL-10 mediated suppression of the innate immune response may be an early viral evasion strategy. These data suggest that both liver inflammation and viral load may have an important role in IL-10 induction. Perhaps comparison of these patients with a cohort of individuals with non-viral liver disease with the presence of liver inflammation could further help to define these associations.

In this study we have investigated the role of regulatory B cells as a potential source of IL-10 production in CHB. These regulatory B cells have been shown in murine models of chronic inflammation to suppress T cell responses either directly or indirectly through interference with APC function (Moore et al. 2001). Depletion of these regulatory B cells resulted in exacerbation of Th1 type inflammatory cascades in collagen induced arthritis (Mauri et al. 2003) and EAE (Fillatreau et al. 2002) and IL-1 dependent inflammatory cascades in the GALT of mice with chronic intestinal inflammation (Mizoguchi et al. 2002). Therefore, in these settings, regulatory B cells had a protective role. What role regulatory B cells may play in HBV infection in humans, as well as in the setting of chronic viral infection as a whole is as yet unclear and was investigated here.

We showed firstly that upon stimulation with the toll like receptor ligand CpG, there was a greater proportion of IL-10 producing B cells in patients compared to healthy donors. The ability of TLR ligands to induce IL-10 producing B cells has been shown by Lampropoulou et al in a mouse model of EAE. In this study it was shown that upon CpG or LPS stimulation, naïve B cells produced IL-10 and were able to suppress Th1/Th17 mediated inflammation indirectly by downregulating production of cytokines (IL-6, IL-12, IL-23) by dendritic cells (Lampropoulou et al. 2008). Therefore it is feasible in our model that TLR stimulation, especially in the liver where there is an enrichment of ligands derived from gut derived bacteria, could be inducing a similar regulatory IL-10 producing B cell population in vivo. However, this is unlikely the sole mechanism of IL-10 induction as it cannot explain the preferential enrichment of these B cells in patients with HBV infection. This suggested the presence of an HBV-specific stimulus also.

Previous reports have shown that the HBV core antigen, an icosahedral nucleocapsid made up of 180-240 core protein, was able to activate naïve B cells in mice by binding directly to and cross-linking B cell receptors (Lazdina et al. 2001). This was however not the case for HBeAg, a secreted protein produced by the virus at high quantities. Similarly, Cao et al

showed in human peripheral blood leucocyte NOD/SCID mice that HBcAg could bind human B cell receptor in a T cell independent fashion (Cao et al. 2001). Although these studies were not directly looking at the ability for HBcAg to induce IL-10, they showed at least that HBV particles had the potential to activate B cells. Taking this one step further, we investigated whether HBcAg could act as an antigen specific stimulus to induce IL-10 production in vitro by B cells. Our results showed that upon stimulation with HBcAg for 4 days and a short restimulation with PMA/ionomycin, there was production of a low frequency of IL-10 producing single positive cells, and TNF-α producing single positive B cells but no dual positive populations by HBcAg, indicating the presence of antigen specific B cells in HBV. Of note, stimulation with HBsAg at different concentrations was not able to induce IL-10 production either by ELISA or by intracellular cytokine staining, further suggesting the preferential effect of HBcAg on B cells. As both core and surface antigen stocks were produced by the same company at the same time, it is unlikely therefore that the effects seen with HBcAg were due to LPS contamination, as the same effect would have been seen with HBsAg, and LPS does not induce regulatory B cell IL-10 production (Mauri C. personal communication). The ability of HBcAg to stimulate IL-10 by PBMC has been demonstrated in HBV. In these studies, however, T cells and monocytes were the main producers of IL-10 production, whereas only a minority of HBcAg specific IL-10 was from B cells (Hyodo et al. 2003;Hyodo et al. 2004).

It is unlikely therefore that HBcAg alone could be stimulating IL-10 production in B cells, and other viral factors in addition to the cytokine milieu and inflammatory mediators could be having an effect. IL-10, which is raised during flares of HBV, has been shown to induce IL-10 production by B cells and other lymphocytes. Interestingly, Lampropoulou have suggested a two step model of induction for IL-10 regulatory cells in EAE, by which initial stimulation with TLR ligands induced a pool of IL-10 producing B cells which were then further stimulated with autoantigen and CD40 to become antigen-specific IL-10 regulatory B cells, required for remission of EAE. In chronic HBV infection, ability of HBcAg to induce IL-10 may similarly partially be dependent on whether B cells have been primed towards IL-10 production, perhaps by TLR stimulation (Lampropoulou et al. 2008).

In order to better characterise these regulatory B cell populations, we have in addition determined the phenotypic profile of these cells. Studies by Carsetti et al. have shown that human peripheral B cells can be subdivided into fractions; mature, transitional and

memory, dependent on surface expression of markers CD24 and CD38 (Carsetti et al. 2004). Based on this classification we showed that following stimulation with CpG as well as directly ex vivo, the majority of IL-10 producing cells were of transitional or mature phenotype. This is similar to the observations by Lampropoulou et al who also determined that TLR stimulation induced mainly naïve B cell IL-10 production and Duddy et al who have showed that naïve, not memory, B cells produced IL-10 on stimulation with CD40 and through the B cell receptor (Duddy et al. 2007;Lampropoulou et al. 2008). Making use of serially stored PBMC collected before, during and after chronic flares of liver inflammation, we next studied the temporal correlations of these cell types over chronic flares of disease. This analysis showed that the transitional B cells, but not the mature or memory correlated temporally with disease flares, suggesting that these cells were the putative regulatory subset.

To address whether these IL-10 B cells regulate immune responses in HBV, we selectively depleted out B cells which we had previously identified phenotypically as the regulatory B cells, and showed that HBV-specific responses were rescued in the three patients studied. Thus, these transitional B cell subsets had the capability to directly suppress virus-specific responses. Similar phenotypic populations of marginal zone B cells upon stimulation with CpG have been shown to suppress SLE in vivo via IL-10 (Brummel & Lenert 2005;Lenert et al. 2005), and transitional zone MZ precursor 2 B cells mediate the same suppressive effects in collagen induced arthiritis (Evans et al. 2007). How they might mediate this effect in vivo within the liver, the site of highest liver inflammation and viral load, is still open to speculation. In previous reports, IL-10 production from regulatory B cells has been shown to both directly and indirectly suppress T cell responses by interference with expression of costimulatory molecule expression on APC and cytokine and chemokine production (Brummel & Lenert 2005). Thus it is likely that regulatory B cells use a multi-faceted approach in their regulation. Firstly, these cells have been shown to induce regulatory T cell populations, which have previously been shown to be enriched in patients with severe chronic HBV infection (Xu et al. 2006). This could potentially be through IL-10 production, although previous studies have shown that B cells can induce T cell regulatory activity instead by acting as inefficient antigen presenting cells (Reichardt et al. 2007). In the anterior chamber of the eye, for example, simultaneous presentation of antigens on MHC Class I and II has been shown to induce both CD4 and CD8 regulatory T cell activity associated with peripheral tolerance. (Ashour & Niederkorn 2006).

Furthermore, in a model of colitis, protective transfer of mesenteric lymph node B cells was associated with increased CD3+NK1.1+T cells in the mesenteric lymph nodes and an increase in regulatory CD4+CD8a+T cells among intraepithelial lymphocytes (Wei et al. 2005). Therefore, induction of these populations may amplify the regulatory potential of B cells, although Fillatreau et al show in a bone marrow chimeric model in EAE where only B cells but not T cells are IL-10 deficent, that there is no resolution of infection. Therefore, B cells may indeed have their own direct effects as well (Fillatreau et al. 2002). Overall, from the previous literature it is evident that multiple sources of IL-10 are available, especially within the HBV infected liver and not only resident Kupffer cells but infiltrating monocytes, macrophages and T cells can respond to TLR ligands within the local environment to elaborate IL-10 (Boonstra et al. 2006). Therefore, although B cells are not the dominant source, they may still have potent action by amplifying their function by recruiting other cells within the regulatory framework. Furthermore, greater study is needed to characterise the proportion of regulatory B cells in livers with high viral load and ongoing liver inflammation, as from the evidence in peripheral B cells, it is at these timepoints that regulatory B cells are enriched over other B cell fractions, and may contribute significantly.

We have established that IL-10 is raised in patients with CHB, but what are the functional implications of this? To investigate this, we next studied the impact of IL-10 blockade in vitro on the magnitude of virus-specific responses. We observed that blockade of IL-10 in vitro was able to rescue HBV-specific CD8 T cell responses. A more comprehensive blockade with IL-10 receptor as well had a synergistic effect on the enhancement of responses. Furthermore, in some cases, new HBV-specific CD8 T cell responses were revealed whereas they were previously undetectable. This suggested that CD8+ specific responses may be under constitutive suppression by the elevated IL-10 levels in these individuals. Interestingly, in resolved individuals, HBV specific responses were rescued preferentially to CMV or FLU responses from the same individuals. In these individuals, serum IL-10 levels were not significantly different compared to healthy donors. This may imply that HBV specific responses are more sensitive to the effects of IL-10, possibly due to differential expression of IL-10 receptor although this remains to be investigated. Thus in resolved individuals, any IL-10 present could possibly modulate HBV specific responses but not be sufficient to significantly modulate the more robust responses to CMV and FLU viruses.

In previous chapters we showed that CD8 T cells from patients with high viral load displayed a marked global dysfunction in their ability to produce IL-2 and proliferate. Based on our observations that blockade of IL-10 can rescue not only HBV-specific but other viral responses, we suggest that IL-10 may be a possible mechanism for this pan-CD8 T cell dysfunction seen. In support of these data, a study in HIV infection showed that IL-10-producing gag-specific CD8 T cells could suppress IL-2 production and cytolysis from antigen-stimulated CD8 T cells. Furthermore, depletion of these IL-10-producing CD8 T cells resulted in a recovery of IL-2 but not IFNγ production by both CMV and HIV specific CD8 T cells (Elrefaei et al. 2007). This is congruent with our identification of CD8 T cells that maintain their ability to produce IFN-γ, in the face of IL-2 loss. Similarly, in the LCMV mouse model, blockade of IL-10 production was associated with recovery of T cell IL-2 production and subsequent viral clearance (Brooks et al. 2006).

We have also shown that CD8 T cells isolated from the liver of patients with HBV, have the greatest impairment in their production of IL-2. This disparity in function between the peripheral blood and intahepatic lymphocytes could again be partially explained by IL-10. In recent reports, it has been shown that Kupffer cells may constitutively produce IL-10 in the liver in responses to Toll like receptor ligands such as LPS derived from gut derived bacteria (Tu et al. 2008a). This resting state of IL-10, we hypothesize, could further induce IL-10 production by regulatory B cells initiating a regulatory cascade which could contribute to heightened local suppression of HBV-specific CD8 T cell responses. Indeed, localised IL-10 mediated tolerance of anti-viral responses has been associated with viral persistence in other organs such as the salivary glands and the spleen. Persistence of MCMV in salivary glands has been associated with the presence of IL-10 producing CD4 T cells specifically within this organ, but not elsewhere. Blockade of IL-10R resulted in an increased accumulation of IFNγ producing CD4 T cells and concurrent clearance of infection (Humphreys et al. 2007). Similarly, high levels of IL-10mRNA was detected in splenic CD25 negative T cells, and associated with persistence of human Visceral leishmaniasis in the spleen (Nylen et al. 2007). These data support the idea that IL-10 mediated local tolerance may facilitate loss of viral control, however this is only a contributory mechanism, and other factors have been described. For example, in previous chapters we have alluded to the role of L-arginine depletion and resultant CD3ζ chain downregulation which could influence the capacity of global CD8 T cell IL-2 production

and proliferation. Additionally, there is accumulating evidence for the role of other cytokines such as TGF-β in the regulation of immune responses. In patients with HIV/HCV coinfection or HCV monoinfection for example, blockade of TGF β enhanced both CD4 and CD8 T cell production of IFN-γ on ELISPOT analysis in response to a pool of HCV core peptides. In this case, HCV specific CD8+CD25- T cells were a potential source of this regulatory cytokine. Interestingly, blockade of TGF-β or IL-10 did not significantly alter IFN-γ responses following stimulation with CEF (comprised for a pool for 23 major histocompatability compex class I restricted T cell 11-18mer peptides from CMV, EBV and FLU viruses). This was against global CD8 T cell dysfunction, however due to the fact that the magnitude of responses (number of spot forming units) was upto 1000 times greater with this more robust stimulus, effect of IL-10/TGF-β may have been more negligible and difficult to detect (Alatrakchi et al. 2007b).

We have shown in this study that IL-10 actively fluctuates in chronic HBV infection and is associated temporally with disease flares. Blockade of IL-10 and its receptor significantly rescues virus specific responses thus suggesting that IL-10 is able to suppress T cell responses within these individuals. This could serve as a partial mechanism for the global functional defects we have observed in previous chapters. Furthermore, we show that IL-10 producing B cells exist in chronic HBV infection and correlate temporally with disease activity. These B cells have direct suppressive capacity in vitro at least implicating them as potential suppressors of viral control and mediators of pathogenesis in HBV.

Future directions

The data presented in this thesis contribute to the understanding of CD8 T cell inefficiency and potential mechanisms for perpetuation of chronic HBV infection. In this section, I will discuss some of the future plans for this work, as well as its potential implications for the design of immunotherapeutic agents, and its limitations.

In chapter three, we identified a global CD8 T cell defect in IL-2 production and proliferation in HBeAg positive patients with high viral load and liver inflammation. Since the majority of patients with high viral load frequently also have co-existing liver inflammation and are HBeAg positive, it is difficult to distinguish cause from effect in the observed association between disease variables in CHB and the functional/phenotypic parameters investigated in CD8 T cells. It would be useful to dissect this relationship further and determine whether one disease variable was more influential than the others at inducing loss of IL-2. One potential way to test this would be to compare the function of intrahepatic global CD8 T cells isolated from HBV inflamed livers vs. non-HBV inflamed liver. Patients with alcoholic hepatitis may be recruited, as there is no underlying viral pathology, yet liver inflammation is still present. This would help to determine whether inflammation alone can induce loss of CD8 T cell function, and what contributory role virus/secreted viral antigens may have in inducing T cell hyporesponsiveness.

Alternatively, it is also feasible to study longitudinal changes in CD8 T cell IL-2 production before, during and after HBeAg negative flares of liver disease, in which there are intermittent peaks of disease activity over a relatively condensed timeframe. These data may reveal whether fluctuations in CD8 T cell effector function are related temporally to HBV DNA, ALT or both.

An ongoing study in our group (Khanna et al.) is comparing the function of global and virus-specific CD8 T cells in treated vs. untreated patients with CHB. Anti-viral treatments, to date, had the capacity to suppress HBV viral load in patients, however this was not maintained on cessation of therapy suggesting that immune function was not recovered. A range of novel more potent anti-viral drugs are now licensed for use; their efficacy in reversing or boosting immune function will be a focus of this study. This knowledge will facilitate the timely delivery of the most potent agents to maximally boost the immune response, with an aim of regaining immune control and viral clearance.

In the initial cross-sectional study design, parameters being compared between the groups include CD8 and CD4 T cell production of cytokines (IL-2, TNF, IFNγ), cytolytic ability and proliferation. Furthermore, the frequency of virus-specific CD8 T cells expressing the pro-apoptotic molecule Bim, which is upregulated in HBV-specific CD8 T cells and associated with their attrition during CHB (Lopes et al. 2008), is being compared between the groups. Effector function and Bim expression will also be characterised longitudinally in serial PBMC samples isolated from patients at pre and post treatment timepoints, to determine whether impaired effector function in CD8 T cells can be recovered upon sustained reduction of viral load and ALT on antiviral therapy.

In chapter four, we showed that in the absence of L-arginine, CD8 T cells were unable to upregulate CD3ζ in vitro. Furthermore, serum L-arginine levels were depleted in vivo in patients with high liver inflammation, and arginase activity was elevated in the livers of patients with CHB compared to non-viral liver disease. We hypothesized therefore that arginase-1 mediated L-arginine depletion could be partially responsible for hyporesponsiveness of CD8 T cells in CHB. These data raise the following questions;

1. What is the source of arginase-1 in the HBV inflamed liver?
2. Can depletion of other non-essential amino acids have the same effect on T cell responses?

One potent source of arginase which has not been investigated in this study is myeloid-derived suppressor cells. These cells were first described in the 1970's as natural-suppressor cells, and have the capability to suppress T cell responses, partially by upregulation of arginase-1 and catabolism of extracellular arginine (Bronte & Zanovello 2005). They have been shown to accumulate in cancer (Zea et al. 2005), autoimmune disease, in situations of immune stress or trauma (Serafini et al. 2004), and during certain acute or chronic fungal (Mencacci et al. 2002) or parasitic infections (Goni et al. 2002), where they mediate immunosuppression. They are regarded as immature cells from the myeloid lineage, and in mice, are phenotypically heterogenous. In mice with subcutaneous implants of colon carcinoma, MDSC were CD11b+GR1+ and upregulated iNOS and arginase-1 which can both catabolise L-arginine (Ochoa et al. 2007). In murine lung

carcinoma however, MDSC expressed macrophage markers CD11b+GR1-CD68+ and had high arginase-1 activity (Rodriguez et al. 2004).

In humans, Filipazzi et al. found that a population of CD14+HLA DR (negative/low) cells were enriched in all metastatic melanoma patients but undetectable in healthy donors and were potently capable of suppressing lymphocyte function. However, this was mediated by TGF–β and not NOS/arginase (Filipazzi et al. 2007). In a more recent study however, Hoechst et al. demonstrated that the same phenotypic subset of MDSC were enriched in the periphery of patients with hepatocellular carcinoma compared to healthy donors, had high arginase activity, and suppressed autologous T cell proliferation (Hoechst et al. 2008). Although these studies agree on the phenotypic characterisation of a subset of MDSC in humans, there is some debate as to whether their influence on T cell responses is mediated by arginase activity or not.

In patients with metastatic renal cell carcinoma, Zea et al. showed that PBMC had a 6-8 fold higher arginase activity compared to those isolated from healthy donors. This correlated with reduced serum L-arginine levels, high plasma ornithine (the breakdown product of L-arginine when metabolised by arginase-1), and low cytokine production and CD3ζ downregulation in T cells. Cell separation studies showed that elevated arginase activity was limited to a subset of CD11b+CD14-CD15+ cells with a polymorphonuclear granulocyte morphology, and the percentage of these cells directly correlated with arginase activity in PBMC (r^2=0.9327). Phenotypically, these cells also expressed CD80, CD83, CD86, MHC II and CD11a. Importantly, depletion of these MDSC upregulated CD3ζ and recovered proliferative capacity in T cells, suggesting that MDSCs were suppressing T cell function through arginase (Zea et al. 2005).

It is feasible that selective accumulation of the MDSC in the liver (possibly recruited by IFNγ induced chemokine release (Kakimi et al. 2001b)) and periphery of patients with CHB may contribute to suppression of global CD8 T cell IL-2 production and proliferation, and thus potentially impair viral control. According to the above literature, MDSC in humans may be identified by their expression of markers such as CD14 and CD11b, as shown in mice. Using a cross-sectional study design, the frequency of these cells could be quantitated by surface staining and flow cytometry in peripheral blood and intrahepatic lymphocytes obtained from patients with CHB and non-viral liver disease or

healthy donors. Furthermore, the capacity of these cells to suppress both HBV-specific and other control virus-specific CD8 T cell responses could be determined by co-culture of isolated MDSC with PBMC stimulated with HLA-A2 restricted viral peptides. Addition of arginase inhibitors (nor-NOHA) may further help to determine whether any observed suppression was mediated through arginase activity or not. These experiments would help strengthen our current findings and provide additional mechanisms for global and HBV-specific CD8 T cell dysfunction during CHB.

In addition to depletion of L-arginine, studies have shown that depletion of tryptophan and accumulation of its toxic metabolites is associated with CD3ζ downregulation and impaired T cell proliferation (Fallarino et al. 2006;Frumento et al. 2002), although this is not true for all non-essential amino acids, as depletion of L-glutamine does not have this effect (Rodriguez et al. 2002). By obtaining tryptophan depleted medium, it would be possible to study whether depletion of this amino acid may also contribute to CD8 T cell dysfunction during CHB.

Normal serum concentrations of L-arginine range from 50-150μmol/L (Ochoa et al. 2007). During trauma, studies have reported that rapid release of arginase can significantly deplete serum L-arginine levels to below 50μmol/L, which is associated with CD3ζ downregulation and T cell anergy (Bernard et al. 2001;Ichihara et al. 1999). In our cross-sectional study, 3 patients had serum L-arginine levels below 50μmol/L, and in a further 6 patients, serum L-arginine levels were at the lower end of normal range, below 75μmol/L (figure 4.9). L-arginine levels may be even lower in the liver, where we have shown arginase to be upregulated. In these individuals, an insufficient supply of L-arginine may hamper the ability of HBV-specific CD8 T cells to respond to antigenic stimulation through the TCR. This could potentially hinder the efficacy of immunotherapeutic vaccines which aim to boost HBV-specific CD8 T cell responses. Dietary L-arginine has been shown to protect against infection in trauma patients, however in one study, it was also shown to stimulate tumours in patients with breast cancer (Park et al. 1992). Other potential therapies may include drugs such as sildenafil, which have known safety and pharmokinetics, and may boost T cell responses by downregulating arginase and iNOS activity, primarily in MDSC (Serafini et al. 2006).

In chapter five, we showed that global CD8 T cells were more differentiated in patients with CHB and had shorter telomeres within the CD27 negative fraction. Furthermore, telomere length was even shorter in HBV-specific CD8 T cells. These latter experiments were done in a limited number of patients for the following reasons;

1. Ex vivo circulating frequencies of HBV-specific CD8 T cells are barely detectable in patients with high viral load, therefore are difficult to study.

2. Expansion of these HBV-specific CD8 T cells in vitro grossly increases telomere length in all CD8 T cells, suggesting that this was an artefact of cell culture.

3. Virus-specific CD8 populations have been identified by measuring the production of IFN-γ production by these cells. Staining the cells with IFN-γ occurred prior to hybridisation of PBMC in the flow FISH assay. Heating of the conjugated antibody to 82°C results in degradation of the fluorochrome, weakening the fluorescence signal and making it even more difficult to detect HBV-specific populations.

In future experiments, these assays could be repeated with the use of Quantum Dot nanocrystal primary antibody conjugates, which are more heat stable, brighter and photostable than organic fluorophores. These reagents are being routinely used now by our collaborators at the Department of Medicine, Addenbrooke's Hospital, Cambridge.

In addition to studying telomere length in CD8 T cells, an additional marker associated with replicative senescence is phosphorylated histone H2AX. When telomeres reach a critical length, DNA double strand breaks trigger cell cycle arrest. This DNA damage response involves phosphorylation of histone H2AX, which initiates a cascade of events resulting in cell senescence (d'Adda et al. 2003). Accumulation of phosphorylated histone H2AX has been found in colonocytes in patients with Ulcerative Colitis compared to controls in a recent study and this DNA damage signal can be measured by flow cytometry (Risques et al. 2008). It is feasible therefore in future studies to determine the fluorescence of phosphorylated histone H2AX in PBMC from patients with CHB, as a measure of DNA damage and replicative senescence in these cells.

Telomere shortening and replicative senescence can be delayed by the action of telomerase, which can add telomeric repeats back on to the ends of chromosomes (Akbar

& Vukmanovic-Stejic 2007;Zajac et al. 1998). In future studies, we aim to study whether aberrant expression of telomerase in lymphocytes may partially explain why telomere length is shorter in CD8 T cells from patients with high viral load compared to patients with low viral load and healthy donors. This would involve extending the preliminary cross-sectional study shown in figure 5.10. In addition, future studies would also investigate the in vitro effect of IFNα on telomerase activity on CD8 T cells from patients with CHB, as this has been previously reported to inhibit telomerase (Reed et al. 2004). Interestingly, previous data from our group has demonstrated that IFNα levels in the serum correlated temporally with disease activity during flares of HBeAg negative hepatitis (Dunn et al. 2007). Longitudinal determination of telomerase activity derived from PBMC cell extracts available from timepoints before, during and after such flares, would allow correlation of telomerase activity with IFNα levels in vivo. Furthermore, the effect of IFNα therapy on telomerase activity in patients with CHB may also be determined.

In chapter six we suggested a novel role for B cells in regulating the immune response during human chronic viral infection through production of IL-10. We showed that a subset of transitional B cells fluctuated with disease activity ex vivo in patients undergoing HBeAg negative flares of liver disease, and depletion of these cells rescued HBV but not CMV-specific CD8 T cell responses in vitro. Additionally, suppression by transitional cells was mediated partially by IL-10 production; blockade of IL-10 and its receptor rescued HBV-specific CD8 T cell responses. A number of questions are raised by our findings which can be addressed in future work.

The ability of B cells to regulate human viral infections is a new emerging field of research. No phenotypic markers have yet been published to identify IL-10 producing regulatory B cells in humans. In mice, Evans et al. showed that MZ-transitional 2 B cells were selectively responsible for IL-10 mediated suppression of autoimmunity (Evans et al. 2007). Work from the same group has provided evidence that human transitional B cells may have the same regulatory capabilities (Paul Blair et al, manuscript submitted). On the basis of this work, and our own observations that IL-10 is produced exclusively by transitional and mature B cells upon CpG stimulation, we investigated CD24hiCD38hiCD19+ B cells as the putative regulatory subset. Additional phenotypic markers which may be useful to characterise these cells in the future include CD1d; a marker found of CD19+ positive B cells which were enriched in gut-associated lymphoid

tissue of mice and regulated inflammation through production of IL-10 (Mizoguchi et al. 2002).

In this work, we have studied the phenotypic properties of B cells after 4 days stimulation with CpG. These data have also been repeated in a limited number of patients ex vivo and show a trend towards an increase in transitional cells with viral load (work by Gidon Ellis). In future work, this phenotypic study can be extended. Phenotypic markers may also be used to look at the proportion of regulatory B cells within HBV and non-HBV livers. Through our collaborators, we have access to a large library of paraffin embedded samples, which may be stained by immunohistochemistry for B cell and T cell markers.

We have functionally identified regulatory B cells through their ability to produce IL-10 following stimulation with CpG and the mitogen PMA. In our cross-sectional study, the proportion of IL-10 producing B cells was raised in CHB compared to controls. However, it is not clear whether HBV signals through toll like receptors, and whether it contains unmethylated CpG motifs which would be capable of ligating TLR9 on B cells in vivo. It would be important in future studies to investigate whether IL-10 could be induced in these subsets by a more physiological stimulus. Candidate stimuli include the recombinant surface and core antigens of HBV. HBsAg is released at milligram quantities in patients with CHB, and HBeAg (the cleaved form of HBcAg), is produced during active disease and has no known role in viral replication, but instead has been suggested to be a tolerogen (Milich et al. 1990). In preliminary data, we have shown that stimulation of PBMC with HBcAg for four days induced IL-10 and TNFα single positive subsets in CD19+ B cells from patients with CHB. HBsAg did not have the same effect in our study. Observations by Dunn et al., who quantified supernatant IL-10 by ELISA following stimulation of PBMC from acute patients with the same HBcAg or HBsAg preparations (Dunn et al. manuscript submitted), also found IL-10 induction only by nucleocapsid antigen. Comparison of the proportion of IL-10 producing B cells stimulated with HBcAg between patients and healthy donors may help to confirm our observations with the CpG stimulus.

In addition to the above experiments, there are further lines of work which may be pursued. They are outlined in brief here.

1) We observed that HBV-specific CD8 T cell responses may be more sensitive to IL-10 blockade than CMV or FLU-specific responses from the same individuals. This

differential sensitivity to the effects of IL-10 could reflect distinct expression of IL-10 receptor on different populations of virus-specific CD8 T cells. In future studies, we could therefore study expression of IL-10R on the surface of CD8 T cells directed against different viruses.

2) We now have access to longitudinal PBMC and serum samples taken from patients before and after initiation of anti-viral therapy. In these individuals, it will be possible to study the effect of therapy and reduction in viral load on serum IL-10 concentration and ex vivo frequencies of transitional B cells.

3) Regulatory B cells may additionally suppress HBV-specific CD8 T cell responses by mechanisms independent of IL-10. This was suggested by our observation that transitional cells partially suppressed HBV-specific responses upon IL-10 blockade. We hypothesize that this could be mediated through release of cytokines such as TGF-β, blockade of which can rescue both HBV (Lopes and Khanna unpublished data) and HCV-specific CD8 T cell responses in vitro (Alatrakchi et al. 2007a), or through triggering of a regulatory network, including regulatory T cells, which may futher amplify suppression. B cell deficiency, during EAE infection of mice, delayed the emergence of Foxp3+ IL-10 producing regulatory T cells in the CNS. Regulation of CD4+CD25+ T regs by B cells was mediated through the costimulatory molecule B7 (Mann et al. 2007).

4) We aim to investigate the potential for HBV-specific B cells to regulate the immune response during CHB. Recently it was demonstrated by Ward et al. that HBsAg specific memory B cells could be isolated ex vivo with a novel two-step immunomagnetic protocol (Ward et al. 2008). This method could be used to quantify and compare the frequency of HBV-specific B cells in patients with low and high levels of serum IL-10, and also to determine their functional capacity in vitro.

Two recent studies in murine models of LCMV have shown that blockade of IL-10 receptor can mediate clearance of virus in vivo (Brooks et al. 2006;Ejrnaes et al. 2006). More recently, the same group showed that blockade of IL-10 enhanced the function of a previously ineffective therapeutic DNA vaccine to boost antiviral immune responses

(Brooks et al. 2008). In our study, we have shown in vitro that blockade of IL-10 and its receptor can rescue HBV-specific CD8 T cell responses. Neutralising antibodies against IL-10 may potentially boost HBV-specific CD8 T cell responses in vivo. However, the kinetics of IL-10 administration may be critical in determining its efficacy. In a normal immune response, IL-10 production may serve as a physiological brake to prevent an over-reactive immune response (Martinic & von Herrath 2008). Any potential side effects also have to be taken into account. IL-10 deficient mice have been shown to develop enterocolitis in the presence of normal gut flora (Kuhn et al. 1993).

In addition to blockade of IL-10 signalling, studies in mice with chronic LCMV infection have also highlighted other promising, potentially complementary, strategies; blockade of PD-1/PD L1 and treatment with FTY720, a novel class of immunosuppressive drug. As discussed in chapter five, PD-1 is a negative regulator of T cell signalling and has been shown to be hyper-expressed on exhausted LCMV specific CD8 T cells (Barber et al. 2006). In patients with CHB, PD-1 expression was shown to be raised both on HBV-specific (Boni et al. 2007) and global CD8 T cells (Evans et al. 2008;Peng et al. 2008b). Blockade of PD-1/PD-L1 signalling was able to recover core and polymerase, but not envelope specific CD8 T cell responses in vitro (Boni et al. 2007). Antiviral treatment reduced PD-1 expression on both global and HBV-specific CD8 T cells and was associated with recovery in the frequency of IFN-γ producing T cells (Evans et al. 2008). Medarex and Ono Pharmaceuticals have tested the safety of MDX-1106, a fully human anti-PD-1 antibody, in a Phase I study in patients with recurrent or treatment refractory cancer. More recently, they launched a double-blind, randomized, placebo controlled, single-dose, dose-escalation, safety and pharmokinetic study of MDX-1106 for the treatment of HCV infection.

In the last month, Premenko-Lanier et al. have showed that FTY720, an immunosuppressive agent trialed in humans for treatment of relapsing multiple sclerosis and prevention of kidney transplant rejection was able to clear already established chronic LCMV infection in mice (Premenko-Lanier et al. 2008). During murine LCMV infection, it is well characterised that infection with the Armstrong strain of virus results in acute infection and viral resolution, whereas infection with the clone 13 strain instead favours viral persistence. Premenko-Lanier et al. noted that following infection with the Armstrong strain, there was a profound lymphopenia 1-3 days post infection which was much less

marked during infection with the clone 13 variant. These lymphocytes were found to be sequestered within the lymph nodes during this period. By treating clone 13 variant mice with FTY720, which is known to sequester lymphocytes to lymph nodes and Peyer's patches, the authors observed that by day 8, clone 13 titres were undetectable in brain and kidneys, and virus was cleared by day 30. Furthermore, administration of FTY720 at days 30, 31 and 32 of established infection, resulted in clearance of virus by day 60. FTY720 did not have a direct anti-viral effect, and required the presence of CD4 T cells, suggesting that it had an immunomodulatory role. Both glycoprotein (GP)33-41 and nucleoprotein (NP)396-404 specific CD8 T cells in FTY720 treated mice were functional, whereas (NP)396-404 specific CD8 T cells have previously been described to be deleted during chronic LCMV infection with clone 13 variant (Zajac et al. 1998). This study has used a drug which is already used in humans for the clearance of chronic LCMV infection. Such therapy may also have efficacy in patients with CHB, although the mechanism of action is not yet fully clear. In the interim period, it would be interesting to investigate patients who are currently under FTY20 therapy for diseases such as multiple sclerosis, who may be also co-infected with chronic persistent viruses such as HBV/HIV or HCV.

A number of new targets for immunotherapy have been identified in mice and human chronic viral infections. Development of some of these agents for treatment of human disease is in progress, and will be complimentary to the existing anti-viral therapy regimes. Together, these strategies may be more efficacious and provide a means to help reduce the global burden of CHB worldwide.

We have described three novel mechanisms that may contribute to inefficient T cell function in CHB; downregulation of the TCR-associated signalling molecule CD3ζ, partially attributable to arginase-1 mediated L-arginine depletion, regulation by transitional B cell IL-10 and premature ageing of global CD8 T cells. We propose that these mechanisms in combination may act to disable CD8 T cell responses, and in addition, contribute to the pro-inflammatory cytokine environment and immune mediated pathology. In conclusion, these data provide new avenues for immunotherapeutic intervention in CHB, which may boost the body's natural ability to control this virus.

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List of Publications

Functional skewing of the global CD8 T cell population in chronic hepatitis B virus infection.

Das A, Hoare M, Davies N, Lopes AR, Dunn C, Kennedy PT, Alexander G, Finney H, Lawson A, Plunkett FJ, Bertoletti A, Akbar AN, Maini MK. Journal of Experimental Medicine (2008) 205(9):2111-24.

Bim-mediated deletion of antigen-specific CD8 T cells in patients unable to control HBV infection.

Lopes AR, Kellam P, Das A, Dunn C, Kwan A, Turner J, Peppa D, Gilson RJ, Gehring A, Bertoletti A, Maini MK. Journal of Clinical Investigation (2008) 118(5):1835-45.

Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage.

Dunn C, Brunetto M, Reynolds G, Christophides T, Kennedy PT, Lampertico P, Das A, Lopes AR, Borrow P, Williams K, Humphreys E, Afford S, Adams DH, Bertoletti A, Maini MK. *Journal of Experimental Medicine (2007) 204(3):667-80.*

T-lymphocyte telomere attrition in patients with chronic hepatitis C virus infection.

Matthew Hoare, William TH Gelson, Abhishek Das, Jean M Fletcher, Susan E Davies, Martin Curran, Sarah L Vowler, Mala K Maini, Arne N Akbar, Graeme JM Alexander (Submitted August 2008)

Role of IL-10 producing regulatory B cells in the pathogenesis of chronic HBV infection.

A. Das, G. Ellis, P. Blair, M. Brunetta, A.R. Lopes, P. Khanna, G. Dusheiko, D. Peppa, C. Mauri, M. Maini. (Manuscript in preparation)

Premature Senescence of CD8 T cells in patients with chronic HBV infection.

A. Das, P. Khanna, M. Vukmanovic-Stejic, A.R. Lopes, R.J. Gilson, G. Dusheiko, A.Akbar, M.K. Maini. (Manuscript in preparation)

List of Abstracts

International Meeting on the Molecular Biology of Hepatitis B Viruses:

2004 Poster Presentation

Downregulation of the CD3ζ chain: a mechanism for T cell dysfunction in patients with chronic HBV infection?

A.Das, A.R. Lopes, C.Dunn, J. Gotto, R.J. Gilson, A. Bertoletti, M.K. Maini.

*2005 **Oral Presentation***

A reversible defect in CD8 T cell IL-2 production with CD3z chain downregulation in chronic HBV infection.

A.Das, P. Kennedy, A.R. Lopes, C. Dunn, R. Johnstone, R.J. Gilson, A. Bertoletti, M.K. Maini.

2006 Poster Presentation

Global biasing of CD8+ T cells in patients with high level chronic HBV infection towards 'end-stage' effectors.

A. Das, A.R. Lopes, C. Dunn, M. Vukmanovic-Stejic, A.Akbar, R.J. Gilson, A. Bertoletti, M.K. Maini.

2007 Poster Presentation

Role of IL-10 producing regulatory B cells in the pathogenesis of chronic HBV infection.

A. Das, G. Ellis, L. Vialino, P. Blair, C. Mauri, M. Maini.

2008 Poster Presentation

Role of IL-10 producing regulatory B cells in the pathogenesis of chronic HBV infection.

A. Das, G. Ellis, P. Blair, M. Brunetta, A.R. Lopes, P. Khanna, G. Dusheiko, D. Peppa, C. Mauri, M. Maini.

British Society of Immunology and Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry: John Humphrey Advanced Summer Programme in Immunology

*2007 **Oral Presentation***

CD8+ T cells from patients with chronic HBV infection display a 'functionally exhausted' phenotype associated with telomere shortening

A.Das, M.Vukmanovic-Stejic, J. Fletcher, A.R. Lopes, C. Dunn, R.J. Gilson, M.K. Maini.

British Society of Immunology Joint London Immunology Group/Differentiation and Immunosenescence Meeting. Leucocyte differentiation and regulation in disease.

2008 Poster Presentation.

Premature Senescence of CD8 T cells in patients with chronic HBV infection.

A. Das, P. Khanna, M. Vukmanovic-Stejic, A.R. Lopes, R.J. Gilson, G. Dusheiko, A.Akbar, M.K. Maini.